國立臺灣大學獸醫專業學院獸醫學系

博士論文

Department of Veterinary Medicine

School of Veterinary Medicine

National Taiwan University

Doctoral Dissertation

芬普尼透過誘導粒線體凋亡、介白素-7 基因調控的失調及干擾 GABA 基因介導之免疫調節作用以擾亂免疫功能的恆定

Fipronil Disrupts Immune Homeostasis Through Mitochondrial Apoptosis, Dysregulated IL-7 Signaling, and GABAergic Signaling Alterations

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中華民國 114 年 6 月

June, 2025

doi:10.6342/NTU202501708

國立臺灣大學博士學位論文
口試委員會審定書

芬普尼透過誘導粒線體凋亡、介白素-7 基因調控的失調及干擾 GABA 基因介導之免疫調節作用以擾亂免疫功能的恆定

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本論文係郭瑞芳君（D06629001）在國立臺灣大學獸醫學系、所完成之博士學位論文，於民國 114 年 6 月 18 日承下列考試委員審查通過及口試及格，特此證明

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系主任、所長　（簽名）

doi:10.6342/NTU202501708

聲明

本篇論文全文皆由博士生郭瑞芳負責研究執行及結果撰寫。其中 Chapter 1 以及 Chapter 3 至 5 之部分著作內容已發表刊登於 SCI 期刊中。研究題目及期刊名稱如下：

- **Jui-Fang Kuo**, Yin-Hua Cheng, Chun-Wei Tung, and Chia-Chi Wang* "Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in the ovalbumin-immunized BALB/c mice" BMC Veterinary Research. 2024, 20:30. https://doi.org/10.1186/s12917-024-03878-3
- **Jui-Fang Kuo**, Hsin-Ying Wu, Chun-Wei Tung, Wei-Hsiang Huang, Chen-Si Lin, and Chia-Chi Wang* "Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes" Chem. Res. Toxicol. 2024, 37, 1488−1500. https://doi.org/10.1021/acs.chemrestox.4c00060
- **Jui-Fang Kuo**, Yai-Ping Hsiao, Yao-De Wang, Hsin-Pei Weng, and Chia-Chi Wang* "Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes" Toxics 2025, 13, 204. https://doi.org/10.3390/toxics13030204

本篇論文引用已取得發表期刊之同意，無抄襲疑慮。

另 Abstract、Chapter 2、Chapter 6 及 7 段落內文經著作原創性系統 Turnitin 比對後，相似度為 14%。

doi:10.6342/NTU202501708

致謝

八年。沒想到這段旅程，遊蕩了八年才終於靠岸。

博士班的歷程，既不平靜也格外顛簸。其中，二年級下學期更是我博士生涯的最低潮。就在我幾乎放棄之際，王家琪老師恰巧回到台大任教，如同在茫茫汪洋中，為我投下了一根救命浮木，老師義無反顧地將我拉起，並傾其所能地教導我、扶持我。

家琪老師的指導看似輕鬆愜意，實則要求嚴謹，她始終叮嚀我要秉持科學精神與研究倫理，細心完成每一次實驗。即使結果未必如願，老師總是不厭其煩地鼓勵我，陪我從無數次的失敗中，逐步萃取出成功的結晶。家琪老師重視學生的自我管理與獨立思考，給予我極大的自由與空間發揮，在寬容與信任中培養我自主學習與探索的能力。除了學術上的指導外，她也特別關心我的身心狀態，總能察覺我的情緒與身體變化，在我低落時適時地給予關懷與建議，幫助我走出陰霾。在老師的帶領下度過的這幾年，心懷無限的感謝，也感到無比幸運。

我也要誠摯感謝詹東榮老師。儘管我並非老師的正式學生，他仍視我如己出，無私地給予我學術與生活上的支持與指導。老師總在適當時候給予我鼓勵，每每讓我感到溫暖與堅定。

多年來，我最感謝的，是我的家人，特別是我的媽媽與姊姊。她們撐起了整個家庭的經濟重擔，讓我得以無後顧之憂地繼續堅持學位之路。雖然從博士四年級開始，她們常常催促我何時畢業，那些一再的關心，有時也變成巨大的壓力。隨著時間推進，壓力成倍增長，但如今，我終於要畢業了。未來如何尚未可知，但至少，應該可以讓她們安心一些了吧。

在漫長又繁重的實驗室生活中，若沒有小夥伴們的陪伴與扶持，我真的撐不下去。我非常幸運，擁有一群與我同甘共苦的實驗室夥伴。特別感謝雅萍學姐，自我進入獸醫系攻讀博士以來，她一直細心照顧我，並在加入詹老師與家琪老師的實驗室後，更是手把手教導我

doi:10.6342/NTU202501708

各種實驗技術與博士必備的能力。謝謝靖樺學妹、浩余、盈方學妹，與我一同完成無數次的活體實驗，在實驗室中你們也總是主動積極、自我管理，，不需我費太多心神操心。沒有你們，我真的無法應對實驗室裡的種種挑戰，與妖魔鬼怪們。

此外，還有一群與我一樣為博士學位奮戰的小夥伴：羅勻、浭鈺、澤恩、豐傑、昀儒、淑慧，謝謝你們總是能感同身受，理解我所處的難境，並在最需要的時候，給我最實在的（酒精）慰藉。這些年來，若沒有彼此的打氣與吐苦水，我們都無法熬過這些日復一日的挑戰與無理。

最特別要感謝的人是欣貝。這幾年來，無數個夜晚與假日無怨無悔地陪伴著我，並在我實驗不順時給予協助、幫我解決研究難題、陪我度過每一個低潮，同仇敵愾地與我一同出氣。沒有妳的陪伴，我無法走到今天，我很慶幸有你在。

這一路上，要感謝的人真的太多太多。謝謝口試委員張猷忠老師、張芳嘉老師、梁有志老師、林英琦老師、曾湘文副總經理，感謝您們在口試中的寶貴建議與指導。謝謝童俊維老師長期以來對實驗室的支持。也感謝那些為科學奉獻的小鼠們，沒有你們，我無法完成這篇博士論文。

最後，獸醫系的地縛靈終於要退場了，大家也不要彼此留戀了。

爸，我終於畢業了！你會開心的吧？

2025/7/25 郭瑞芳 筆

doi:10.6342/NTU202501708

中文摘要

芬普尼是一種苯基吡唑類殺蟲劑，廣泛應用於農業、家庭害蟲防治與獸醫治療領域，傳統上被認為對脊椎動物毒性較低。然而，越來越多研究指出，芬普尼暴露可能對肝臟、生殖系統與神經系統造成預期之外的不良影響。儘管如此，其對免疫功能，特別是 T 淋巴細胞反應的影響仍未被充分探討。本研究使用體內與體外兩種模型，系統性評估芬普尼對成熟與發育中 T 淋巴細胞的免疫毒性。結果顯示，在免疫卵白蛋白小鼠中口服投予芬普尼後，對卵白蛋白的抗原特異性免疫反應顯著增強，包括促進脾臟細胞代謝活性上升、增加細胞激素 IL-2、IL-4 與 IFN-γ 分泌量，以及同時增加抗原專一性 OVA-IgG_1 與 OVA-IgG_{2a} 血清抗體濃度。基因表現的分析顯示，GABA 調控相關的基因受到影響，其中 *Gad67* 的基因表現下降，以及 GABA 受體次單元（*β2* 和 *δ*）的基因表現則上升。這些結果顯示，芬普尼可能藉由干擾 GABA 路徑基因調控 T 淋巴細胞的免疫抑制作用，從而增強抗原特異性免疫反應不正常的活化。

由於胸腺細胞的生成與 T 細胞的成熟分化是後天免疫系統發育的重要作用，我們進一步評估芬普尼對胸腺發育的影響。結果發現，芬普尼會引起胸腺明顯萎縮、雙陽性胸腺細胞比例與數量下降，T 細胞成熟受到抑制與 IL-7 與其受體表現下降密切相關。IL-7 是胸腺早期發育中不可或缺的細胞激素，支持雙陰性階段後 T 淋巴細胞的的存活、增殖和分化。研究亦發現芬普尼抑制 IL-7 軸相關基因和蛋白（包括 FOXN1、LYL1、SCF 和 c-KIT）的表現，進一步破壞了細胞增生所需的胸腺微環境。除胸腺發育訊號受到抑制外，芬普尼也誘發胸腺細胞的氧化壓力，包括細胞內活性氧含量上升、粒線體膜電位去極化、脂質過氧化、鈣離子與穀胱甘肽濃度下降等現象。同時，BCL-2 家族基因表現失衡，抗凋亡基因抑制、促凋亡基因如 *Bim* 與 *Bnip3* 表現異常，顯示芬普尼誘發粒線體凋亡機制，最終導致胸腺細胞死亡。綜

doi:10.6342/NTU202501708

合我們的研究結果所示，芬普尼具有兩種機制，其一是干擾 GABA 相關基因包含合成與傳遞 GABA 的機制失調，進而誘發 Th1/Th2 細胞激素同時過度分泌，造成 T 細胞的過度活化；另外是抑制 IL-7 訊號及維持胸腺微環境所需之轉錄因子和誘發氧化性傷害以破壞胸腺 T 淋巴細胞發育，最終造成免疫細胞調控的失調。我們的研究成果突顯出暴露芬普尼對 T 細胞功能與發育的潛在毒性作用，全面地重新評估芬普尼對於青春期脊椎動物的安全性為重要的課題。

關鍵字

芬普尼、免疫毒性、抗原特異性免疫反應、GABAergic 基因、胸腺細胞發育、胸腺萎縮、介白素-7、細胞凋亡、Bcl-2 家族、氧化壓力、粒線體膜電位、穀胱甘肽、脂質過氧化

doi:10.6342/NTU202501708

Abstract

Fipronil (FPN) is a phenylpyrazole pesticide widely used in agriculture, household pest control, and veterinary medicine. It is traditionally considered to have low toxicity in vertebrates. However, growing evidence suggests that FPN exposure may lead to unexpected adverse effects on the liver, reproductive system, and nervous system. Despite these findings, its influence on immune function, particularly on T cell responses, remains poorly understood. In this study, I systematically investigated the immunotoxic effects of FPN on both mature and developing T cells using *in vivo* and *ex vivo* models. Oral administration of FPN in ovalbumin-sensitized mice enhanced antigen-specific immune responses, as indicated by increased splenocyte metabolic activity, elevated production of IL-2, IL-4, and IFN-γ, and higher serum levels of OVA-IgG_1 and OVA-IgG_{2a}. Gene expression analysis revealed that GABAergic signaling was altered, with a significant decrease in *Gad67* and an increase in GABA receptor subunits (*β2* and *δ*). These findings suggest that FPN may interfere with the inhibitory role of GABAergic pathways in T cell regulation, thereby enhancing antigen-specific immune activation.

Because T cell lineage commitment and thymopoiesis are fundamental to functional adaptive immunity, I further examined the impact of FPN on thymic development. FPN exposure induced marked thymic atrophy, reduced the proportion of double-positive thymocytes, and impaired T cell maturation. These effects were closely linked to the suppression of IL-7 and IL-7 receptor expression. As a key cytokine in early thymocyte development, IL-7 supports survival, proliferation, and differentiation beyond the double-negative stage. Downregulation of IL-7 axis-related genes and proteins, including FOXN1, LYL1, SCF, and c-KIT, further disrupted the thymic microenvironment required for progenitor cell expansion. In addition to impairing developmental signaling, FPN exposure triggered oxidative stress in thymocytes. Elevated reactive oxygen species, mitochondrial membrane depolarization, lipid peroxidation, calcium depletion, and glutathione reduction were observed. Moreover, an imbalance in BCL-2 family gene expression was detected, with

doi:10.6342/NTU202501708

downregulation of anti-apoptotic genes and dysregulation of pro-apoptotic regulators such as *Bim* and *Bnip3*. These alterations activated mitochondrial apoptosis and led to thymocyte death. Taken together, our results demonstrate that FPN compromises immune system integrity through a dual mechanism involving enhanced peripheral T cell activity and disrupted thymic development *via* GABAergic imbalance, IL-7 signaling suppression, transcriptional factors for supporting thymic microenvironment, and oxidative damage. Our findings highlight the potential risk to immune system integrity from contaminant exposure to FPN and the need for a more comprehensive reassessment of the safety of fipronil in pubertal vertebrates.

Keywords

fipronil, immunotoxicity, antigen-specific immune responses, GABAergic signaling, thymocyte development, thymus atrophy, interleukin-7, apoptosis, Bcl-2 family, oxidative stress, mitochondrial membrane potential, glutathione, lipid peroxidation

doi:10.6342/NTU202501708

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List of Abbreviations

Abbreviations	Full name
ADEs	Adverse drug events
BSA	Bovine Serum albumin
c-Kit	Tyrosine-protein kinase KIT
CNS	Central nervous system
ConA	Concanavalin A
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
DN	Double-negative
DP	Double-positive
ELISA	Enzyme-linked immunosorbent assay
ETP	Early thymic progenitors
FBS	Fetal Bovine Serum
FPN	Fipronil
Foxn1	Forkhead box protein N1
Foxo	Forkhead box-O
$GABA_A$	γ-aminobutyric acid
GABAR	GABA receptor
GABPα	GA-binding protein α
GAD	Glutamate decarboxylases
GAT	GABA transporter
GSH	Glutathione
H & E	Hematoxylin and eosin

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H_2O_2	Hydrogen peroxide
HSC	Hematopoietic stem cells
$^{\bullet}OH$	Hydroxyl radicals
IFN	Interferon
Ig	Immunoglobins
IHC	Immunohistochemistry
IL	Interleukin
Iono	Ionomycin
KLH	Keyhole Limpet Hemocyanin
LD_{50}	Lethal dose 50
LPO	Lipid peroxidation
Lyl1	Lymphoblastic leukemia 1
MCU	Mitochondrial calcium uniporter
MMP	Mitochondrial membrane potential
mRNA	Messenger ribonucleic acid
NA	Naïve
NAC	N-acetylcysteine
NO	Nitric oxide
NOAEL	No-observed-adverse-effect level
$O2^{\bullet-}$	Superoxide anions
OVA	Ovalbumin
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species

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RT	Room temperature
SCF	Stem cell factors
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sox13	SRY-box transcription factor 13
SP	Single-positive
T cell	T lymphocyte
TCR	T-cell receptor
TDAR	T-cell dependent antigen response
TEC	Thymic epithelial cells
Th cell	T helper cells
TSH	Thyroid-stimulating hormone
VH	Vehicle
WB	Western blotting
WHO	World Health Organization

doi:10.6342/NTU202501708

Chapter 1. Background and literature review

1.1 Introduction and applications of fipronil

1.1.1 Fipronil: chemical properties and broad applications

Fipronil (FPN; $C_{12}H_4Cl_2F_6N_4OS_3$; CAS number: 120068-37-3) is a second-generation broad-spectrum insecticide belonging to the phenylpyrazole chemical family, introduced by Rhône-Poulenc in 1993 (Tingle et al., 2003). Its chemical structure features a central pyrazole ring substituted with various functional groups, including chlorine, fluorine, nitrile, and thioether moieties, which contribute to its insecticidal activity and physicochemical properties. These properties include moderate water solubility, lipophilicity, and relative stability under normal environmental conditions, which contribute to its efficacy and persistence (PubChem, n.d.).

Due to its potent insecticidal activity against a wide range of pests, FPN has been extensively utilized in various sectors globally. In agriculture, it is employed to control soil-dwelling insects, foliar pests, and seed-treating applications for crops such as rice, corn, potatoes, and cotton (Simon-Delso et al., 2015; Tingle et al., 2003). Furthermore, its effectiveness against household pests like cockroaches, ants, and termites has led to its inclusion in household and professional pest control products, making it a valuable tool in integrated pest management strategies across different environments (Gondhalekar and Scharf, 2012; Ibrahim et al., 2003; Jiang et al., 2014; Zhao et al., 2003). The broad efficacy and relatively long residual activity of FPN contribute to its widespread use in these diverse settings.

However, despite its utility, FPN is classified as a Class II moderately hazardous pesticide by the World Health Organization (WHO), signifying potential risks to non-target organisms and various biological systems (Authority (EFSA), 2006). Growing concerns regarding its environmental impact and toxicity have been substantiated by findings of cumulative pollution and adverse effects within

doi:10.6342/NTU202501708

natural ecosystems. Notably, FPN has been implicated in detrimental effects on beneficial insects, including pollinators like bees and predatory insects such as dragonflies, raising ecological concerns about biodiversity and ecosystem services (Pisa et al., 2015).

The documented risks associated with FPN have prompted regulatory actions in several regions. In recognition of its potential environmental and ecological hazards, the European Union (EU) imposed a ban on its use in 2013 (Pisa et al., 2015). Furthermore, stringent prohibitions on the use of FPN pesticides in food-producing animals have been implemented in the United States, the EU, and other countries to mitigate potential residues in the food chain and protect human health ("FSS_Fipronil_QA," 2017; Stafford et al., 2018). These regulatory measures underscore the significant concerns surrounding the widespread use of FPN and highlight the necessity for a thorough understanding of its toxicological effects on various biological systems, including the immune system.

1.1.2 Fipronil metabolism and pharmacokinetics

FPN is readily absorbed *via* the gastrointestinal tract and undergoes hepatic biotransformation primarily through cytochrome P450-mediated oxidation. Its principal metabolite, fipronil sulfone, is significantly more persistent and toxic than the parent compound (Tingle et al., 2003). This metabolite undergoes substantial enterohepatic recirculation, thereby prolonging its systemic retention. In humans, the elimination half-life of FPN is approximately 7–8 hours, whereas fipronil sulfone has a markedly longer half-life of 7–8 days (Maddison et al., 2008).

Fipronil sulfone exhibits slow elimination and stable tissue accumulation following oral exposure. Morgane Cam et al. (2018) demonstrated that in mice administered 10 mg/kg FPN by oral gavage, plasma concentrations of fipronil sulfone remained detectable for up to 56 days, with an estimated half-life of approximately 26 days. Even under intermittent dosing regimens (5 days/week for 3 weeks), fipronil sulfone accumulated in plasma, brain, and epididymal adipose tissue, with

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concentrations reaching 3.7 μg/mL, 2.7 μg/brain, and 13.5 μg/adipose pad, respectively (Cam et al., 2018). These findings indicate that FPN metabolites can achieve a steady-state concentration with repeated exposure and persist in tissues long after dosing cessation, reinforcing the likelihood that fipronil sulfone may exert prolonged biological effects, including on immune function, even under non-daily exposure conditions.

Species-specific differences in FPN metabolism have also been observed. Compared to rodents, dogs and cats exhibit reduced hepatic metabolic capacity, rendering them more susceptible to toxic effects associated with unmetabolized FPN and its sulfone derivative. This interspecies variability highlights the need for caution in veterinary applications and toxicological risk assessments (Suzuki et al., 2021).

Environmental persistence of FPN and its metabolites further compounds its toxicological relevance. In aerobic soils, FPN degrades slowly, with reported half-lives of 122 to 128 days. In contrast, under photolytic conditions in surface waters, degradation is more rapid (half-life of 4–12 hours). However, metabolites such as fipronil sulfone remain environmentally persistent, with reported aquatic half-lives ranging from several months to over a year depending on temperature, pH, and light exposure (D. et al., 2009; Shi et al., 2021). Such environmental persistence, combined with high lipid solubility and bioaccumulation potential, underscores the need for careful evaluation of chronic exposure risks in both target and non-target species.

1.1.3 Sustained bioaccumulation of fipronil metabolites under intermittent exposure regimens

To accurately reflect realistic environmental exposure scenarios and account for the metabolic accumulation of FPN metabolites, our Ovalbumin (OVA)-specific immune model experiment employed a discontinuous dosing strategy, informed by both toxicokinetic evidence and established toxicological testing guidelines. Specifically, mice were orally administered FPN five consecutive

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days per week, followed by two days of rest, for a total of eleven doses. This regimen aimed to mimic intermittent, real-world human exposure, which, while not daily, can still lead to cumulative toxicity over time.

This dosing approach is further supported by the European Medicines Agency's guideline on repeated dose toxicity testing (CPMP/SWP/1042/99), which recommends adjusting dosing frequency based on a compound's pharmacokinetic and pharmacodynamic properties (European Medicines Agency, 2010). Although FPN undergoes rapid metabolism following oral administration, its major bioactive metabolite, fipronil sulfone, exhibits a significantly prolonged biological half-life. As reported by Cam et al. (2018), the half-life of fipronil sulfone in mice can extend up to 26 days, strongly suggesting its potential for bioaccumulation even under intermittent exposure conditions.

Cam et al. (2018) further elucidated this accumulative nature in their time-course study. Following a single oral administration of FPN (10 mg/kg), fipronil sulfone levels in plasma and adipose tissue peaked within 1–2 days and gradually declined over a 56-day period. In contrast, repeated oral administration (10 mg/kg, 5 days/week for 3 weeks) led to a stable and sustained increase in fipronil sulfone concentrations in plasma and various tissues, with plasma levels maintaining a consistent elevation over 21 days (Cam et al., 2018). These findings unequivocally highlight the bioaccumulative properties of fipronil sulfone even with intermittent dosing, directly supporting our hypothesis that FPN metabolites can persist long enough to significantly influence immune function.

Beyond these pharmacokinetic considerations, our discontinuous dosing regimen also aligns with established practices in toxicology research. For example, Chapter 42 of Veterinary Toxicology (3rd Edition) documents a dermal toxicity study in which rabbits were exposed to FPN five days per week for three weeks. In that study, a no-observed-adverse-effect level (NOAEL) of 5 mg/kg was derived based on decreased food intake and weight gain at higher doses, a finding consistent with the

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dose range utilized in our current investigation (*Veterinary Toxicology*, 2018).

Furthermore, the intermittent 5-day-per-week design has been validated in other toxicology studies, such as the subchronic MX exposure study in Wistar rats (Vaittinen et al., 1995). There, significant toxicological endpoints were observed after just two weeks of exposure using a similar schedule, reinforcing the biological relevance and practical feasibility of this administration pattern for investigating cumulative effects.

In summary, our discontinuous FPN dosing strategy represents a scientifically justified and environmentally relevant experimental model. Despite incorporating intermittent rest periods, the significant bioaccumulation profile of fipronil sulfone remains sufficient to exert measurable immunotoxic effects, including those on T-cell-dependent antibody production. This design not only leverages critical toxicokinetic data but also closely mimics real-world human exposure scenarios, ensuring the translational relevance of our findings.

1.1.4 Regulatory toxicological endpoints and human risk assessment of fipronil

Regulatory toxicological benchmarks are critical in assessing human health risks associated with pesticide exposure. For FPN, various exposure scenarios have been extensively evaluated to establish reliable toxicological endpoints, including NOAEL, lowest observed adverse effect levels (LOAEL), and reference doses (RfD).

For acute dietary exposure, particularly for sensitive populations such as infants and children, the acute reference dose (RfD) of FPN has been set at 0.025 mg/kg/day, derived from a NOAEL of 2.5 mg/kg/day, based on acute neurotoxic effects observed in rodents, specifically decreased hind leg coordination at a LOAEL of 7.0 mg/kg. Chronic dietary exposure standards are even more stringent, with a chronic RfD of 0.0002 mg/kg/day, reflecting chronic and carcinogenic endpoints identified at a LOAEL of 0.059 mg/kg/day, associated with seizures, mortality, thyroid dysfunction, and

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biochemical alterations in rodents (PubChem, n.d.).

Residential exposure through oral, dermal, and inhalational routes has also been assessed. Short-term (1–7 days) oral exposure has established a maternal LOAEL of less than 0.1 mg/kg/day due to developmental toxicity in rabbits, characterized by decreased maternal body weight and reduced food consumption. Dermal exposures have indicated a NOAEL at 5 mg/kg/day based on rabbit studies showing systemic effects such as decreased body weight gain and food intake at a LOAEL of 10 mg/kg/day. Additionally, inhalational assessments have defined a NOAEL of 0.05 mg/kg/day, considering developmental neurotoxicity effects observed at a LOAEL of 0.90 mg/kg/day, highlighting the sensitivity of developing organisms to FPN exposure (Environmental Protection Agency, 2007).

Furthermore, FPN is classified as a Group C (possible human) carcinogen, based on increased incidences of thyroid follicular cell tumors observed in animal studies following long-term exposure. This classification underscores the need for careful long-term monitoring and risk management practices to protect public health (Jackson et al., 2009).

Human biomonitoring studies have further reinforced the potential for significant human exposure to FPN and its metabolites, particularly via dietary intake and indoor dust exposure, suggesting the importance of comprehensive risk assessments encompassing all potential exposure pathways. Given the persistent and bioaccumulative properties of FPN and its metabolites, ongoing research and stringent regulatory oversight remain essential to minimize human health risks, especially in vulnerable populations such as infants, pregnant women, and occupationally exposed individuals.

In addition to toxicological evaluations, regulatory agencies have established specific pesticide tolerances to manage human exposure to FPN residues in food. For instance, the U.S. Environmental Protection Agency (EPA) has set maximum permissible levels for combined residues

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of FPN and its metabolites in various food commodities. In 2007, the EPA established tolerances for FPN residues in potatoes and wheat, as well as time-limited tolerances for turnip and rutabaga, in response to emergency exemptions under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). These tolerances are designed to ensure that the levels of pesticide residues in food remain within safe limits for human consumption (Environmental Protection Agency, 2007).

The establishment of these tolerances involves comprehensive risk assessments that consider various factors, including the toxicity of the pesticide, the amount and frequency of pesticide application, and the potential for residue accumulation in food. By setting these tolerances, regulatory agencies aim to protect public health by ensuring that exposure to pesticide residues through food consumption does not pose significant health risks.

1.1.5 Veterinary clinical applications of fipronil

In veterinary practice, FPN is primarily used for the topical treatment of external parasite infestations such as fleas, ticks, and chewing lice on companion animals, including dogs and cats. It is typically administered as a spot-on formulation or spray, distributing rapidly into the sebaceous glands and hair follicles, from which it is gradually released onto the coat over several weeks (Richardson and Little, 2012). Its high lipophilicity enables this rapid partitioning and sustained release, providing residual flea and tick control for up to four weeks following a single application. FPN-based products are available in various formulations, including spot-on treatments, sprays, and impregnated collars, to accommodate different durations of efficacy, application methods, and animal sizes (“A review of Animal Safety Studies for Fipronil in the dog and cat,” n.d.).

FPN exerts its ectoparasiticidal activity by non-competitively blocking GABA-gated chloride channels in arthropods, leading to neuronal hyperexcitation and rapid parasite death (Simon-Delso et al., 2015). It exhibits a markedly higher affinity for insect GABA receptors than for mammalian homologues, conferring a wide safety margin when used according to label instructions in companion

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animals (Bhatt et al., 2023). In addition, its compatibility with oil-based vehicles and stability in sebaceous secretions ensure consistent drug levels at the skin surface and along hair shafts, making it well suited for once-monthly topical administration in both cats and dogs (Gupta and Doss, 2024).

When applied as directed, FPN is generally considered safe for dogs and cats, with minimal systemic absorption and limited off-target distribution. However, despite an extensive safety profile, adverse drug events (ADEs) associated with FPN-containing products have been reported in target and non-target animal species, as well as in humans exposed during application or handling of treated animals. Monitoring and reporting of these ADEs remain important to ensure ongoing safe use in veterinary medicine.

ADEs following the use of FPN-containing products have been reported in both cats and dogs, with dermatological reactions being the most frequent observation in both species. In addition, a number of reports of ADEs following off-label use, particularly in rabbits and guinea pigs, have been noted (Webster, 1999).

In cats, the most commonly reported ADE involved alopecia, often accompanied by pruritus and erythema, primarily at or around the application site. These signs are indicative of local irritation or contact-type dermatitis. Other reported ADEs in cats included neurological signs such as inappetence, lethargy, and salivation, as well as gastrointestinal signs, primarily vomiting. Distinguishing between behavioral responses to intense local skin reactions and primary neurological effects was challenging in some cases, particularly reports of distress and intense pruritus (Pesticides and Authority, 2011, n.d.).

Dogs exhibited a similar pattern of frequent dermatological reactions to FPN products, with pruritus and erythema at the application site being common. However, reports of alopecia alone were less frequent in dogs, and more than half of the skin reactions were categorized as severe, including acute moist dermatitis, possibly secondary to self-trauma. It has been suggested that self-trauma or

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primary skin reactions may compromise the stratum corneum, potentially increasing systemic FPN absorption (Roberts et al., 2002). The rapid onset of many skin reactions following application strongly implicates FPN as a direct cause of skin irritation, further supported by reports of dogs avoiding subsequent applications. Neurological signs in dogs included ataxia and lethargy, similar to cats, but also included instances of biting or aggression. Gastrointestinal signs reported in dogs consisted of vomiting and diarrhea. The possibility of gastrointestinal issues arising from ingestion of the product concentrate has been raised, although the precise etiology, whether direct irritation or a systemic reaction to FPN, remains unclear (Pesticides and Authority, 2011, n.d.).

Off-label use of FPN was associated with severe neurological ADEs and mortality, particularly in rabbits and guinea pigs. In rabbits, 32 deaths were reported following application of FPN concentrate or spray, with 13 animals recovering. All affected rabbits displayed severe lethargy, depression, and inappetence. One report also documented ADEs in nine guinea pigs, with six deaths occurring after the onset of neurological signs. The limited published information on off-label use, with only one report found in rabbits (Webster, 1999), and frequently cited, highlights the need for further investigation into the safety of FPN in non-approved species.

1.1.6 Global usage and potential exposure pathways of fipronil

FPN's extensive application in agriculture and veterinary medicine worldwide leads to significant environmental release, contaminating various environmental matrices and the food chain. Studies have detected FPN residues in a substantial proportion of households, highlighting the potential for widespread human exposure (Bonneau et al., 2015; Chen et al., 2022; Cochran et al., 2015; Dumont et al., 2015). Notably, approximately 40% of FPN residues found in American households have been linked to contact with pets treated with FPN-containing products (Jennings et al., 2002; Lee et al., 2010). The U.S. EPA has intensified its scrutiny of these spot-on insecticides due to a surge in adverse outcome reports in treated pets, underscoring the potential for direct exposure

doi:10.6342/NTU202501708

and harm.

FPN exposure in humans can be categorized as occupational, unintentional, or, rarely, intentional. Occupational exposure occurs among pesticide handlers (Herin et al., 2011). Unintentional exposure results from contact with treated environments or pets, with FPN and its metabolite found in a significant percentage of sampled serum (Chen et al., 2022; Jennings et al., 2002; McMahen et al., 2015). Self-poisoning represents the rare intentional route (Leghait et al., 2009). Health effects resulting from short-term FPN exposure vary depending on the route of exposure. Direct, short-term contact with the skin can lead to mild skin irritation. Ingestion of FPN has been associated with reported effects including sweating, nausea, vomiting, headache, abdominal pain, dizziness, weakness, and seizures (Mohamed et al., 2004). Generally, the signs and symptoms of short-term FPN exposure resolve without medical intervention. Alarmingly, FPN contamination has also been reported in food products, such as the widespread egg contamination incident across 45 countries in 2017 due to illegal use in food-producing animals, clearly demonstrating the risk of dietary exposure (Chen et al., 2022; Cordeiro et al., 2019; Guo et al., 2018; Munoz-Pineiro and Robouch, 2018). It is noteworthy that while FPN is authorized for pest control on pets in many regions, its use is strictly prohibited in food-producing animals in the EU and other countries to prevent food contamination.

Furthermore, FPN can be rapidly absorbed through the gastrointestinal tract ("Fipronil Risk Characterization Document," n.d.; World Health Organization, 2022), and its primary metabolite, fipronil sulfone, exhibits even greater toxicity and can persist in the body for an extended period, up to 7 days in cases of acute self-poisoning in humans (Mohamed et al., 2004). Worryingly, Fipronil sulfone has been detected in the serum of newborns, indicating placental transfer from exposed mothers during pregnancy. This prenatal exposure raises concerns about potential adverse effects on fetal development, including thyroid function and Apgar scores (Kim et al., 2019).

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The increasing prevalence of FPN in the environment and the multiple pathways of human exposure, coupled with reports of acute illnesses and potential long-term health consequences, underscore the significant public health concerns associated with its widespread use. The potential for toxic effects on non-target species and various organ systems in vertebrates, as previously discussed, further necessitates comprehensive mechanistic toxicity studies to elucidate the hazards posed by FPN exposure fully.

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1.2 Toxicological mechanisms of fipronil

1.2.1 Insecticide modes of action: selective antagonism of insect GABA-gated chloride channels

FPN's primary mode of action is the non-competitive antagonism of γ-aminobutyric acid type A ($GABA_A$)-gated chloride channels in the insect central nervous system. Normally, $GABA_A$ receptor activation facilitates the influx of chloride ions, resulting in neuronal hyperpolarization and inhibition of excitatory signaling. FPN disrupts this inhibitory pathway by binding to an allosteric site on the GABA receptor complex, thereby preventing the opening of the chloride channel (Bloomquist, 2001). This inhibition leads to sustained neuronal excitation, manifesting as tremors, convulsions, paralysis, and ultimately death in target insects (Zhao et al., 2004, 2003).

Although GABA-gated chloride channels are also essential in vertebrate neurophysiology, FPN demonstrates a significantly higher affinity for insect isoforms of the receptor, likely due to differences in subunit composition and receptor pharmacodynamics, particularly the presence of the RDL (resistance to dieldrin) subunit in insects, which confers heightened sensitivity to noncompetitive antagonists like FPN (Badgujar et al., 2015; Li et al., 2021; Stehr et al., 2006). This disparity of selectivity is further amplified in radioligand binding assays using 4'-ethynyl-4-n-[2, 3-3H2]propylbicycloorthobenzoate ([^{3}H]EBOB), where FPN and its metabolites display the half maximal inhibitory concentration (IC_{50}) values of 3–12 nM in insects versus 110–1103 nM in vertebrates, resulting in a selectivity index exceeding 500-fold (Hainzl et al., 1998).

Interestingly, human recombinant β3 homooligomeric $GABA_A$ receptors bind FPN with similar affinity to insect receptors, suggesting that the insecticide binding site is conserved. However, such β3 homooligomers are not typically expressed in native mammalian systems. The inclusion of α and γ subunits in heterooligomeric assemblies, common in vertebrate neurons, modulates insecticide sensitivity, often reducing binding affinity. Therefore, receptor subunit composition plays

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a pivotal role in determining FPN susceptibility across species and likely explains its preferential toxicity in insects (Ratra and Casida, 2001). Notably, FPN also targets glutamate-gated chloride channels, which are absent in vertebrates, further enhancing its insect-specific toxicity (Narahashi et al., 2010, 2007; Simon-Delso et al., 2015).

Beyond $GABA_A$ and GluCl receptors, FPN's potential vertebrate toxicity may also involve glycine receptors, another major class of ligand-gated chloride channels. These receptors mediate fast inhibitory neurotransmission in the spinal cord and brainstem and are among the most widely distributed inhibitory receptors in the vertebrate central nervous system (Lynch, 2004). Comparative binding studies have shown that the selectivity of FPN for cockroach GABA receptors is approximately 59 times greater than for rat receptors (Zhao et al., 2005). This pharmacological selectivity is reflected in acute toxicity profiles, with the median lethal dose (LD_{50}) in rats reported at 91 mg/kg, compared to values as low as 0.07 mg/kg in corn rootworms and 0.13 mg/kg in houseflies, indicating a 700- to 1300-fold greater sensitivity in insects (Hainzl and Casida, 1996; Zhao et al., 2005, 2003).

The unique mechanism of FPN, which differentiates it from other insecticide classes such as acetylcholinesterase inhibitors (e.g., organophosphates, carbamates), sodium channel modulators (e.g., pyrethroids), and nicotinic acetylcholine receptor disruptors (e.g., neonicotinoids), underscores its value in pest management. Its selective toxicity, attributed to the structural and functional divergence between insect and mammalian GABA receptor subtypes, has been a cornerstone in its development and application as a safer alternative for vertebrate exposure under controlled usage scenarios.

1.2.2 Acute toxicity of FPN in mammals

FPN exhibits species- and exposure route-dependent variations in acute toxicity among mammals. Technical-grade FPN demonstrates moderate oral toxicity, with LD_{50} of approximately

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91–95 mg/kg in mice and 97 mg/kg in rats (Gupta and Doss, 2024). The photodegradation metabolite, fipronil-desulfinyl, presents even higher oral toxicity in rats, with LD_{50} values of 15 mg/kg in females and 18 mg/kg in males. In rodents, acute neurotoxic symptoms such as decreased hindlimb coordination, reduced food intake, and body weight loss have been observed at doses as low as 7.5 mg/kg (LOAEL), whereas the NOAEL has been established at 2.5 mg/kg (Jackson et al., 2009).

Dogs display comparatively lower oral sensitivity ($LD_{50} \approx 640$ mg/kg); however, chronic exposure to significantly lower doses (0.2–0.3 mg/kg/day) can result in pronounced neurotoxic effects, including hyperactivity and convulsions. While specific oral LD_{50} values for cats remain limited, clinical reports indicate feline sensitivity comparable to or possibly greater than canine susceptibility, similarly characterized by neurological manifestations such as tremors or convulsions (Jackson et al., 2009).

Dermal exposure generally results in lower systemic toxicity, with rat dermal LD_{50} values exceeding 2000 mg/kg due to limited absorption through intact skin. In contrast, rabbits exhibit greater susceptibility, with a dermal LD_{50} of approximately 354 mg/kg (Gupta and Doss, 2024). Repeated dermal exposure studies (0.5–10 mg/kg/day for 21 days) in rabbits demonstrated systemic effects, including decreased body weight gain and reduced food consumption, establishing a systemic NOAEL at 5 mg/kg/day. While FPN can cause mild, transient skin and eye irritation, it shows no significant skin sensitization potential.

Inhalation exposure demonstrates moderate toxicity, with rat LC_{50} values ranging from 0.390 to 0.682 mg/L following a 4-hour exposure period, underscoring the potential for significant respiratory absorption and acute neurotoxic effects. Human toxicity data, primarily derived from accidental or occupational exposures, indicate that acute oral poisoning results in gastrointestinal disturbances, convulsions, and central nervous system depression (Jackson et al., 2009). Although explicit human LD_{50} values remain unavailable, clinical manifestations align closely with animal

doi:10.6342/NTU202501708

neurotoxicity profiles indicative of GABA receptor antagonism.

Collectively, these acute toxicity profiles highlight notable route-dependent and species-specific susceptibilities to FPN. The differential metabolic capacities between species, particularly the reduced metabolism in companion animals, emphasize the importance of cautious interpretation in toxicological risk assessments and veterinary applications.

1.2.2 Non-target toxicity of fipronil in vertebrates

Although developed for its selective toxicity against insect pests by targeting insect-specific $GABA_A$-gated chloride channels, FPN poses significant concerns regarding its adverse effects on non-target organisms. While acute neurotoxicity in mammals is generally lower than in insects due to the relatively higher affinity of FPN for insect GABA receptors, both FPN and its metabolites, particularly fipronil sulfone, can interfere with mammalian GABA receptors and accumulate in various tissues. This interference leads to a range of toxicities in non-target organs, including liver and kidney damage, thyroid dysfunction, and reproductive toxicity, observed in various non-target species, including mammals, birds, amphibians, and aquatic species.

Neurotoxicity

The potential for FPN to induce neurotoxicity in both target and non-target species has been documented in several studies. In rats, acute neurotoxicity studies involving single oral administration of FPN reported varying NOAELs depending on the dose and duration of observation. A study with single oral doses up to 25 mg/kg bodyweight established a NOAEL of 2.5 mg/kg, with clinical signs resolving within 14 days. However, a subsequent study using single oral doses up to 50 mg/kg bodyweight reported a lower NOAEL of 0.5 mg/kg, based on histopathological findings and clinical signs observed up to 16 days post-treatment. Longer-term exposure in rats (oral administration up to 11 mg/kg bodyweight for 13 weeks) yielded an even lower NOAEL of 0.3 mg/kg for neurotoxicity.

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In a limited study in dogs, oral administration of 20 mg/kg/day induced neurotoxicity within 5 to 13 days of treatment initiation, accompanied by a decrease in food consumption from day 1 to 2. These signs resolved within 12 days following cessation of treatment, leading the authors to conclude that the clinical manifestations resulted from systemic pharmacological modulation as FPN was eliminated ("A review of Animal Safety Studies for Fipronil in the dog and cat," n.d.).

While acute FPN exposure primarily results in transient neurological effects, accumulating evidence suggests that long-term and repeated exposure may contribute to persistent neurological disorders, such as Alzheimer's and Parkinson's diseases. *In vitro* studies using SH-SY5Y neuroblastoma cells have demonstrated that even non-cytotoxic concentrations of FPN can induce neurite outgrowth shortening and apoptosis involving caspase-6, an effector implicated in neurodegenerative diseases (Wang et al., 2015). Furthermore, sub-apoptotic concentrations of FPN have been shown to cause mitochondrial dysfunction and autophagic vacuole formation, accompanied by a significant upregulation and altered isoform expression of vimentin, a protein potentially involved in cellular repair mechanisms under FPN-induced stress (Ruangjaroon et al., 2017). Notably, *in vitro* research has also indicated that FPN, along with other pyrazole insecticides, can stimulate the production of the neurotoxic amyloid β 1-42 peptide, suggesting a potential pathway linking FPN exposure to the development of Alzheimer's disease (Cam et al., 2018). *In vivo* studies corroborate these findings, with long-term, low-dose FPN treatment in mice leading to behavioral perturbations, specifically hyperlocomotion observed after 43 weeks of exposure, consistent with the compound's known effects on the GABAergic system and indicating an accumulative neurotoxic effect of sustained exposure (Koslowski et al., 2020).

Hepatotoxicity

Fipronil exhibits significant hepatotoxic potential, as evidenced by a range of biochemical, cellular, and histological alterations in the liver. In rat liver microsomes, FPN dose-dependently

doi:10.6342/NTU202501708

enhances the activity of several cytochrome P450 (CYP) enzyme subfamilies (CYP2E, CYP1A, CYP2A, CYP2B, and CYP3A), which may contribute to its metabolism and potentially the formation of more toxic metabolites (Caballero et al., 2015). At the cellular level, FPN induces marked changes in hepatocytes, including autophagic processes, steatosis, and necrotic cell death, along with Kupffer-cell proliferation, hepatocyte hypertrophy, and disrupted intracellular homeostasis (De Oliveira et al., 2012). Mechanistically, FPN impairs mitochondrial function, reducing respiration, dissipating membrane potential, and disrupting ATP and calcium levels, with increased toxicity observed in hepatocytes from normal rats, suggesting bioactivation enhances its toxic potential (Guelfi et al., 2015). These cellular changes are reflected in increased serum levels of liver enzymes such as AST, ALT, and ALP, indicative of liver damage (Kartheek and David, 2018). Furthermore, FPN exposure leads to oxidative stress, as shown by altered activities of antioxidant enzymes (catalase, glutathione S-transferase, superoxide dismutase, and glutathione peroxidase) and elevated malondialdehyde levels, and induces structural irregularities in the liver, including impaired portal vein structure and hepatocyte hypertrophy. These findings collectively demonstrate that FPN exerts its hepatotoxicity through multiple pathways, encompassing altered enzyme activity, cellular damage, mitochondrial dysfunction, and oxidative stress.

Nephrotoxicity

Fipronil exhibits significant nephrotoxic potential, inducing a range of functional and structural alterations in the kidneys. A hallmark of FPN-induced nephrotoxicity is the dose-dependent elevation of serum creatinine and blood urea nitrogen (BUN) levels, indicative of impaired renal function (Badgujar et al., 2015; Mossa et al., 2015). Histopathological examination reveals a spectrum of damage, varying with the dose of FPN. At lower doses, mild congestion, tubular cast formation, and mild vacuolation of tubular epithelial cells may be observed. However, higher doses induce severe lesions, including tubular necrosis, glomerular shrinkage and atrophy, interstitial fibrosis, dilatation of collecting tubules, vacuolation, focal hemorrhage, and inflammatory cell infiltration.

doi:10.6342/NTU202501708

Ultrastructural changes encompass reduction in glomerular size, widening of Bowman's spaces, dilated blood vessels, and pyknotic nuclei in tubular epithelium (Mossa et al., 2015; Sakr et al., 2022). The increased levels of kidney injury molecule-1 (KIM-1) further corroborate the damage to the kidney tissue (Sakr et al., 2022). Mechanistically, FPN disrupts normal kidney function, leading to increased levels of total protein and uric acid in serum. Notably, studies have demonstrated the potential of antioxidants like vitamins E and C, as well as betaine, to mitigate FPN-induced nephrotoxicity, reducing the severity of both biochemical and histological changes, suggesting that oxidative stress and inflammation may play a role in the pathogenesis of this toxicity.

Endocrine disruption

In addition to its potent neurotoxic effects, FPN has emerged as a potential endocrine-disrupting chemical (EDC), raising concerns about its unintended effects on non-target organisms ("A review of Animal Safety Studies for Fipronil in the dog and cat," n.d.). Evidence from rodent studies suggests that FPN may interfere with reproductive endocrinology. For instance, topical administration of high doses of FPN (280 mg/kg) significantly reduced pregnancy rates in rats, while hormonal alterations were observed at lower exposures (70 mg/kg), indicating its dose-dependent interference with endocrine function (Ohi et al., 2004). Further *in vitro* analyses using estrogen receptor alpha (ERα)-positive MCF-7 breast cancer cells demonstrated that FPN downregulates ERα and its downstream gene CDC2, possibly by suppressing the upstream regulator PES1, suggesting anti-estrogenic properties (Okazaki et al., 2016). Consistent with these findings, reporter gene assays in CHO-K1 cells confirmed that both FPN and fipronil sulfone exert antagonistic effects on ERα without displaying agonistic activity. Fipronil sulfone also exhibited anti-thyroid hormone activity *via* the thyroid hormone receptor (TR), as supported by molecular docking studies indicating favorable binding to TR (Lu et al., 2015).

Importantly, thyroid-disrupting potential has been observed not only in experimental animal

doi:10.6342/NTU202501708

models but also in human-relevant systems. A recent study using the Nthy-ori 3-1 human thyroid follicular cell line showed that environmentally relevant concentrations of FPN metabolites (FPM), including fipronil sulfone, fipronil sulfide, and fipronil desulfinyl, modulate key regulators of thyroid hormone synthesis and metabolism. FPM exposure led to oxidative stress-mediated NRF2 activation and altered the expression of thyroid-specific proteins such as TPO, DIO I/II, and NIS, alongside increased tetraiodothyronine (T4) secretion, implying a disruption of thyroid homeostasis (Zhuang et al., 2023).

Reproductive Toxicity

Although the effects of FPN on mammalian fertility are not yet fully elucidated, accumulating evidence indicates that it may exert detrimental effects on sperm physiology, which in turn may compromise fertilization capacity and early embryonic development.

Recent *in vitro* studies using mouse spermatozoa have shown that exposure to FPN at concentrations ranging from 0.1 to 300 μM significantly reduces sperm motility, motion kinematic parameters, and intracellular ATP levels in a dose-dependent manner. Concurrently, FPN treatment was found to enhance the acrosome reaction, a critical event required for oocyte penetration, suggesting a premature activation that could lead to failed fertilization. At the molecular level, the expression of sperm function-related proteins was altered, including decreased phosphorylation of protein kinase A (phospho-PKA) and tyrosine substrates, both of which are essential for capacitation and flagellar activity. These changes indicate that FPN interferes with signal transduction pathways crucial for sperm maturation and function (Bae and Kwon, 2024, 2020). Moreover, although the total levels of $GABA_A$ receptor β-3 remained unchanged following FPN exposure, the phosphorylated form (pS408/pS409) was significantly reduced at higher doses. Given the established role of GABA and its receptors in modulating sperm capacitation, motility, and acrosome reaction via chloride ion channels, such alterations may contribute to functional deficits observed in spermatozoa (Bae and

doi:10.6342/NTU202501708

Kwon, 2020).

Importantly, these disruptions in sperm physiology translated into adverse reproductive outcomes. Embryological assessments revealed significantly reduced cleavage and blastocyst formation rates following fertilization with FPN-exposed sperm, highlighting the compound's negative impact on fertilization competence and early embryonic viability (Bae and Kwon, 2024). Taken together, these findings support the notion that FPN can directly and indirectly impair sperm function, likely through mitochondrial dysfunction, aberrant protein phosphorylation, and premature acrosome reaction.

1.2.3 Fipronil-induced oxidative damage in mammals

Notably, one of the principal mechanisms underlying FPN off-target toxicity is the induction of oxidative stress, which has been implicated in a wide spectrum of tissue and organ damage (Awad et al., 2021; Bano and Mohanty, 2020a; Khan et al., 2015; Mossa et al., 2015; Vidau et al., 2011; Wang et al., 2016). FPN perturbs the endogenous antioxidant defense network by promoting the accumulation of reactive oxygen species (ROS) and concurrently depleting intracellular antioxidant reserves such as SOD, CAT, GST, and GSH (Badgujar et al., 2016, 2015). This oxidative imbalance has been linked to vascular disturbances, including congestion, ischemia, and hypoxia, ultimately triggering lipid peroxidation and genotoxic effects (Kartheek and David, 2018). Sustained redox disruption also compromises mitochondrial integrity, resulting in membrane potential loss, inhibition of complex I-linked respiration, suppression of ATP synthesis, and activation of apoptotic cascades (Cenini et al., 2019; Saleh et al., 2020).

In neuronal cells, FPN-induced ROS production has been implicated in the release of pro-inflammatory mediators and apoptosis-related signals, thereby contributing to neuronal degeneration (Koslowski et al., 2020). Key indicators of FPN-induced neurotoxicity include increased expression of cleaved caspase-3, upregulation of glial fibrillary acidic protein (GFAP), and overexpression of

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inducible nitric oxide synthase (iNOS), which collectively signify ongoing neuroinflammation and apoptotic cell death (Awad et al., 2022; Khalaf et al., 2019; Romero et al., 2016).

Oxidative stress elicited by FPN exposure also affects multiple organ systems. *In vivo* studies demonstrated that subacute oral administration of FPN (10 mg/kg for 28 days) results in significant downregulation of antioxidant gene expression and enzymatic activity in the liver, brain, and kidneys, reinforcing the systemic nature of oxidative damage. These effects are accompanied by increased lipid peroxidation, which has been shown to be attenuated by antioxidant supplementation such as vitamin E and vitamin C, suggesting that lipid peroxidation is a critical downstream mechanism in FPN-induced oxidative injury (Badgujar et al., 2016, 2015).

Consistent with these findings, in Wistar rats treated with FPN at 5 and 10 mg/kg body weight, significant elevations in sperm ROS levels and malondialdehyde (MDA) concentrations were observed (Khan et al., 2015). Similar increases in lipid peroxidation have been reported in liver, kidney, and brain tissues of FPN-exposed mice (Badgujar et al., 2016, 2015), as well as in erythrocytes of FPN-treated calves and in multiple tissues of fish exposed to sublethal field-relevant doses of FPN (Clasen et al., 2012; Gill and Dumka, 2016).

In vitro studies further confirm that FPN induces ROS in a concentration-dependent manner across various cell models. SH-SY5Y cells exhibited significant ROS accumulation after 6 h exposure to FPN at doses ranging from 25 to 100 μM (Ki et al., 2012; S.-J. Lee et al., 2011; Park et al., 2016; Zhang et al., 2015). Similar dose-responsive increases in ROS were observed in Drosophila S2 cells and Sf9 insect cells (Wang et al., 2013; Zhang et al., 2015). Mechanistic studies using isolated rat liver mitochondria suggest that FPN can directly increase mitochondrial $O_2^{\bullet -}$ production and oxygen consumption (Vidau et al., 2011).

In addition to its cytotoxic effects, the oxidative stress generated by FPN may interfere with immune cell function. ROS are known to play a vital role in T lymphocyte activation, clonal

doi:10.6342/NTU202501708

expansion, and effector differentiation (Pearce and Pearce, 2013; Sena et al., 2013; Wang and Green, 2012), and proper ROS regulation is essential for normal thymocyte development (Peng et al., n.d.). Therefore, this study focuses on evaluating whether FPN exposure disrupts redox homeostasis in developing thymocytes and elucidating the mechanisms by which oxidative stress contributes to immune dysfunction.

1.2.4 N-acetylcysteine (NAC): biochemical properties and rationale for use

N-acetylcysteine (NAC) is an acetylated derivative of the amino acid cysteine that functions both as a direct scavenger of ROS and as an efficient intracellular donor of cysteine, the rate-limiting substrate for glutathione (GSH) biosynthesis. In cell culture systems, NAC at low concentrations (approximately 0.1 to 2.5 mM) has been shown to effectively restore intracellular GSH levels, reduce lipid peroxidation, preserve mitochondrial membrane potential, and suppress redox-sensitive signaling pathways such as NF-κB in lymphoid and other primary cell types (Puerto et al., 2002; Ezeriņa et al., 2018; Zhitkovich, 2019). NAC (1mM) protects THP-1-derived macrophages from oxidative stress during efferocytosis and enhances leukocyte viability and proliferation in culture, and is reported as the most efficacious dose for stimulating leukocyte proliferation without cytotoxicity (De la Fuente et al., 2011; Tsai et al., 2022).

Compared to other antioxidants, NAC has several unique biochemical features. Unlike vitamin C (ascorbate), which mainly works through electron donation in watery environments (Padayatty et al., 2003), or vitamin E (α-tocopherol), which protects against lipid peroxidation within membranes (Brigelius-Flohé and Traber, 1999), NAC provides a dual action: directly neutralizing ROS through its thiol group and regenerating GSH by supplying intracellular cysteine (Zafarullah et al., 2003). While α-lipoic acid is also a thiol-containing antioxidant that can undergo redox cycling, it needs enzymatic reduction to become active as dihydrolipoic acid, which can delay its effects (Packer et al., 1995). Additionally, because exogenous GSH is poorly taken up by most cells due to

doi:10.6342/NTU202501708

limited membrane permeability, NAC is often a more practical and efficient agent for replenishing GSH in many *in vitro* systems.

doi:10.6342/NTU202501708

1.3 Immunotoxicity of fipronil and T cell development

The potential for FPN to exert toxic effects beyond the nervous system has raised significant concerns about its impact on the immune system. The neuroendocrine and immune systems are now understood to have a complex bidirectional communication (Ansar Ahmed, 2000), highlighting the possibility of FPN affecting immune responses through direct or indirect pathways. Immunotoxicity, the adverse effects of chemical agents on the immune system, can lead to a spectrum of outcomes. These outcomes include immunosuppression, increasing susceptibility to infections and cancer, as well as immune dysregulation, potentially contributing to allergic and inflammatory disorders.

Unfortunately, despite the well-established neurotoxicological profile of FPN, fewer studies have specifically focused on investigating its immunotoxic effects. However, existing research provides evidence of FPN's ability to interfere with immune function. For instance, oral administration of a sub-lethal dose (10% LD_{50}) of FPN to rats over 30 days resulted in histopathological alterations in key lymphoid organs, namely the spleen and thymus (Aldayel et al., 2021). Furthermore, this sub-chronic exposure was associated with slightly elevated serum levels of IL-4, IL-12, and IgE, suggesting that prolonged FPN exposure could potentially enhance allergic and inflammatory responses (Aldayel et al., 2021). In another study, exposure of young mice to a low dose of FPN (0.5% LD_{50}) from 4 to 13 weeks of age did not alter spleen weight, but it did slightly decrease the mitogenic proliferation of splenocytes stimulated with ConA or LPS (Bano and Mohanty, 2020b). Notably, *in vitro* studies using human lymphocytic Jurkat cells demonstrated that FPN can directly inhibit the production of key T cell cytokines, IL-2 and IFN-γ, even at non-cytotoxic concentrations (Sidiropoulou et al., 2010), indicating a direct impact on T cell function. While these data collectively suggest adverse effects of FPN on the immune system, our understanding of its immunomodulatory effects on the T helper 1 (Th1) and T helper 2 (Th2) immune balance *in vivo* remains limited. Moreover, the underlying mechanisms of FPN's immunotoxicity on T cell-dependent

doi:10.6342/NTU202501708

immune responses urgently require further clarification.

The immune system, particularly the thymus, exhibits heightened sensitivity to the toxic responses induced by various chemicals (Ashwell et al., 2000; Drela, 2006; Nohara et al., 2008; Shanker, 2004; Zoller and Kersh, 2006). The thymus, a vital primary immune organ, plays a pivotal role in orchestrating the maturation, selection, and differentiation of the majority of naive T cells, which are crucial for adaptive immunity (Ladi et al., 2006). Despite its functional decline with age, the thymus remains essential for T-cell-repertoire reconstitution, ensuring effective immune responses throughout adulthood (Shanker, 2004). The thymus's susceptibility to atrophy upon exposure to immunosuppressive drugs and environmental chemicals makes it a sensitive indicator of the immunotoxicity of toxicants. Therefore, potential threats to immune function arising from compounds that induce thymic atrophy, such as FPN, are of significant concern.

Given the critical role of the thymus in establishing a functional T cell repertoire and the emerging evidence suggesting FPN's ability to disrupt immune function, including direct effects on T cells and potential thymic alterations, investigating the impact of FPN on thymic function and T cell development is a crucial area of research. Elucidating the underlying mechanisms of FPN-induced immunotoxicity, particularly its effects on T cell development and the Th1/Th2 balance, is essential for a comprehensive risk assessment and for developing strategies to mitigate potential adverse health outcomes associated with FPN exposure.

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1.4 The role of GABAergic signaling in immune regulation

Gamma-aminobutyric acid (GABA) is widely recognized as the principal inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates (Bhandage and Barragan, 2021). However, evolutionary and comparative studies have revealed that GABA functionally predates the emergence of the vertebrate CNS (Roberts and Frankel, 1950), acting as a bioactive molecule in stress and metabolic responses in bacteria, plants, and invertebrates (Steward, 1949). In recent years, increasing attention has been given to its immunomodulatory roles in mammals, particularly through the discovery that GABAergic signaling machinery, including receptors, transporters, and metabolic enzymes, is expressed by a variety of immune cells.

Mononuclear phagocytes and lymphocytes, such as dendritic cells, microglia, T cells, and NK cells, have been shown to express functional GABA receptors and related signaling components (Barragan et al., 2015; Bhandage and Barragan, 2021). More surprisingly, immune cells are capable of synthesizing and releasing GABA themselves, suggesting the existence of both autocrine and paracrine signaling loops. GABA has been implicated in the modulation of several key immune processes, including cytokine production, cellular proliferation, cytotoxic responses, and directed cell migration (Bhat et al., 2010; M. Lee et al., 2011; Reyes-García et al., 2007; Xia et al., 2021). Notably, GABA concentrations in peripheral tissues, such as the blood, lymph nodes, and pancreatic islets, are sufficient to activate $GABA_A$ receptors, supporting the relevance of peripheral GABAergic signaling outside the CNS (de Groote and Linthorst, 2007; Petty and Sherman, 1984; Semyanov et al., 2003).

This expanding body of evidence reflects an emerging view of the immune system as not only a passive target but also an active participant in neurotransmitter signaling. GABAergic pathways have been increasingly linked to immune responses during infection, inflammation, and autoimmunity. For instance, altered GABA signaling has been associated with diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis (Bhat et al., 2010; Shan et al., 2023; Tian

doi:10.6342/NTU202501708

et al., 2004). Additionally, certain pathogens, including protozoan parasites, appear to exploit host GABAergic pathways to facilitate colonization, suggesting a role for GABA in interspecies communication (Fuks et al., 2012; Kim et al., 2018; Zhu et al., 2017).

1.4.1 GABAergic components and their mediated effects in T-cells

The immunomodulatory role of GABA is supported by the expression and function of key components of the GABAergic signaling machinery in immune cells. These components include GABA-synthesizing enzymes, receptors, metabolic enzymes, and transporters, which together coordinate the synthesis, signaling, degradation, and reuptake of GABA in both autocrine and paracrine contexts. Below, I describe the known functions of each component in the context of immune regulation.

Glutamate decarboxylases (GAD)

GABA is synthesized from glutamate via the action of glutamate decarboxylase, primarily the isoforms GAD65 and GAD67. Both isoforms have been identified in various immune cells, including macrophages, dendritic cells, and T lymphocytes. In these cells, GAD activity contributes to the intracellular production of GABA, which can be subsequently secreted or act in an autocrine manner (Jin et al., 2013). Upregulation of GAD expression has been observed during immune cell activation, and GAD-derived GABA may play a role in buffering inflammatory responses. For instance, GAD expression in pancreatic islets has been implicated in the modulation of autoimmune responses in type 1 diabetes (Bhandage and Barragan, 2021).

GABA Receptors

GABA exerts its immunomodulatory effects primarily through two classes of receptors: the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors, both of which are functionally expressed in various immune cell types, including T lymphocytes. The $GABA_A$ channel is an

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asymmetric pentameric structure. Nineteen distinct $GABA_A$ channel subunit genes have been identified (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3), representing eight classes. The diversity of these subunits, further increased by splice variants, suggests a large potential for $GABA_A$ channel heterogeneity. However, the number of functional $GABA_A$ channel subtypes is constrained (Olsen and Sieghart, 2008), with the most common subtypes assembled from two α, two β, and a single additional subunit (γ, δ, or ε) (Sieghart and Sperk, 2002). $GABA_A$ receptors, which are ligand-gated chloride channels, mediate hyperpolarization upon activation, leading to decreased intracellular calcium influx and suppression of pro-inflammatory cytokine production such as IFN-γ and IL-2 (Fenninger et al., 2019; Jha et al., 2009; Omilusik et al., 2011; Soltani et al., 2011; Tian et al., 1999). In contrast, $GABA_B$ receptors are G-protein-coupled receptors that inhibit adenylate cyclase activity, modulate downstream signaling, and have been shown to suppress T cell proliferation and influence antigen presentation in dendritic cells (Marshall et al., 1999).

Notably, the expression pattern of $GABA_A$ receptor subunits in T cells is highly variable across species, strains, and even individual donors. In rodent models, multiple subunits, including α1-α4, β2-β3, γ1, δ, and ρ1-ρ3, have been identified in $CD4^+$ and $CD8^+$ T cells isolated from lymphoid tissues (Bjurström et al., 2008; Mendu et al., 2011; Tian et al., 2004). Similar variability has been observed in human T cells, with studies identifying different combinations of subunits such as α1, α3, α6, β2-β3, γ2, δ, π, and ρ2 depending on the tissue source and individual (Alam et al., 2006; Dionisio et al., 2011). Despite this heterogeneity, functional $GABA_A$ channels have been demonstrated in T cells and are implicated in regulating proliferation, cytokine release, and intracellular Ca^{2+} dynamics (Bergeret et al., 1998; Tian et al., 1999; Wheeler et al., 2011).

The downstream effects of GABA receptor activation in T cells may also depend on the expression of cation-chloride cotransporters, such as NKCC1 and KCC family proteins, which determine the direction of chloride flow and thus whether GABAergic signaling results in depolarization or hyperpolarization (Bhandage et al., 2015; Köchl et al., 2016). However, the specific

doi:10.6342/NTU202501708

chloride transporter expression profile in T cells remains to be elucidated. Similarly, while some studies suggest the presence of functional $GABA_B$ receptors in immune cells, conclusive evidence of their expression in T cells is still lacking.

GABA transporters (GATs)

GABA transporters (GATs) are membrane-bound proteins responsible for the reuptake of GABA from the extracellular space, thereby regulating its local availability and signaling duration. Among the four-known plasma membrane GABA transporters (GAT1 to GAT4) only GAT1 and GAT2 have been identified in immune cells, including both mouse and human T lymphocytes (Bhat et al., 2010; Dionisio et al., 2011; Wang et al., 2009). These transporters mediate ion-coupled secondary active transport of GABA, and their activity plays a key role in maintaining the immunosuppressive microenvironment by controlling extracellular GABA levels.

In T cells, GAT1 expression appears to be functionally relevant. Mice deficient in GAT1 exhibit increased T cell proliferation and enhanced production of pro-inflammatory cytokines such as IFN-γ, suggesting that GAT-1 helps restrain excessive immune activation (Wang et al., 2009). Although transcripts for the vesicular GABA transporter VIAAT have also been detected in T cells (Dionisio et al., 2011), there is no current evidence for the formation of synaptic-like vesicles in these cells. The expression of GATs may also be activation-dependent, with some studies detecting GAT mRNA only upon stimulation, indicating that GABA uptake mechanisms in T cells may be dynamically regulated.

To summarize the functions and physiological roles of GABAergic signaling components in T cells, I constructed a schematic diagram to illustrate the key mechanisms and better clarify the role of GABAergic signaling in T cell regulation (Fig. 1).

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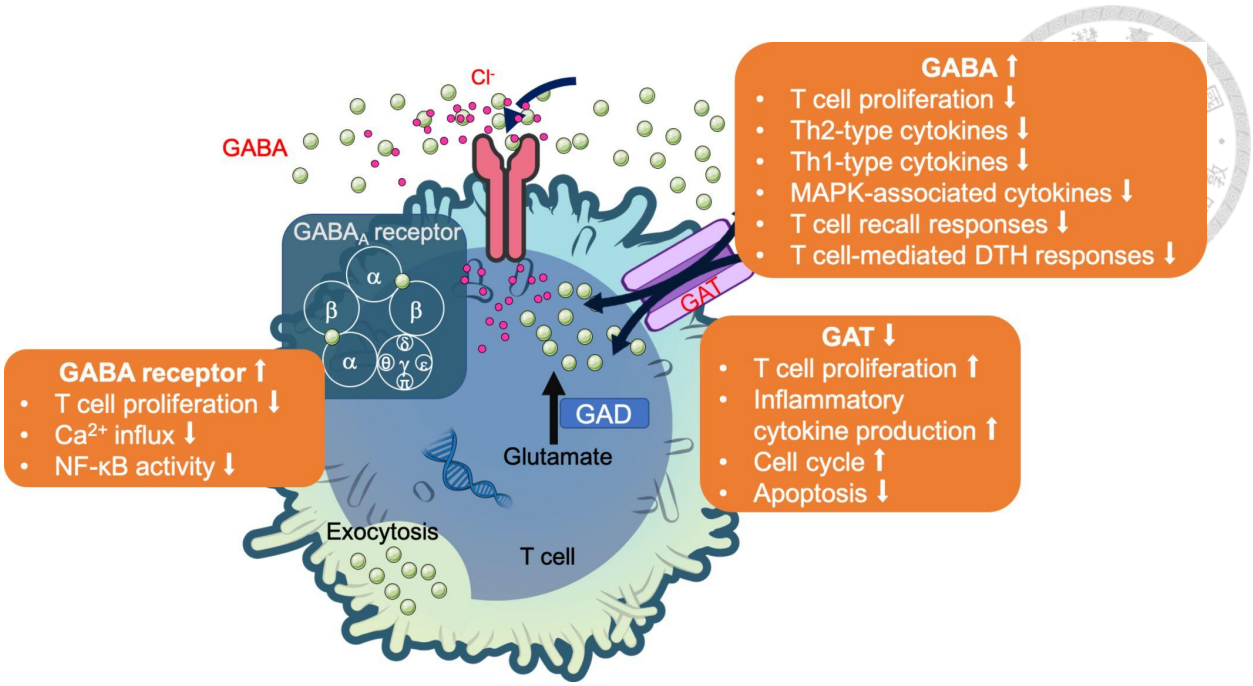

Figure 1. Schematic illustration of GABAergic signaling components and their immunomodulatory roles in T cells

Glutamate is converted to GABA *via* GAD, and GABA is released into the extracellular space through exocytosis and/or GAT. GABA interacts with $GABA_A$ receptors, leading to chloride ion (Cl^-) influx, reduced calcium (Ca^{2+}) signaling, and suppression of NF-κB activity. Upregulation of GABA or $GABA_A$ receptors has been shown to suppress T cell proliferation, Th1- and Th2-type cytokine production, MAPK-related cytokine expression, and T cell-mediated responses, including delayed-type hypersensitivity (DTH). In contrast, downregulation of GAT enhances T cell proliferation, inflammatory cytokine production, cell cycle progression, and reduces apoptosis (Bhandage and Barragan, 2021; Bhat et al., 2010; Dionisio et al., 2011; Fenninger et al., 2019; Jin et al., 2013; Olsen and Sieghart, 2008; Sieghart and Sperk, 2002; Wheeler et al., 2011).

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1.5 T cell development and lineage commitment in the thymus

T cell development begins with the migration of hematopoietic stem cells (HSCs) from the bone marrow to the thymus, where they differentiate into lymphoid-primed multipotent progenitors (LMPPs). Thymus-seeding progenitors (TSPs) migrate *via* blood circulation into the thymic microenvironment. Here, their interaction with thymic stromal (non-haematopoietic) cells in distinct regions is crucial for initiating the T cell developmental program. This process is driven by thymic stromal signals, including Notch ligand-receptor interactions and cytokine signaling (Yang et al., 2010).

Once within the thymus, TSPs progress through a series of well-defined stages known as double-negative (DN) stages, which are characterized by the absence of CD4 and CD8 surface expression. The DN stages are further subdivided into DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) phases, reflecting sequential changes in surface marker expression and developmental potential (Godfrey et al., 1993; Wu et al., 1991). During the DN2 to DN3 transition, thymocytes undergo T cell receptor (TCR) β chain gene rearrangement. Cells that successfully rearrange a functional TCRβ chain pair pair it with the pre-Tα chain to form the pre-TCR complex, triggering the β-selection checkpoint. This event is essential for survival, proliferation, and further differentiation (Germain, 2002).

Following β-selection, thymocytes progress to the double-positive (DP) stage, during which they express both CD4 and CD8 co-receptors. At this stage, cells undergo positive and negative selection processes based on the affinity of their newly rearranged TCRs for self-peptide-MHC complexes presented by thymic epithelial cells (TEC). Thymocytes with appropriate TCR signaling

doi:10.6342/NTU202501708

strength are selected to survive, whereas those with too weak or overly strong self-reactivity undergo apoptosis.

Successful completion of positive selection leads to lineage commitment into either $CD4^+$ helper or $CD8^+$ cytotoxic T cells, depending on whether the TCR interacts with MHC class II or MHC class I molecules, respectively. These single-positive (SP) thymocytes then exit the thymus and populate peripheral lymphoid organs as functionally mature, naïve T cells (Germain, 2002).

1.5.1 IL-7 and IL-7R expression in the thymus

Interleukin-7 (IL-7) is a non-redundant cytokine essential for T cell development. Within the thymus, IL-7 is predominantly produced by TECs and other stromal populations. Its receptor, IL-7R, is composed of the IL-7R alpha chain (IL-7Rα, also known as CD127) and the common gamma chain (γc, CD132), which is shared by several cytokine receptors including those for IL-2, IL-4, IL-9, IL-15, and IL-21 (Plumb et al., 2017).

IL-7Rα expression is tightly regulated during thymocyte differentiation. It is highly expressed in early DN stages, particularly DN2 and DN3, when cells undergo TCRβ rearrangement. Following successful β-selection, IL-7Rα is transiently downregulated in the DP stage, potentially to prevent excessive survival signals. Expression is re-induced during the transition to the SP stage, where IL-7R again becomes critical for thymocyte survival and homeostasis (Puel et al., 1998).

1.5.2 Downstream signaling pathways of IL-7 and IL-7R interaction

Upon IL-7 binding, IL-7R recruits and activates Janus kinases JAK1 and JAK3, which phosphorylate the intracellular domain of IL-7Rα, leading to recruitment and phosphorylation of STAT5. Activated STAT5 dimerizes and translocates to the nucleus, where it drives the expression of genes involved in cell survival, proliferation, and differentiation (Rochman et al., 2009).

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Key STAT5 targets include *Bcl-2*, *Mcl-1*, and *Bcl-xL*, which inhibit apoptosis and promote survival of developing thymocytes (Opferman et al., 2003; Ribeiro et al., 2018; Sprent and Surh, 2011). IL-7 signaling also enhances the expression of *c-Myc* and *Cyclin D3*, facilitating cell cycle progression in early thymocytes (Sicinska et al., 2003). In the DN2 to DN3 transition, IL-7 signaling is indispensable for the expression and rearrangement of TCRβ genes *via* upregulation of *Rag1*, *Rag2*, and *Il7r* itself, forming a positive feedback loop (Kuo and Schlissel, 2009). Moreover, IL-7 regulates metabolic pathways, including glucose uptake and mitochondrial activity, thereby supporting the energy demands of proliferating thymocytes.

1.5.3 Functional roles of IL-7 signaling in T cell development

IL-7 plays a central role in T cell development within the thymus, particularly during the early stages of thymocyte differentiation. IL-7 is produced primarily by TECs and exerts its effects through the IL-7R. IL-7 signaling is essential for supporting thymocyte survival, proliferation, and differentiation through tightly regulated, stage-specific mechanisms (Winer et al., 2022).

During the DN stages, IL-7 is crucial for maintaining survival and promoting the expansion of thymocyte progenitors. This is primarily achieved via activation of the JAK-STAT, PI3K-Akt, and Bcl-2-mediated anti-apoptotic pathways. In addition to its role in cell survival, IL-7 facilitates TCR β-chain gene rearrangement during the DN2-DN3 transition by promoting accessibility of the *Tcrb* locus through modulation of chromatin structure and RAG expression. Adequate IL-7 signaling at this stage ensures successful β-selection, a critical checkpoint that allows for progression to the DP stage.

Although IL-7R expression is transiently downregulated in DP thymocytes, recent studies have shown that low-level IL-7 signaling remains functionally relevant. In DP cells, IL-7 contributes to cellular metabolism and survival, including the maintenance of mitochondrial function and glucose uptake. Furthermore, IL-7 indirectly supports the maturation of SP thymocytes. It has been implicated

doi:10.6342/NTU202501708

in maintaining $CD8^+$ SP cell homeostasis and responsiveness by sustaining IL-7R expression and downstream signaling cascades.

Altogether, IL-7 functions as a stage-specific modulator of thymocyte development, orchestrating a fine balance between survival, proliferation, gene recombination, and metabolic programming. Disruption of IL-7 signaling can lead to profound defects in thymopoiesis and peripheral T cell repertoire formation.

1.5.4 Disruption of IL-7 signaling

Proper IL-7 signaling is indispensable for normal T cell development, and its disruption can lead to severe immunological consequences. One of the most well-characterized examples is severe combined immunodeficiency (SCID), a group of rare congenital disorders marked by profound defects in T cell development. Mutations in the genes encoding the IL-7 receptor α chain or the common γ chain impair IL-7 signaling and result in failed thymocyte survival and maturation, ultimately causing a near-complete absence of functional T cells (Puel et al., 1998). Importantly, IL-7's effects are stage- and lineage-specific during T cell development. The number of early thymic progenitors (ETPs) is significantly reduced in mice with defective IL-7 signaling, whereas IL-7 overexpression expands the ETP pool, underscoring its critical role at the onset of thymic differentiation (Plumb et al., 2017). Furthermore, the diminish in IL-7 production could result in thymic involution and decrease T-cell lymphopoiesis, where the IL-7 treatment might gradually accelerate recovery of thymic function (Bullenkamp et al., 2021; Mackall and Gress, 1997; Perales et al., 2012; Sheikh et al., 2016; Trédan et al., 2015).

Disruption of IL-7 signaling also compromises cellular metabolism in developing and mature T cells (Michalek and Rathmell, 2010). IL-7 has been shown to promote glucose metabolism by enhancing Glut1 trafficking and glycolysis through the STAT5-Akt axis. This metabolic support prevents T cell atrophy and death, especially under lymphopenic conditions. In IL-7-deficient

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environments, T cells exhibit reduced glycolysis, cellular shrinkage, and compromised viability, even when glucose transporter expression remains relatively unchanged (Rathmell et al., 2001; Wofford et al., 2008). This illustrates that IL-7 is not only a survival factor but also a key metabolic regulator essential for maintaining T cell homeostasis.

1.5.5 Transcription factors regulating T cell lineage commitment

T cell lineage commitment is a highly orchestrated process that occurs within the thymus, requiring dynamic interactions among developing thymocytes, thymic stromal cells, and TECs (THAPA and FARBER, 2019). These interactions are governed not only by cytokines such as IL-7 and stem cell factor (SCF), but also by a tightly regulated transcriptional network that ensures proper progression through thymopoiesis (Chung et al., 2011; Politikos et al., 2015). Transcription factors such as GABPA, FOXO1, FOXO3, Lyl1, Foxn1, as well as genes related to microenvironmental support like Scf, c-Kit, and Sox13, play pivotal roles in defining T cell identity, maintaining progenitor survival, and supporting thymic architecture.

Among these, Foxn1 is a master regulator of thymic organogenesis and epithelial cell maturation. Expressed specifically in TECs, Foxn1 governs the development and maintenance of the thymic microenvironment essential for thymocyte survival, proliferation, and differentiation. Loss of Foxn1 function leads to an arrest in TEC differentiation at the progenitor stage, resulting in thymic hypoplasia, impaired T cell development, and severe immunodeficiency (Amorosi et al., 2008; Romano et al., 2013; Žuklys et al., 2016).

SCF, the ligand for the c-Kit receptor, is another key microenvironmental factor produced by TECs and other stromal cells. SCF synergizes with IL-7 to promote the expansion and differentiation of immature thymocytes, particularly during early stages such as the DN phase (Politikos et al., 2015; Shichkin and Antica, 2022). The c-Kit receptor is highly expressed on ETPs and plays an indispensable role in their proliferation and lineage commitment (Frumento et al., 2019; Massa et al.,

doi:10.6342/NTU202501708

2006; Rodewald et al., 2001; Wei et al., 2017).

Intrinsic transcriptional regulators also dictate T cell fate decisions. Lyl1, a basic helix-loop-helix (bHLH) transcription factor, is crucial for lymphoid specification and the survival of ETPs. Deficiency in Lyl1 leads to increased apoptosis, blocked differentiation at the DN stage, and impaired population expansion of thymocyte progenitors. Moreover, Lyl1 has been implicated in the pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL), highlighting its dual roles in normal and malignant hematopoiesis (Youssef et al., 2018; Zohren et al., 2012a, 2012b).

GABPA, an ETS family transcription factor, directly regulates IL-7 receptor expression in developing thymocytes. GABPA deficiency results in impaired IL-7 responsiveness, reduced thymic cellularity, and developmental arrest at the DN3 stage, accompanied by downregulation of TCRβ expression in DN4 thymocytes. These findings underscore its essential role in coordinating cytokine receptor expression and TCR gene rearrangement (Luo et al., 2017; Xue et al., 2007; Yu et al., 2010).

The Forkhead box O (FOXO) family of transcription factors, particularly FOXO1 and FOXO3, integrates metabolic, survival, and differentiation signals during thymocyte development. FOXO1 enhances the expression of IL-7Rα and anti-apoptotic molecules like BCL-2, promoting the survival and homeostasis of naive T cells. In contrast, FOXO3 is often associated with pro-apoptotic effects under conditions of growth factor deprivation. Both FOXO1 and FOXO3 are negatively regulated by the PI3K-AKT pathway, which inactivates their transcriptional activity via phosphorylation. Moreover, FOXOs maintain T cell quiescence and inhibit aberrant activation, partly by repressing pro-inflammatory Th1 and Th17 differentiation and supporting regulatory T cell (Treg) generation (Bupp et al., 2009; Fu and Tindall, 2008; Kerdiles et al., 2010, 2009; Lin et al., 2004).

Finally, Sox13, a transcription factor from the SRY-related HMG-box family, contributes to the specification of γδ T cells, representing a lineage branch distinct from conventional αβ T cells. Sox13 thus adds further complexity to the transcriptional control of T cell lineage divergence (Spidale

doi:10.6342/NTU202501708

et al., 2018).

Taken together, these transcription factors not only orchestrate T cell fate determination at the cellular and molecular levels but also interact with extrinsic signals from the thymic microenvironment. Disruption of this finely tuned network, whether by genetic mutation or environmental insult, can compromise T cell development and immune competency.

1.5.6 The regulatory roles of ROS and apoptosis in thymocyte development

Reactive oxygen species are increasingly recognized as critical modulators of T-cell development, where precise regulation of oxidative balance determines the fate of developing thymocytes (Peng et al., n.d.). Physiological levels of ROS are necessary for normal thymocyte differentiation, acting as secondary messengers in signaling pathways that guide lineage commitment and progression through developmental checkpoints (Peng et al., n.d.; Zhang et al., 2005). However, excessive ROS disrupts this balance, leading to oxidative stress and activation of apoptotic pathways that can impair thymopoiesis (Gülow et al., 2024; Mosmann, 1983; Zhang et al., 2005).

Apoptosis plays a pivotal role in shaping the T-cell repertoire by eliminating thymocytes that are either non-functional or autoreactive. This process is tightly regulated by the BCL-2 family of proteins, including anti-apoptotic members such as BCL-2 and MCL-1, which are essential for cell survival during specific developmental stages (Aldayel et al., 2021; Veis et al., 1993). MCL-1, for example, is indispensable during the DN to DP transition, while BCL-2 ensures the survival of positively selected cells (Akashi et al., 1997; Matsuzaki et al., 1997; Opferman et al., 2003). When oxidative stress overwhelms the cell's antioxidant defenses, mitochondria-mediated apoptosis is initiated, often *via* activation of BAX/BAK and subsequent cytochrome c release (Awad et al., 2022; Matsuzaki et al., 1997; Nakayama et al., 1994; Niizuma et al., 2009). This cascade culminates in caspase activation and DNA fragmentation, leading to programmed cell death.

doi:10.6342/NTU202501708

Given this context, the redox state of thymocytes is not merely a byproduct of cellular metabolism but an active determinant of T-cell fate. Disruption of ROS homeostasis, whether through environmental toxicants or genetic perturbations, can skew thymocyte survival and selection, ultimately compromising immune competence (Akashi et al., 1997).

IL-7 and its receptor play pivotal roles in promoting the proliferation and survival of early thymocyte subsets, particularly at the DN2 stage, thereby sustaining the DN cell pool and facilitating transition beyond the DN stage. Downregulation of IL-7 or its receptor leads to impaired thymopoiesis, reduced thymic cellularity, and accelerated thymic involution. The transcription factors FOXN1, FOXO, GABPα, and LYL1 contribute to the maintenance of the thymic microenvironment, T-cell lineage commitment, and cellular homeostasis. FOXN1 supports TEC function and T-cell differentiation, while GABPα regulates IL-7R expression, and LYL1 is involved in population expansion and apoptosis resistance. Anti-apoptotic proteins Bcl-2 and Mcl-1 mediate thymocyte survival, particularly during early developmental checkpoints. This coordinated network of cytokine signaling and transcriptional regulation is critical for the establishment of a functional and self-tolerant T-cell repertoire.

In summary, literature has indicated the critical roles of IL-7 signaling, key transcription factors that maintain the thymic microenvironment, and anti-apoptotic genes of the BCL-2 family in thymic development and T lymphocyte maturation. The following schematic diagram illustrates the developmental stages at which these factors exert their effects, as well as their associated physiological functions (Fig. 2).

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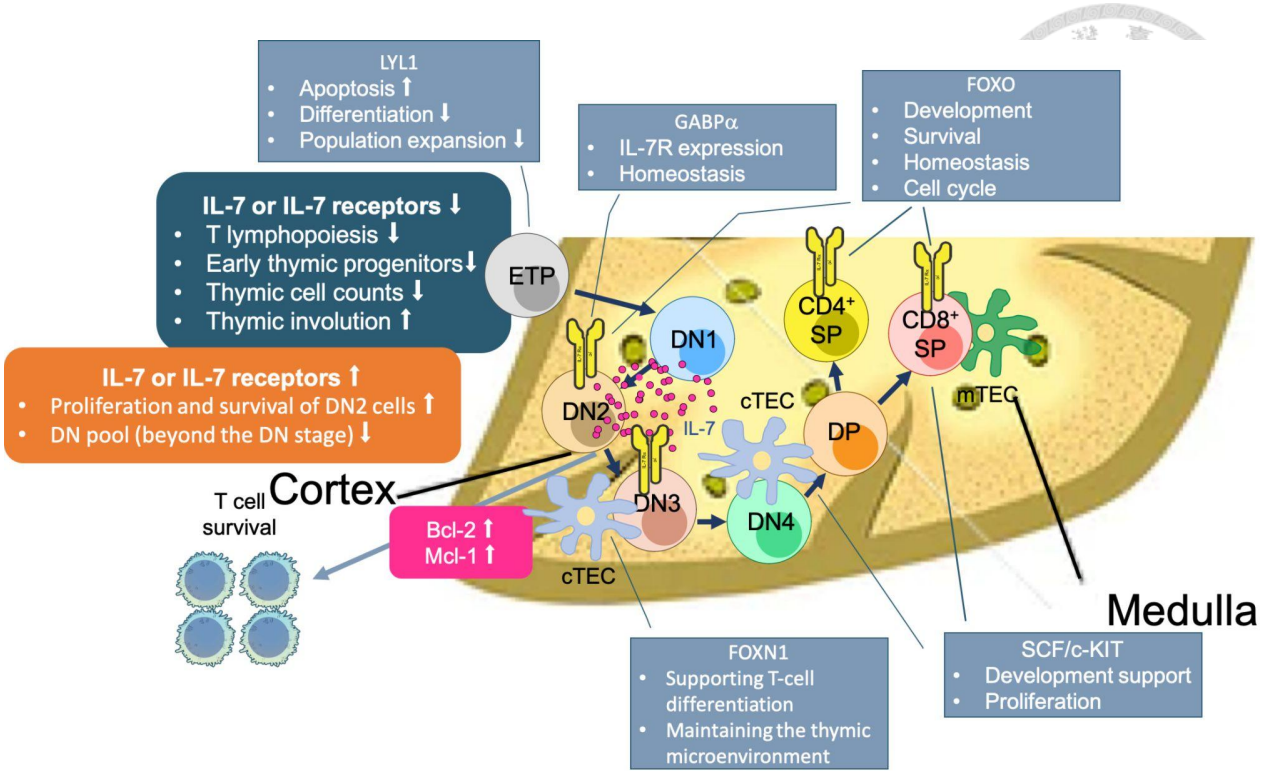

Figure 2. Schematic representation of IL-7 signaling and transcriptional regulation during thymocyte development

(Bullenkamp et al., 2021; Frumento et al., 2019; Luo et al., 2017; Mackall and Gress, 1997; Perales et al., 2012; Plumb et al., 2017; Politikos et al., 2015; Puel et al., 1998; Sheikh et al., 2016; Shichkin and Antica, 2022; THAPA and FARBER, 2019; Youssef et al., 2018; Žuklys et al., 2016)

doi:10.6342/NTU202501708

Chapter 2. Rationale

Fipronil is a broad-spectrum phenylpyrazole insecticide widely used for pest control in agriculture, veterinary medicine, and household applications. Despite its effectiveness against insect pests, concerns have been raised regarding its ecological and human health impacts. As described in previous sections, FPN is not selective to target pests. Instead, it poses significant risks to non-target organisms, including pollinators such as bees and beneficial insects like dragonflies, contributing to biodiversity loss in agricultural ecosystems. Due to its relatively high-water solubility and persistence in aquatic environments, FPN contamination has been frequently detected in water bodies, raising substantial concerns about its toxic effects on aquatic organisms.

Although regulatory authorities in several countries, including Taiwan, have restricted the use of FPN in food-producing animals, residue monitoring still reveals excessive levels of FPN in imported agricultural products. Moreover, humans may be exposed to FPN through various routes, including environmental spraying, topical application for flea and tick control in pets, and, in rare instances, deliberate ingestion in self-harm incidents. Clinical symptoms such as seizures, hepatotoxicity, and renal impairment have been reported following acute or chronic exposure, emphasizing its potential threat to human health.

FPN was initially developed as an insecticide with high specificity toward insect $GABA_A$ receptors, based on structural differences between insect and mammalian receptor subtypes. However, subsequent studies have demonstrated that FPN can also interact with vertebrate $GABA_A$ receptors, leading to neurotoxicity, hepatotoxicity, nephrotoxicity, endocrine disruption, and even reproductive toxicity. These findings have considerably undermined the presumed safety of FPN and raised broader concerns regarding its potential health effects on non-target species, including mammals.

Given the well-established interactions among the nervous, endocrine, and immune systems, the immunotoxic potential of FPN is a critical, yet underexplored, area of toxicological research.

doi:10.6342/NTU202501708

While neurotoxicity and endocrine-disrupting properties of FPN have been increasingly documented, evidence regarding its impact on the immune system remains scarce. Considering the crosstalk between neuroendocrine and immune networks, it is reasonable to hypothesize that FPN exposure may disrupt immune homeostasis, particularly during developmentally sensitive periods.

Emerging evidence suggests that FPN and its metabolite fipronil sulfone can cross the placental barrier and be detected in the cord blood of neonates born to exposed mothers. Given that the thymus is the central organ for T-cell development during fetal and early postnatal life, any disruption during this critical window may result in long-lasting immune dysfunction. Thymocyte development relies on tightly regulated signaling pathways, including IL-7 signaling and transcription factors essential for TEC-thymocyte crosstalk, which are potentially vulnerable to xenobiotic interference.

In light of the aforementioned concerns pertaining to the potential immunotoxicity of FPN, this study aims to comprehensively investigate the immunotoxic effects of FPN and delineate its underlying mechanisms. The following are three experimental approaches proposed.

1. **Assessment of adaptive immune function in adult mice**

Using an ovalbumin (OVA)-immunized murine model, I aim to determine whether FPN exposure impairs the T-cell-dependent antibody response (TDAR), a well-established endpoint for adaptive immune function. Additionally, I will evaluate T-helper cytokine profiles and examine the involvement of GABAergic signaling, which FPN's primary target, in modulating immune responses.

2. **Investigation of thymic development in juvenile mice**

I will employ 3-week-old mice, a stage during which thymic development remains active, to explore FPN’s impact on thymocyte maturation. Particular attention will be given to IL-7 signaling and key transcriptional regulators such as FOXN1 and GABPA, which are critical for maintaining thymic architecture and function.

3. **Evaluation of oxidative damage in the thymus and primary thymocytes**

doi:10.6342/NTU202501708

Considering that oxidative stress is a key mechanism underlying FPN's non-target toxicity, I will implement both short-term *in vivo* and acute *in vitro* models to assess the compound's effects on cellular redox balance. Mitochondrial membrane potential, glutathione activity, and ROS production will be monitored across multiple time points to elucidate the dynamics of FPN-induced oxidative stress and potential mitochondrial dysfunction in immune cells.

Collectively, these approaches aim to provide mechanistic insights into the immunotoxic profile of FPN, particularly its impact on thymic development, redox homeostasis, and adaptive immune function. Given the potential for early-life exposure through environmental and maternal routes, understanding FPN's immunotoxic mechanisms is essential for refining current risk assessments and informing public health policies.

doi:10.6342/NTU202501708

Chapter 3. Materials and Methods

3.1 Reagents

Unless otherwise specified, all other reagents were acquired from Sigma (MO, USA). Fipronil (FPN, > 97%) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). RPMI 1640 medium (Cat. No. SH30027.02) was purchased from Hyclone (UT, USA). Fetal bovine serum (FBS, Cat. No. 10437-028) and cell culture reagents were purchased from GIBCO BRL (MD, USA) and GE Healthcare (Chicago, IL). Reagents for ELISA analysis were provided by BD Biosciences (San Jose, CA).

3.2 Experimental animals

The male BALB/c mice (5-week-old mice for splenocyte isolation and 3-week-old mice for thymocyte isolation) were supplied by the BioLASCO Experimental Animal Center (BioLASCO, Taipei, Taiwan). After arrival, mice were randomly assigned to groups and weighed for randomization. To minimize initial weight differences within each category, the mice were then categorized based on their combined weight into five groups. Individual housing was provided, maintaining controlled conditions, including a 12-hour light/dark cycle, temperature (22 ± 2°C), humidity (40% ± 15%), and unrestricted access to standard laboratory food and water *ad libitum*. Animal experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026).

3.3 Protocol of fipronil administration and murine model

3.3.1 Ovalbumin (OVA)-specific immune model

In immunotoxicity assessment, the U.S. Food & Drug Administration advocates for the use

doi:10.6342/NTU202501708

of the T-cell dependent antigen response (TDAR) assay, a widely employed functional assay designed to detect potential immunotoxic effects of drugs. The TDAR assay relies on triggering T-cell-dependent immune responses through the administration of an exogenous protein antigen, such as ovalbumin (OVA) or Keyhole Limpet Hemocyanin (KLH) (Bugelski and Kim, 2007). The resulting immune response is then analyzed to evaluate immunological function, specifically the efficiency of critical processes including antigen uptake and presentation, T cell help, B cell activation, and antibody generation (Morokata et al., 2000, p. 2; Nohara et al., 2002).

Before a one-week acclimatization period, 5-week-old mice (N=3-5 per group) were randomly assigned to one of five treatment groups: a naïve group (NA; no treatment), a vehicle control group (VH; corn oil), and three groups receiving FPN *via* oral gavage at doses of 1, 5, or 10 mg/kg, with FPN suspended in corn oil. FPN was administered for a total of 11 doses according to the schedule described below (Fig. 3). Based on previous studies, 10 mg/kg of FPN (equivalent to 1/10 of the oral LD_{50} in mice) was selected to minimize the risk of acute toxicity and mortality while still inducing sub-chronic toxic effects over a seven-dose treatment period (Kuo et al., 2024a; Tingle et al., 2003). The other doses, 1 mg/kg (1/100 LD_{50}) and 5 mg/kg (1/20 LD_{50}), were chosen to assess the dose-dependent effects of FPN.

Except for the NA group, mice were sensitized twice *via* intraperitoneal injection with 100 μg ovalbumin and 1 mg alum (adjuvant) in 0.1 mL saline on days 5 and 15. Body weight was monitored daily. On day 16, serum samples were collected from each mouse before sacrifice. Spleens were then harvested and processed into single-cell suspensions for subsequent analyses. Due to the need for clinical monitoring of the mice following FPN exposure, blinding of the experimenter to treatment groups was not feasible.

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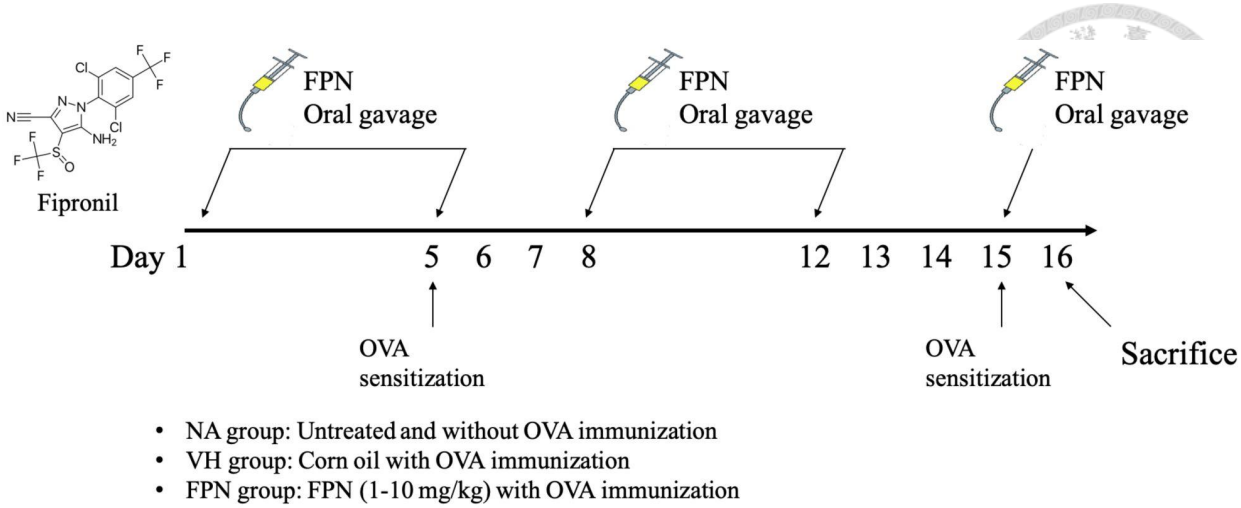

Figure 3. Protocol of FPN administration and ovalbumin (OVA) immunization

Mice were randomly assigned to one of five treatment groups (N=3-5 per group): naïve (NA), vehicle control (VH) with OVA immunization, and FPN-treated groups (1, 5, and 10 mg/kg) with OVA immunization, which was administered *via* oral gavage. Except for the NA group, mice were immunized intraperitoneally with 100 μg ovalbumin and 1 mg alum (adjuvant) in 0.1 mL saline on days 5 and 15 (two immunizations total). Body weight was monitored daily. On day 16, serum samples were collected from each mouse before sacrifice. Spleens were then harvested and processed into single-cell suspensions for subsequent analyses.

doi:10.6342/NTU202501708

3.3.2 Thymus developing murine model

Before a one-week acclimatization period, 3-week-old mice (N=3-5 per group) were randomly assigned to one of five treatment groups: a naïve group (NA; no treatment), a vehicle control group (VH; corn oil), and three groups receiving FPN *via* oral gavage at doses of 1, 5, or 10 mg/kg, with FPN suspended in corn oil. FPN was administered for a total of 7 doses according to the schedule described below (Fig. 4). Body weight was monitored daily. On day 10, thymi were then harvested and processed into single-cell suspensions for subsequent analyses. Due to the need for clinical monitoring of the mice following FPN exposure, blinding of the experimenter to treatment groups was not feasible.

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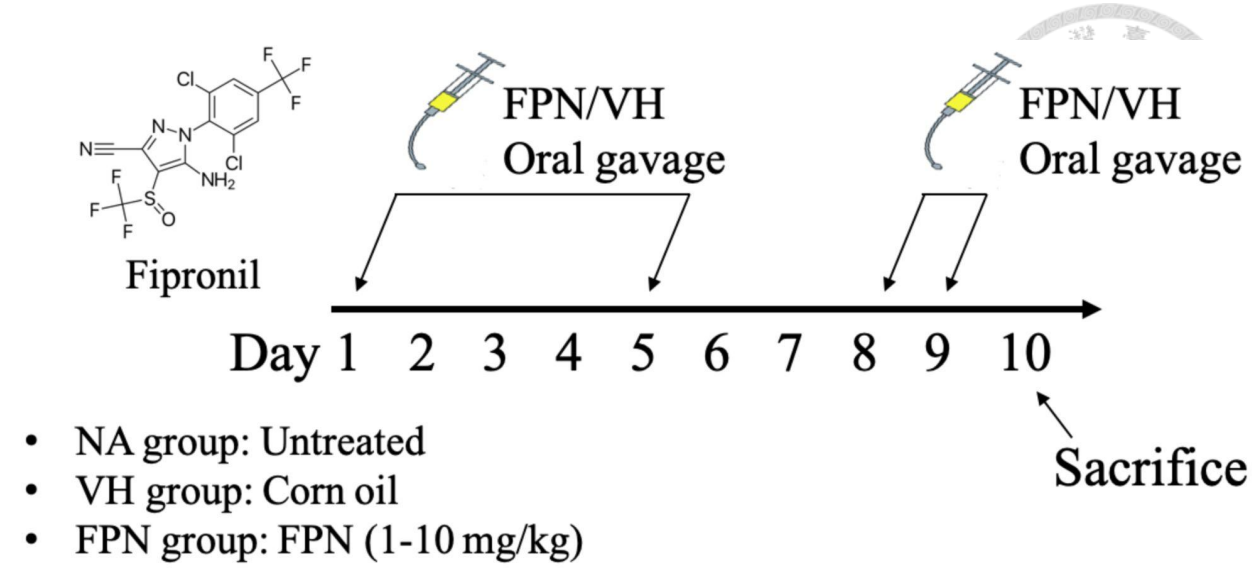

Figure 4. Protocol of FPN administration for the thymus developing study

Mice were randomly assigned to one of five treatment groups (N=3-5 per group): naïve (NA), vehicle-treated group (VH), and FPN-treated group (1, 5, and 10 mg/kg), which was administered *via* oral gavage. Body weight was monitored daily. On day 10, thymuses were then harvested and processed into single-cell suspensions for subsequent analyses.

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3.4 Measurement of spleen enlargement

Splenomegaly was assessed by calculating the spleen index. Spleens were harvested and weighed immediately following euthanasia (N=20 per group). Body weight was measured before sacrifice. The spleen index was then calculated for each mouse by dividing spleen weight (mg) by body weight (g).

3.5 Measurement of thymus enlargement

The thymus from each mouse (N=20 in each group) was aseptically dissected and weighed immediately upon euthanasia. Body weight was measured before sacrifice. The thymus index was calculated as the thymus weight (mg) divided by the mouse's body weight (g).

3.6 Histological Examination

Formalin-fixed, paraffin-embedded thymus tissue sections were stained with hematoxylin and eosin (H&E) for histological evaluation. Slides were visualized using a Zeiss optical microscope (Carl Zeiss AG, Oberkochen, Germany). Morphometric analysis was performed to quantify alterations in the thymic cortex and medulla size. The cortex-to-medulla ratio was calculated using ImageJ software (Bethesda, MD, USA).

3.7 Immunohistochemical (IHC) Analysis

Paraffin-embedded tissue sections were deparaffinized by three 5-minute washes in xylene. Rehydration was performed through a series of graded ethanol washes (100%, 95%, 90%, 80%, and 60% ethanol, 5 minutes each). Slides were then washed three times for 5 minutes each with TBS (0.01 M Tris, 0.15 M NaCl, pH 7.5). Antigen retrieval was performed by immersing the rehydrated slides in Trilogy™ (Cell Marque, Pleasanton, CA, USA) and autoclaving at 121°C for 15 minutes, followed by a 3-minute hold at 121°C. After cooling to 42°C, slides were immediately washed three

doi:10.6342/NTU202501708

times for 5 minutes each with TBS. Endogenous peroxidase activity was quenched by incubating the slides in ice-cold methanol containing 3% H_2O_2 for 30 minutes in the dark. Slides were then washed three times for 5 minutes each with TBS. Non-specific binding was blocked by incubating the slides with a blocking buffer (2.5% goat serum in phosphate-buffered saline, PBS) for 1 hour at 37°C in the dark. Slides were incubated overnight at 4°C with a primary anti-mouse IL-7 antibody (Thermo Fisher Scientific, MA, USA; OriGene Technologies, MD, USA). The following day, slides were incubated for 30 minutes with the ImmPRESS HRP Goat Anti-Rabbit Polymer (Vector Laboratories, CA, USA). Visualization was achieved by incubating the slides with 3,3'-diaminobenzidine (DAB) substrate for 37 minutes, followed by a 1-minute hematoxylin counterstain. Quantification of positive signals was conducted using thymic tissue sections. For each sample, six to eight fields encompassing either the cortical or medullary regions were randomly selected. Dark brown positive cells were manually counted within these fields. Images were acquired using a Zeiss optical microscope at an original magnification of 400×.

3.8 Splenocyte and thymocyte isolation and culture

Spleens and thymi were aseptically harvested from mice, washed, and processed into single-cell suspensions. Erythrocytes in the splenocyte suspensions were lysed using ACK lysing buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). Splenocytes and thymocytes were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell counts were determined using a Moxi Z Mini Automated Cell Counter (ORFLO, ID, USA).

3.9 Flow cytometric analysis for cellularity of splenocytes

The expression of $CD4^+$, $CD8^+$, $CD11b^+$, $Gr\text{-}1^+$, and $B220^+$ on splenocytes was assessed by

doi:10.6342/NTU202501708

flow cytometry. Splenocytes (1×10^6 cells/mL) were transferred from culture medium to staining buffer (PBS containing 2% FBS and 0.09% sodium azide) by centrifugation at 250 × *g* for 5 minutes. The splenocytes were then incubated for 30 minutes on ice, protected from light, with the following rat anti-mouse antibodies: CD4 (BD Biosciences, San Jose, CA, USA) and Gr-1 (eBioscience, Waltham, MA, USA) conjugated with FITC, and/or CD8 (BD Biosciences) and B220 (BD Biosciences) conjugated with PE-Cy5, and/or CD11b (eBioscience) conjugated with APC. Isotype-matched control antibodies were used to assess non-specific binding. Following washing, the fluorescence of 10,000 single cells per sample was acquired using a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA). Data analysis was performed using FlowJo version 10.4 software (FlowJo LLC, Ashland, OR, USA).

3.10 Flow cytometric analysis for cellularity of thymocytes

The expression of $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ on thymocytes was assessed by flow cytometry. Thymocytes (1×10^6 cells/mL) were transferred from culture medium to staining buffer (PBS containing 2% FBS and 0.09% sodium azide) by centrifugation at 250 × *g* for 5 minutes. The thymocytes were then incubated for 30 minutes on ice, protected from light, with the following rat anti-mouse antibodies: CD4 (BD Biosciences, San Jose, CA, USA) conjugated with FITC, and/or CD8 (BD Biosciences) with PE-Cy5, and/or TCRαβ (BioLegend, San Diego, CA) conjugated with APC, and/or TCRγδ conjugated with PE (BioLegend). Isotype-matched control antibodies were used to assess non-specific binding. Following washing, the fluorescence of 10,000 single cells per sample was acquired using a BD FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using FlowJo version 10.4 software (FlowJo LLC, Ashland, OR, USA).

3.11 Metabolic activity by MTT assay

The metabolic activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

doi:10.6342/NTU202501708

tetrazolium bromide (MTT) assay (Mosmann, 1983). Splenocytes or thymocytes (6×10^6 cells/mL) were cultured in quadruplicate (100 μL/well) in 96-well culture plates, followed by either left unstimulated or re-stimulated, respectively, with phorbol 12-myristate 13-acetate (PMA)/ionomycin (PMA/Iono: 80 nM/1 μM) for 24 hr, concanavalin A (ConA; 5 μg/mL) for 48 h, or OVA (100 μg/mL) for 72 h. Four hours before the end of stimulation, a stock solution of MTT (5 mg/mL) was added. Subsequently, the resultant formazan was dissolved with the Dimethyl sulfoxide (DMSO, 100 μL/well) and incubated for 1 hour. The absorbance was read by an ELISA microplate reader (SpectraMax® M5 Microplate Reader, Molecular Devices LLC, San Jose, California, USA) at $OD_{570\ nm}$, with $OD_{630\ nm}$ utilized as a background reference for accurate measurements.

3.12 Enzyme-linked immunosorbent assay (ELISA)

3.12.1 Measurement of OVA-specific antibodies

Blood samples were collected from the submandibular vein using a lancet and allowed to clot at room temperature (RT) for 30 minutes. Serum was then obtained by centrifugation at 3000 rpm for 15 minutes at 4°C. Serum OVA-specific IgM, IgG_1, and IgG_{2a} levels were measured by enzyme-linked immunosorbent assay (ELISA).

For the ELISA, 96-well plates were coated overnight at 4°C with 50 μL/well of 0.05% OVA in the coating buffer (0.1 M $NaHCO_3$ and 0.034 M Na_2CO_3, pH 9.5). Following three washes with PBST (0.05% Tween 20 in PBS), wells were blocked with 200 μL/well of blocking buffer (1% bovine serum albumin in PBS) for 1 hour at RT. After additional washes with PBST, serum samples, diluted in blocking buffer, were added to wells (50 μL/well) and incubated for 1 hour at RT. The plates were washed again with PBST, and then 50 μL/well of horseradish peroxidase (HRP)-conjugated anti-mouse IgM (1:1500 dilution in blocking buffer), IgG_1 (1:1500 dilution in blocking buffer), or IgG_{2a} (1:2000 dilution in blocking buffer) was added and incubated for 1 hour at RT. After five washes

doi:10.6342/NTU202501708

with PBST, 50 μL/well of TMB substrate solution (3,3',5,5'-tetramethylbenzidine) was added for colorimetric detection of bound peroxidase conjugate. The reaction was stopped by the addition of 150 μL/well of 3 N H_2SO_4. Optical density was measured at 450 nm using a SpectraMax® M5 microplate reader.

3.12.2 Measurement of cytokines

Splenocytes and thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well) and either left unstimulated or stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (PMA/Iono: 80 nM/1 μM) for 24 hr, concanavalin A (ConA; 5 μg/mL) for 48 h, or OVA (100 μg/mL) for 72 h. The supernatants were collected and quantified for IL-2, IL-4, and IFN-γ by ELISA kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions.

For the ELISA, 96-well plates were coated overnight at 4°C with 50 μL/well of capture antibodies specific for mouse IL-2, IL-4, or IFN-γ in coating buffer (0.1 M $NaHCO_3$ and 0.034 M Na_2CO_3, pH 9.5). Following three washes with PBST (0.05% Tween 20 in PBS), wells were blocked with 200 μL/well of blocking buffer (1% bovine serum albumin in PBS) for 1 hour at RT. After washing with PBST, 50 μL/well of biotin-conjugated detection antibodies specific for mouse IL-2, IL-4, or IFN-γ were added and incubated for 1 hour at RT. After further washing, 50 μL/well of HRP-conjugated streptavidin was added and incubated for 1 hour at RT. Following five washes with PBST, 50 μL/well of TMB substrate solution was added. The reaction was stopped by the addition of 150 μL/well of 3 N H_2SO_4. Optical density was measured at 450 nm using a SpectraMax® M5 microplate reader.

3.13 RNA isolation and cDNA synthesis

Total RNA was extracted from isolated splenocytes (stimulated with OVA for 72 hours), thymus tissue, and isolated thymocytes (stimulated with or without ConA for 24 hours) using TRIzol

doi:10.6342/NTU202501708

reagent (Thermo Fisher Scientific, Waltham, MA, USA) and purified with the TriRNA Pure Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturers' instructions. Genomic DNA contamination was removed during the RNA purification process using the DNase I and DNase I Reaction Buffer (Geneaid Biotech Ltd.) according to the manufacturer's recommended protocol. RNA concentration was determined using a NanoPhotometer™ (Implen GmbH, Munich, Germany). RNA quality was assessed by measuring the A260/A230 and A260/A280 absorbance ratios. Acceptable RNA quality was defined as A260/A230 ratios between 1.9 and 2.3, and A260/A280 ratios between 1.8 and 2.0. Purified RNA was eluted in 30 μL of nuclease-free water (Geneaid Biotech Ltd.) and stored at -80°C.

Complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was reverse transcribed in a 20 μL reaction containing 1 μL of iScript™ reverse transcriptase, 4 μL of iScript™ reaction mix, and nuclease-free water. The reverse transcription protocol was as follows: 25°C for 5 minutes, 46°C for 20 minutes, and 95°C for 1 minute.

3.14 Quantitative polymerase chain reaction (qPCR) assay

Quantitative PCR (qPCR) was performed to determine the mRNA expression levels of genes associated with GABAergic signaling, IL-7 signaling, T-cell development, and the *Bcl-2* family. Primer sequences for all target genes are listed in Table 1. Each SYBR Green-based PCR reaction was performed in duplicate in a 20 μL total volume containing 0.5 μ of each forward and reverse primer to a final concentration of 0.25 μM, 1 μL of cDNA, and 10 μL of iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories) using an AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). The PCR protocol consisted of an initial denaturation step at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. A melt curve analysis (65-95°C) was performed after

doi:10.6342/NTU202501708

each run to confirm the specificity of the amplification products. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization to the *Hprt* housekeeping gene.

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Table 1. List of quantitative PCR (qPCR) primers

Gene name	NCBI RefSeq	Primer Sequence	Tm (°C)	Product size (bp)
Il-2	NM_008366.3	F: AGCAGCTGTTGATGGACCTA R: CGCAGAGGTCCAAGTTCA	52	157
Il-4	NM_021283.2	F: GCTAGTTGTCATCCTGCTCTTC R: GGCGTCCCTTCTCCTGT	55	120
Ifng	NM_008337.4	F: GCCAAGTTTGAGGTCAACAAC R: CCGAATCAGCAGCGACTC	53	125
T-bet	NM_019507.2	F: GCCAGGGAACCGCTTATATG R: GACGATCATCTGGGTCACATTCT	54	136
Gata3	NM_001417048.1	F: TACCCTCCGGCTTCATCCT R: TGCACCTGATACTTGAGGCAC	54	170
Gad65	NM_008078.2	F: TCAACTAAGTCCCACCCTAAG R: CCCTGTAGAGTCAATACCTGC	53	134
Gad67	NM_001420099.1	F: CGCTTGGCTTTGGAACCGACAA R: GAATGCTCCGTAAACAGTCGTGC	57	161
Gat1	NM_178703.5	F: CAAGCCCAAAACCCTGGTAGT R: CCACGCAGGACATGAGGAA	54	101
Gabra5	NM_176942.4	F: GATTGTGTTCCCCATCTTGTTTGGC R: TTACTTTGGAGAGGTGGCCCCTTTT	57	100
Gabrb2	NM_008070.5	F: GCTGGTGAGGAAATCTCGGTCCC R: CATGCGCACGGCGTACCAAA	57	70
Gabrb3	NM_008071.3	F: GAGCGTAAACGACCCCGGGAA R: GGGACCCCCGAAGTCGGGTCT	60	100
Gabrd	NM_008072.3	F: TCAAATCGGCTGGCCAGTTCCC R: GCACGGCTGCCTGGCTAATCC	60	147
Il-7	NM_008371.5	F: TCTGCTGCCTGTCACATCATC R: GGACATTGAATTCTTCACTGATATTCA	54	251
Il-7	NM_008	F: CACAGCCAGTTGGAAGTGGATG	56	121

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receptor	372.4	R: GGCATTTCACTCGTAAAAGAGCC		
Scf	NM_001 347156.2	F: CCCTGAAGACTCGGGCCTA R: CAATTACAAGCGAAATGAGAGCC	54	65
C-kit	NM_001 122733.1	F: GAGTTCCATAGACTCCAGCGTC R: AATGAGCAGCGGCGTGAACAGA	56	157
Gabpα	NM_001 416812.1	F: CCGCTACACCGACTACGATT R: ACCTTCATCACCAACCCAAG	53	206
Foxo1	NM_019 739.3	F: CTACGAGTGGATGGTGAAGAGC R: CCAGTTCCTTCATTCTGCACTCG	57	138
Foxo3	NM_019 740.3	F: CCTACTTCAAGGATAAGGGCGAC R: GCCTTCATTCTGAACGCGCATG	57	107
Foxn1	NM_008 238.2	F: TGACGGAGCACTTCCCTTAC R: GACAGGTTATGGCGAACAGAA	53	70
Lyl1	NM_008 535.2	F: CAGCTAACTGCCTTGGGAAG R: CCAGCTCACTATGGCTTGGT	54	151
Sox13	NM_001 420924.1	F: GATGCCACCAACGCTAAAGC R: TTGCGGTTGAAGTCCAGGC	54	131
Bcl-2	NM_009 741.5	F: CCTGTGGATGACTGAGTACCTG R: AGCCAGGAGAAATCAAACAGAGG	56	123
Bcl-6	NM_001 348026.2	F: CAGAGATGTGCCTCCATACTGC R: CTCCTCAGAGAAACGGCAGTCA	57	134
Mcl-1	NM_008 562.3	F: AGCTTCATCGAACCATTAGCAGAA R: CCTTCTAGGTCCTGTACGTGGA	55	125
Bnip3	NM_009 760.4	F: GCTCCAAGAGTTCTCACTGTGAC R: GTTTTTCTCGCCAAAGCTGTGGC	57	98
Bim	NM_207 680.2	F: GGAGATACGGATTGCACAGGAG R: CTCCATACCAGACGGAAGATAAAG	56	156
Hprt	NM_013 556.2	F: TCAGTCAACGGGGGACATAAA R: GGGGCTGTACTGCTTAACCAG	55	142

doi:10.6342/NTU202501708

3.15 Preparation of thymus protein extracts

Thymus tissue samples were weighed and washed with ice-cold PBS to remove any residual blood or debris. For each experimental group, three thymi were pooled and homogenized in 400 μL of ice-cold Mammalian Cell Lysis Buffer (GoldBio, MO, USA) supplemented with a 1× protease inhibitor cocktail (GoldBio). Homogenization was performed using a TissueRuptor® II (QIAGEN, Hilden, Germany) with cycles of 15 seconds on and 5 seconds off for a total of 1 minute. The resulting lysate was incubated on ice for 30 minutes with intermittent vortexing (15 seconds every 5 minutes) to ensure complete lysis and minimize protease activity. Cell debris was removed by centrifugation at 12,000 × *g* for 20 minutes at 4°C. The supernatant containing the protein lysate was collected and transferred to a new tube. Protein lysate aliquots were stored at -80°C for long-term storage.

3.16 BCA™ protein assay

Protein concentrations in the thymus tissue lysates were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. A series of BSA standards (2000, 1500, 1000, 750, 500, 250, 125, 25, and 0 μg/mL) was prepared. The BCA working reagent was prepared by mixing Reagent A and Reagent B at a 50:1 ratio. Thymus protein lysates were diluted 40-fold in PBS. In a 96-well plate, 25 μL of each standard or diluted thymus protein lysate was added in duplicate and mixed with 200 μL of the BCA working reagent. The plate was incubated at 37°C for 30 minutes, protected from light. Absorbance at 562 nm was measured using a SpectraMax® M5 microplate reader. Protein concentrations in the thymus tissue lysates were then calculated based on the BSA standard curve.

3.17 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared for SDS-PAGE by combining 40 μg of protein with 4× Laemmli sample buffer (Bio-Rad Laboratories) containing 2-mercaptoethanol (2-ME) to a final

doi:10.6342/NTU202501708

volume of 25 μL. The mixture was heated at 95°C for 5 minutes to denature the proteins. 20 μL of each prepared sample was then loaded onto a 10-12% 0.75 mm SDS-PAGE gel. The gel was cast using the TGX Stain-Free FastCast Acrylamide Kit (Bio-Rad Laboratories) following the manufacturer's instructions. Briefly, the resolving gel was prepared by combining 2 mL of Resolver A, 2 mL of Resolver B, 20 μL of 10% ammonium persulfate (APS), and 2 μL of *N, N, N′, N′*-tetramethylethylenediamine (TEMED). The stacking gel was prepared by combining 1 mL of Stacker A, 1 mL of Stacker B, 10 μL of 10% APS, and 2 μL of TEMED. Gels were allowed to polymerize for 40 minutes. Electrophoresis was performed at 300 V for 20-30 minutes.

3.18 Western blotting

Following SDS-PAGE, proteins were transferred onto a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) using the Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Mü nchen, Germany). The transfer buffer was prepared by combining 200 mL of 5× transfer buffer (Bio-Rad Laboratories), 600 mL of deionized water, and 200 mL of 95% ethanol. Two stacks of filter paper from the Bio-Rad RTA Transfer Packs (Bio-Rad Laboratories) were soaked in 25 mL of 1× transfer buffer per stack. The PVDF membrane was activated by immersion in methanol. The gel, filter paper stacks, and PVDF membrane were assembled into the transfer cassette according to the manufacturer's instructions. The transfer was performed using the "Turbo" setting on the Trans-Blot Turbo system at 1.3 A for 7 minutes. Following transfer, the PVDF membrane was immediately placed in EveryBlot Blocking Buffer (Bio-Rad Laboratories).

The PVDF membrane was trimmed to the size of the target proteins of interest. The membrane was then blocked with EveryBlot Blocking Buffer for 1 hour at RT. Primary anti-mouse antibodies against IL-7 (Thermo Fisher Scientific, 1:1000), IL-7R (OriGene Technologies, 1:1000), SCF (Thermo Fisher Scientific, 1:1000), c-KIT, FOXO3A, and GABPA (Genetex, Hsinchu, Taiwan, 1:1000), LYL1 (Thermo Fisher Scientific, 1:1000), or β-actin (Genetex, 1:10000) were diluted in

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EveryBlot Blocking Buffer and incubated with the membrane overnight at 4°C. After incubation, the membrane was washed three times for 10 minutes each wash with TBST. The membrane was then incubated with HRP-conjugated goat anti-rabbit secondary antibodies (Bio-Rad Laboratories, 1:5000 in TBST) for 1 hour at RT. Following incubation with the secondary antibody, the membrane was washed again three times for 10 minutes each wash with TBST. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagent (Bio-Rad Laboratories) following the manufacturer's instructions. Briefly, equal volumes of ECL Reagent A and Reagent B were mixed and added to the membrane. After a 5-minute incubation in the dark, the membrane was imaged using a Bio-Rad ChemiDoc XRS+ System. Densitometric analysis of the bands was performed using Bio-Rad Image Lab software with β-actin serving as the loading control.

3.19 *In vitro* model

3.19.1 Assessment of apoptotic/necrotic indicators

Apoptosis in primary thymocytes following FPN treatment was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Dojindo, Kumamoto, Japan) following the manufacturer's protocol. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were used: VH, FPN 10 μM, FPN 25 μM, FPN 50 μM, VH + 1 mM NAC, FPN 10 μM + 1 mM NAC, FPN 25 μM + 1 mM NAC, and FPN 50 μM + 1 mM NAC. Cells were treated for 2, 6, or 18 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following centrifugation at 250 × *g* for 5 minutes and

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removal of the supernatant, the cell pellet was resuspended in 100 μL of staining solution and incubated for 15 minutes at RT in the dark. After staining, 200 μL of additional staining solution was added, and the cells were analyzed immediately. Apoptosis was analyzed by BD FACSCalibur flow cytometry using a 488 nm laser for excitation. FITC fluorescence (for Annexin V) was detected using a 515 nm bandpass filter, and propidium iodide (PI) fluorescence was detected using a filter >600 nm.

3.19.2 Measurement of mitochondrial depolarization

Mitochondrial membrane potential (Δψm) of primary thymocytes was assessed using the JC-1 Mitochondrial Membrane Potential Assay Kit (Invitrogen) according to the manufacturer's instructions. A JC-1 stock solution (2.5 mg/mL) was prepared in DMSO and subsequently diluted to the desired working concentration. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were the same as chapter 3.19.1. Cells were treated for 2, 6, or 18 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following another centrifugation at 250 × *g* for 5 minutes and removal of the supernatant, 150 μL of cell suspension was mixed with pre-prepared JC-1 staining solution (5 μg/mL final concentration) and incubated at 37°C for 30 minutes. After incubation, 1 mL of pre-warmed dPBS was added, and the cells were centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the remaining 200 μL of cell suspension was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

doi:10.6342/NTU202501708

3.19.3 Measurement of intracellular calcium concentration (Ca^{2+})

The intracellular calcium concentration (Ca^{2+}) of primary thymocytes was evaluated using the Fluo-4 AM Calcium Assay Kit (Invitrogen) according to the manufacturer's instructions. A Fluo-4 AM stock solution (1 mM) was prepared in DMSO and subsequently diluted to the desired working concentration. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were the same as chapter 3.19.1. Cells were treated for 2, 6, or 18 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × g for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following another centrifugation at 250 × g for 5 minutes and removal of the supernatant, 100 μL of cell suspension was mixed with pre-prepared Fluo-4 AM staining solution (1 μM final concentration) and incubated at 37°C for 30 minutes. After incubation, 1 mL of pre-warmed dPBS was added, and the cells were centrifuged at 250 × g for 5 minutes. The supernatant was removed, and the remaining 200 μL of cell suspension was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

3.19.4 Detection of glutathione (GSH) activity

Intracellular glutathione (GSH) levels in primary thymocytes were measured using the CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) probe (Invitrogen, Carlsbad, CA, USA). A CMFDA stock solution (10 mM) was prepared in DMSO and subsequently diluted to the desired working concentration. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following

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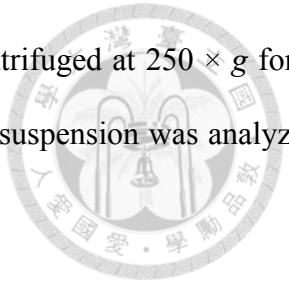
pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were the same as chapter 3.19.1. Cells were treated for 2, 6, or 18 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following another centrifugation at 250 × *g* for 5 minutes and removal of the supernatant, 150 μL of cell suspension was mixed with pre-prepared CMFDA staining solution (0.2 mM final concentration) and incubated at 37°C for 30 minutes. After incubation, 1 mL of pre-warmed dPBS was added, and the cells were centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the remaining 200 μL of cell suspension was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

3.19.5 Quantification of intracellular ROS levels

The levels of intracellular ROS of primary thymocytes were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA; Invitrogen) according to the manufacturer's instructions. A H_2-DCFDA stock solution (5 mM) was prepared in DMSO and subsequently diluted to the desired working concentration. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were the same as chapter 3.19.1. Cells were treated for 0.5, 2, or 6 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following another centrifugation at 250 × *g* for 5 minutes and removal of the supernatant, 150 μL of cell suspension was mixed with pre-prepared DCFDA staining solution (50 μM final concentration) and incubated at 37°C for 30 minutes. After

doi:10.6342/NTU202501708

incubation, 1 mL of pre-warmed dPBS was added, and the cells were centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the remaining 200 μL of cell suspension was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).



3.19.6 Evaluation of lipid peroxidation (LPO)

Lipid peroxidation in FPN-treated thymocytes was quantified using the C11-Bodipy$^{581/591}$ fluorescent probe (Cayman Chemical, MD, USA). This sensor specifically detects LPO by undergoing oxidation in the presence of intracellular lipid peroxides, emitting a distinct bright green fluorescence signal that indicates oxidative stress. Thymocytes (6×10^6 cells/mL) were cultured in triplicate in 48-well plates (300 μL/well). After a 30-minute stabilization period in a humidified incubator at 37°C with 5% CO_2, cells in specific groups were pretreated with 1 mM NAC for 30 minutes. Following pretreatment, cells were treated with FPN at the indicated concentrations (10 μM, 25 μM, and 50 μM) according to the experimental design. Eight experimental groups were the same as chapter 3.19.1. Cells were treated for 0.5, 2, or 6 hours before collection. Cells were collected and transferred to Eppendorf tubes, then centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was washed with 1 mL of pre-warmed dPBS. Following another centrifugation at 250 × *g* for 5 minutes and removal of the supernatant, 150 μL of cell suspension was mixed with pre-prepared C11-Bodipy$^{581/591}$ staining solution (20 μM final concentration) and incubated at 37°C for 30 minutes. After incubation, 1 mL of pre-warmed dPBS was added, and the cells were centrifuged at 250 × *g* for 5 minutes. The supernatant was removed, and the remaining 200 μL of cell suspension was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

3.20 Statistical analysis

Statistical analyses were conducted using GraphPad Prism V9 software (GraphPad Software, Inc., La Jolla, CA). Data were expressed as mean ± standard error (SEM) and were determined for

doi:10.6342/NTU202501708

each treatment group in individual experiments. To assess the impacts of FPN compared to the VH-group, statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed t-test. Additionally, a two-way ANOVA was employed to evaluate the effects of treatment and time, as well as their interactions. Time-dependent effects were specifically assessed by examining the significance of the main effect of time. A p-value < 0.05 was considered statistically significant. All analyses were carried out in a blinded fashion.

3.21 Bioinformatic analysis

To investigate the biological functions, enriched signaling pathways, and potential disease associations of FPN-responsive genes identified in this study, I conducted functional annotation and enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8; https://david.ncifcrf.gov/) and the Comparative Toxicogenomics Database (CTD; https://ctdbase.org/). All gene symbols were uploaded to DAVID for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

To further explore disease associations, I utilized the Gene Set Analyzer function in CTD. Enrichment analysis was performed by intersecting our list of FPN-altered genes with gene-disease association data curated from peer-reviewed literature.

For reference, all bioinformatic queries were performed between May and June 2025 using the most updated versions of each tool at the time of analysis.

doi:10.6342/NTU202501708

Chapter 4. Experimental Results

4.1 Fipronil perturbs antigen-specific immune responses and alters GABAergic gene expression in ovalbumin-immunized BALB/c mice.

Partial reprint from BMC Veterinary Research

Authors: Jui-Fang Kuo, Yin-Hua Cheng, Chun-Wei Tung, and Chia-Chi Wang

Title: Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in the ovalbumin-immunized BALB/c mice

4.1.1 Effects of FPN exposure on body weight, spleen index, and spleen cellularity *in vivo*

To investigate the direct toxic effects of FPN on mice and their secondary lymphoid system, specifically the spleen, I first assessed the general physiological condition, monitored body weight changes, and evaluated the degree of splenomegaly. The BALB/c mice were immunized with OVA to study T-cell-dependent immune responses (Fig. 3). In the OVA-specific immune model, mice exposed to corn oil (VH) or FPN (1, 5, and 10 mg/kg) for a total of 11 doses exhibited no overt clinical symptoms, and no mortality was observed in any FPN-treated group. In OVA-immunized mice, administration of FPN at 5 and 10 mg/kg resulted in a slight reduction in weight gain and an increase in spleen index compared to the VH group (Table 2).

I also investigated whether FPN exposure altered the major splenocyte populations. However, FPN administration did not significantly alter the cellularity of $CD4^+$, $CD8^+$, $CD11b^+$, $Gr\text{-}1^+$, and $B220^+$ splenocytes (Table 2).

doi:10.6342/NTU202501708

Table 2. Effects of FPN exposure on body weight, spleen index, and cellularity of splenocytes

	NA	VH	Fipronil (mg/kg)		
			1	5	10
Body Weight					
Day 1	21.39±0.25	21.56±017	21.24±0.21	21.5±0.21	21.68±0.18
Day 16	23.68±0.31	23.22±0.24	22.46±0.18	22.31±0.23*	21.74±0.19*
Spleen index [a]	3.888±0.07	4.124±0.05	4.205±0.10	4.211±0.96	4.577±0.26
Spleen Cellularity (%) [b]					
$CD4^+$	24.03±0.43	21.9±0.66	17.52±1.63	18.22±1.7	21.21±0.51
$CD8^+$	13.28±0.61	14.23±0.57	13.61±0.70	13.67±0.74	13.22±0.78
$B220^+$	50.94±1.07	51.78±1.4	52.75±1.41	51.87±1.13	49.76±1.33
$CD11b^+$	1.92±0.34	1.49±0.25	1.68±0.30	1.5±0.23	1.72±0.32
$Gr1^+$	1.18±0.25	0.99±0.19	1.03±0.22	0.98±0.20	1.03±0.19
$CD11b^+/Gr1^+$	1.77±0.14	2.29±0.22	2.19±0.21	2.34±0.24	2.4±0.24

[a] Spleen index was calculated as the spleen weight (mg) per body weight (g). Data are expressed as mean ± SEM of 20 samples and are representative of four independent experiments.

[b] Splenocytes were prepared as described in the Materials and Methods section. The percentage of $CD4^+$, $CD8^+$, $B220^+$, $CD11b^+$, and $Gr1^+$ cells was determined by flow cytometry. Data were expressed as mean ± SEM of triplicate samples pooled from four independent experiments. *$p < 0.05$ as compared with the VH group.

Adapted from **Kuo J-F, Cheng Y-H, Tung C-W, Wang C-C** (2024) *Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in ovalbumin-immunized*

doi:10.6342/NTU202501708

BALB/c mice. BMC Veterinary Research 20:30. doi: https://doi.org/10.1186/s12917-024-03878-3

doi:10.6342/NTU202501708

4.1.2 Modulation of antigen-specific antibody production by FPN administration

After a total of 11 doses of administration, the blood samples were collected and separated into serum by centrifugation. To investigate the impact of FPN exposure on antigen-specific immune responses, serum OVA-specific immunoglobulin (IgM, IgG_1, and IgG_{2a}) levels were measured. Serum from the NA group (non-immunized) served as a negative control. As expected, OVA immunization significantly increased serum levels of all three OVA-specific Ig isotypes compared to the NA group (Fig. 5).

FPN administration differentially affected OVA-specific Ig levels. While OVA-specific IgM levels, representing an early antibody response, were not significantly altered by FPN treatment (Fig. 5A), OVA-specific IgG_1 and IgG_{2a} levels were markedly increased in a dose-dependent manner (Fig. 5B and C). In mice, IgG1 production is typically associated with T helper (Th) 2-type immune responses, whereas IgG_{2a} is indicative of Th1-type immune responses. The concurrent elevation of both IgG_1 and IgG_{2a} suggests a broad activation of T cell-dependent B cell responses, potentially reflecting a mixed Th1/Th2 profile or a general augmentation of humoral immunity by FPN.

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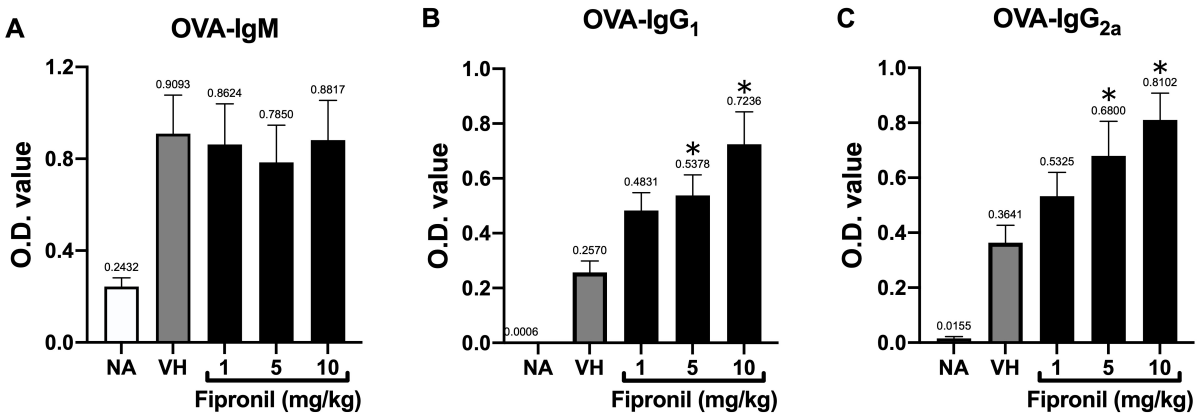

Figure 5. Induction of OVA-specific IgG_1 and IgG_{2a} production *in vivo*

The serum levels of OVA-specific IgM, IgG_1, and IgG_{2a} were determined by ELISA. Data were expressed as mean ± SEM of 20 individuals pooled from 4 independent experiments. *$p < 0.05$ as compared with the VH group. *Adapted from* **Kuo J-F, Cheng Y-H, Tung C-W, Wang C-C** (2024) *Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in ovalbumin-immunized BALB/c mice*. BMC Veterinary Research 20:30. doi: https://doi.org/10.1186/s12917-024-03878-3 © 2024 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

doi:10.6342/NTU202501708

4.1.3 FPN enhanced the cell viability and disturbed IL-2, IL-4, and IFN-γ production *ex vivo.*

To further investigate the immunomodulatory effects of FPN, I examined its influence on the proliferation of OVA-stimulated splenocytes using an MTT assay. FPN administration at 5 and 10 mg/kg robustly enhanced splenocyte viability both in the absence and presence of OVA stimulation (100 μg/mL) (Fig. 6A). This suggests that FPN may directly promote splenocyte survival or proliferation, independent of antigen stimulation.

I next investigated the effects of FPN on the production of key T helper (Th) cytokines. Naïve T cells proliferate and differentiate into effector Th cells, which play a crucial role in adaptive immune responses through their distinct cytokine secretion profiles. Th1 cells are characterized by the production of IL-2 and IFN-γ, while Th2 cells produce IL-4. Maintaining a balance between Th1 and Th2 responses is essential for a properly functioning immune system. Therefore, I assessed the impact of FPN on the production of IL-2 (Th1), IL-4 (Th2), and IFN-γ (Th1) by OVA-stimulated splenocytes. As shown in Figure 6B-D, FPN treatment at 5 and 10 mg/kg significantly increased the production of IL-2, IL-4, and IFN-γ by splenocytes stimulated with OVA (100 μg/mL). This suggests that FPN may skew the immune response towards both Th1 and Th2 cytokine production.

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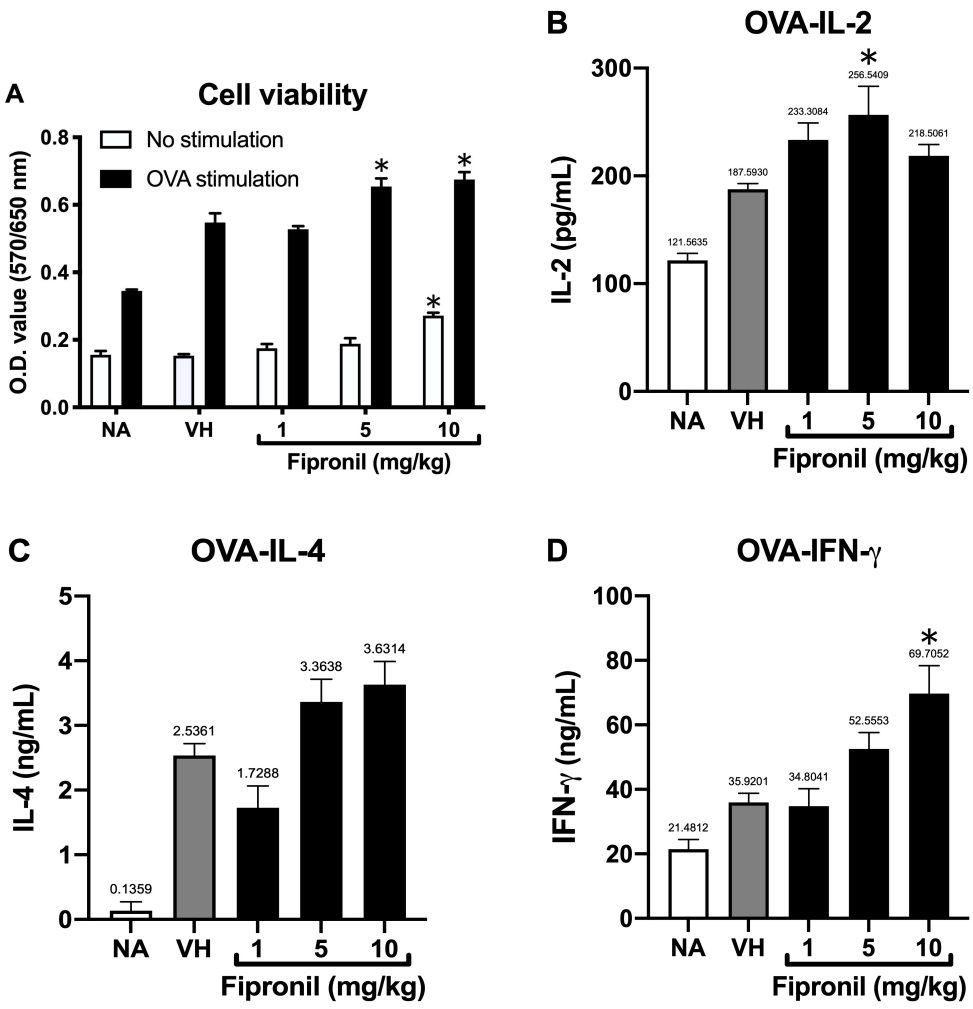

Figure 6. Enhancement of the cell viability and disturbance of IL-2, IL-4, and IFN-γ production

Splenocytes with the same cell concentration were prepared from each group of mice and cultured in the presence of ovalbumin (100 μg/mL) for 72 h. The supernatants were collected to measure the concentration of IL-2, IFN-γ, and IL-4 by ELISA. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments (N=20). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Cheng Y-H, Tung C-W, Wang C-C** (2024) *Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in ovalbumin-immunized BALB/c mice*. BMC Veterinary Research 20:30. doi: https://doi.org/10.1186/s12917-024-03878-3

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4.1.4 FPN slightly down-regulated *Il-2*, *Il-4*, and *Gata3* expression by OVA-stimulated splenocytes

Given the observed increase in antigen-specific cytokine production (Fig. 6B-D), I next examined the effects of FPN on the gene expression of Th1/Th2 cytokines and their upstream transcription factors. Splenocytes stimulated with OVA (100 μg/mL) for 72 hours were analyzed for the relative mRNA expression of *Il2*, *Il4*, *Ifng*, *Gata3*, and *T-bet*. Surprisingly, FPN treatment did not significantly alter the mRNA expression levels of these genes (Fig. 7A-E).

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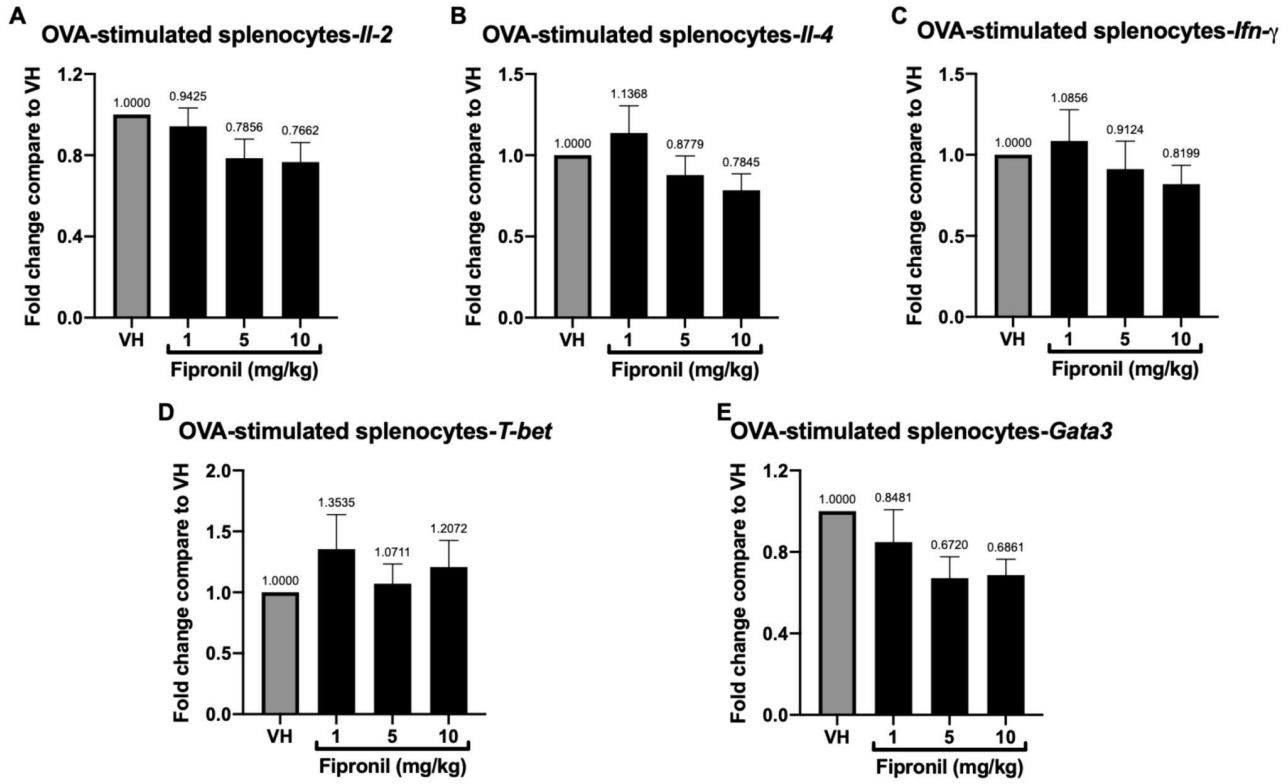

Figure 7. *Il-2*, *Il-4*, and *Gata3* mRNA expression by OVA-stimulated splenocytes

The total RNA of splenocytes (6×10^6 cells/mL) harvested from different treatment groups was extracted to detect the mRNA expression of *Il-2*, *Il-4*, *Ifn-γ*, *T-bet*, and *Gata3* by qPCR after culturing in the presence of ovalbumin (100 μg/mL) for 72 h. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM of pooled data from duplicate pooled from four independent experiments. *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Cheng Y-H, Tung C-W, Wang C-C** (2024) *Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in ovalbumin-immunized BALB/c mice*. BMC Veterinary Research 20:30. doi: https://doi.org/10.1186/s12917-024-03878-3

doi:10.6342/NTU202501708

4.1.5 FPN altered GABAergic signaling gene expression by primary splenocytes

Given the minimal changes observed in Th1/Th2 cytokine and their transcription factor gene expression (Fig. 7), I explored the possibility that FPN's immunomodulatory effects might involve GABAergic signaling, as FPN is known to target $GABA_A$ receptors in insects. To investigate this hypothesis, I examined the expression of key GABAergic signaling genes in splenocytes isolated from FPN-treated mice. These genes major in glutamate decarboxylases (*Gad65* and *Gad67*), the GABA transporter gene (*Gat1*), and $GABA_A$ receptor subunit genes (*Gabra5*, *Gabrb2*, *Gabrb3*, and *Gabrd*).

Our results revealed that FPN exposure notably and dose-dependently reduced the expression of *Gad67* mRNA (Fig. 8B). In contrast, the expression of *Gabrb2* and *Gabrd* mRNA was significantly increased at the highest FPN dose (10 mg/kg) compared to the vehicle control (Fig. 8E and G). While a decrease in *Gat1* mRNA levels was observed at 10 mg/kg FPN, this change was not statistically significant (Fig. 8C). The expression of *Gad65*, *Gabra5*, and *Gabrb3* mRNA was not significantly affected by FPN treatment (Fig. 8A, D, and F).

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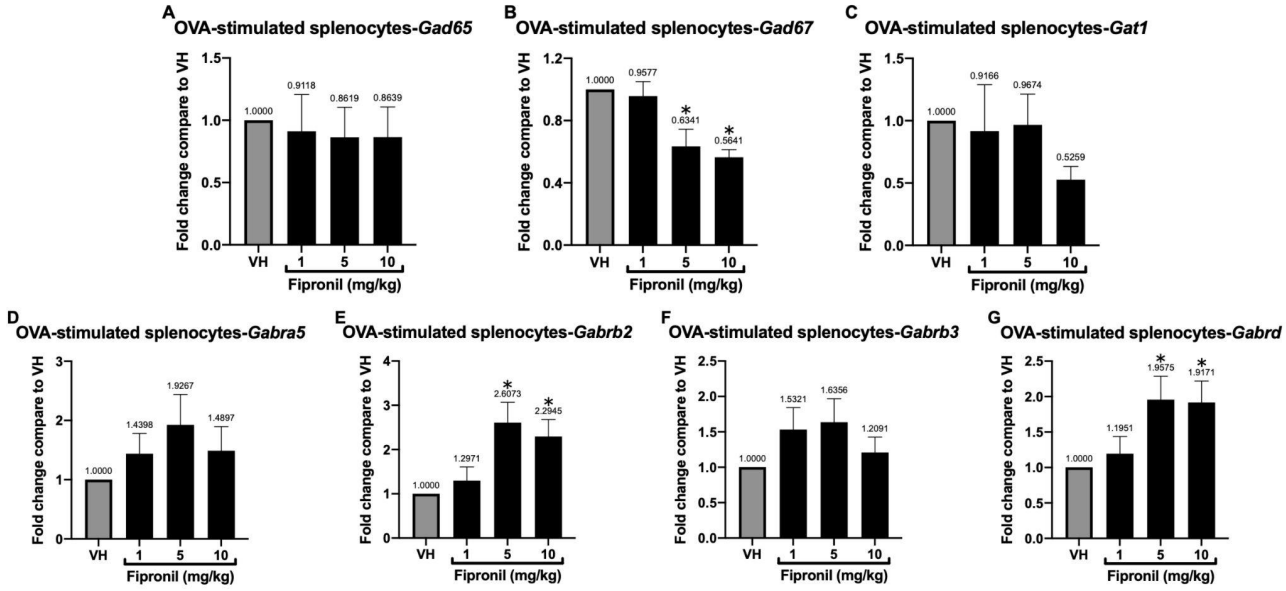

Figure 8. Reduced mRNA expression of *Gad67* and *Gat1* and alternation of the GABA receptor subunit expression

The total RNA of splenocytes (6×10^6 cells/mL) harvested from different treatment groups was extracted to detect the mRNA expression of *Gad65*, *Gad67*, *Gat1*, *Gabra5*, *Gabrb2*, *Gabrb3*, and *Gabrd* by qPCR. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N=20). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Cheng Y-H, Tung C-W, Wang C-C** (2024) *Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in ovalbumin-immunized BALB/c mice*. BMC Veterinary Research 20:30. doi: https://doi.org/10.1186/s12917-024-03878-3

doi:10.6342/NTU202501708

4.2 Investigating the potential immunotoxicity of FPN disrupting IL-7 signaling in a young mouse model of thymic development.

Partial reprint from ACS Chemical Research in Toxicology

Authors: Jui-Fang Kuo, Hsin-Ying Wu, Chun-Wei Tung, Wei-Hsiang Huang, Chen-Si Lin, and Chia-Chi Wang

Title: Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes (Kuo et al., 2024b)

4.2.1 FPN affected body weight, thymus index, and population of thymocytes *in vivo*.

Having investigated the impact of FPN on antigen-specific immune responses using the OVA-sensitized model (Kuo et al., 2024a), I next examined its effects on thymic development in three-week-old mice. Mice were orally administered vehicle (VH) or FPN at 1, 5, or 10 mg/kg for seven doses. No mortality or overt clinical signs were observed in any group. However, mice in the 10 mg/kg FPN group exhibited a significant reduction in body weight by day 10 compared to the vehicle control group. FPN treatment also resulted in a dose-dependent decrease in the thymus index, indicating thymic atrophy (Table 3).

Flow cytometry analysis revealed marked alterations in thymocyte subset distribution following FPN exposure. The percentage of double-positive (DP) thymocytes was significantly reduced, while the proportions of $CD4^+$ or $CD8^+$ single-positive (SP) and double-negative (DN) thymocytes were relatively increased. In contrast, the percentages of TCR α/β^+ and γ/δ^+ thymocytes remained unchanged across groups.

To complement percentage-based analysis and better assess overall cellularity, I further evaluated the

doi:10.6342/NTU202501708

absolute numbers of each thymocyte subset. Notably, the absolute number of DP thymocytes was significantly reduced in the 10 mg/kg FPN group compared to the vehicle control. This was accompanied by modest reductions in $CD4^+$ and $CD8^+$ SP thymocyte counts. Although the proportion of DN thymocytes appeared elevated, their absolute number showed only a mild, non-significant increase. Importantly, the absolute numbers of both TCR α/β^+ and γ/δ^+ thymocytes were significantly reduced in the 10 mg/kg group, despite their relative percentages being unchanged.

These findings suggest that FPN exposure impairs thymopoiesis primarily by depleting the total number of developing thymocytes, especially the DP population, rather than merely altering proportional distribution. The observed reductions in mature TCR-expressing thymocytes further support the hypothesis that FPN interferes with thymocyte differentiation and survival during early T-cell development.

doi:10.6342/NTU202501708

Table 3. Effects of FPN on body weight, thymus index, and cellularity of thymocytes

	NA	VH	Fipronil (mg/kg)		
			1	5	10
Body Weight					
Day 1	17.5±0.72	17.19±0.54	17.52±0.6	17.58±0.54	17.01±0.47
Day 10	21.07±0.64	20.24±0.46	20.69±0.44	20.56±0.35	18.77±0.25*
Thymus index					
Index [a]	3.82±0.16	3.68±0.13	3.26±0.14*	3.06±0.1*	2.81±0.2*
Total number ($\times 10^8$)	1.392±0.58	1.464±0.74	1.217±0.78	1.155±0.64*	0.941±0.6*
Thymus Cellularity (%) [b]					
$CD4^+$	13.54±0.32	12.61±0.48	15.02±0.17*	16.01±0.24*	15.58±0.31*
$CD8^+$	5.80±0.35	6.06±0.47	7.58±0.11*	7.25±0.20*	6.79±0.28
$CD4^+/CD8^+$	76.61±0.71	78.24±0.93	73.84±0.76*	70.98±1.04*	71.18±1.21*
$CD4^-/CD8^-$	3.70±0.19	3.53±0.15	3.73±0.12	4.74±0.28*	5.45±0.32*
TCR α/β^+	34.58±6.6	35±7.08	34.85±6.52	33.01±7.26	34±6.46
TCR γ/δ^+	0.59±0.23	0.39±0.08	0.3±0.05	0.33±0.07	0.4±0.09

doi:10.6342/NTU202501708

Number of different subsets of thymocytes ($\times 10^6$) [c]					
$CD4^+$	17.94±1.02	17.29±1.34	18.28±1.18	19.32±1.22	15.85±1.15
$CD8^+$	7.685±0.43	8.309±0.64	9.228±0.59	8.749±0.55	6.908±0.50
$CD4^+/CD8^+$	101.5±5.79	107.2±8.32	89.9±5.82	85.66±5.44	72.42±5.27*
$CD4^-/CD8^-$	4.902±0.27	4.84±0.37	4.541±0.29	5.72±0.36	5.545±0.40
TCR α/β^+	45.81±2.61	47.99±3.72	42.42±2.75	39.83±2.53	34.59±2.52*
TCR γ/δ^+	0.781±0.044	0.5347±0.041	0.365±0.023*	0.398±0.025*	0.407±0.029*

Data were expressed as mean ± SEM of triplicate samples pooled from four independent experiments (N=20/group). *$p < 0.05$ as compared with the VH group.

[a] Thymus index was calculated as the thymus weight (mg) per body weight (g). Data are expressed as mean ± SEM of 20 samples pooled from 4 independent experiments.

[b] Thymocytes were prepared as described in the Materials and Methods section. The percentage of CD4+/$CD8^+$, TCR α/β^+, and TCR γ/δ^+ cells was determined by flow cytometry.

[c] Percent values and total number of thymus were used to calculate the total number of each cell population in the thymus.

Adapted from **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes.* Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060 © 2024 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence. Reprinted with permission from *Chem. Res. Toxicol.* 2024, 37, 1488−1500. Copyright 2024 American Chemical Society.

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4.2.2 FPN leads to a dose-dependent reduction in thymocyte numbers in mice.

To rigorously investigate the impact of FPN on thymocyte numbers, I performed total thymocyte counts in FPN-treated mice. Our analysis revealed a significant, dose-dependent decrease in total thymocyte numbers following FPN administration (Table 3 and Fig. 9A). Specifically, as the FPN dose increased, I observed a corresponding decrease in the total number of thymocytes, indicating a potential inhibitory effect of FPN on thymocyte development or survival.

At the lower dose of 5 mg/kg, FPN treatment resulted in a noticeable reduction in thymocyte numbers compared to the vehicle control group. However, the most pronounced effect was observed at the highest dose of 10 mg/kg. At this dose, thymocyte numbers were significantly reduced to 9.41 $\times 10^7$ cells/thymus, representing a substantial 35.7% decrease compared to the VH group (1.46×10^8 cells/thymus). This marked reduction in thymocyte numbers at the highest FPN dose suggests a potentially significant impact of FPN on thymic cellularity.

These findings raise several important questions regarding the mechanism by which FPN exerts its effects on thymocyte numbers. One possibility is that FPN may directly or indirectly induce thymocyte apoptosis. Alternatively, FPN could be interfering with thymocyte proliferation or differentiation. Further investigation will be required to elucidate the precise mechanisms underlying the observed decrease in thymocyte numbers following FPN treatment.

4.2.3 Effects of the cortex, medulla ratio, and thymus atrophy

In addition to the observed changes in total thymocyte numbers, histological analysis of H&E-stained thymic sections revealed distinct alterations in thymic architecture following FPN treatment. Specifically, assessment of the cortex/medulla ratio (Fig. 9B) demonstrated a significant shift in the 5 mg/kg FPN group. I observed a decrease in the proportion of the medulla, accompanied by a corresponding increase in the cortical proportion. This alteration in the cortex/medulla ratio suggests

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a potential impact of FPN on the distribution and organization of thymocyte populations within these distinct thymic compartments.

At the higher dose of 10 mg/kg, while I again observed a marked decrease in total thymocyte numbers (as described previously), the cortex and medulla ratio remained relatively unchanged. However, this group exhibited a substantial reduction in the overall thymic area (Fig. 9D), with the total thymic area decreasing to approximately 80.06% of that observed in the VH group. This reduction in overall thymic area, coupled with the previously noted decrease in thymocyte numbers, provides strong evidence of FPN-induced thymic atrophy. The fact that the cortex/medulla ratio is maintained in this group, despite the overall shrinkage, suggests that both cortical and medullary regions are affected proportionally at this higher dose.

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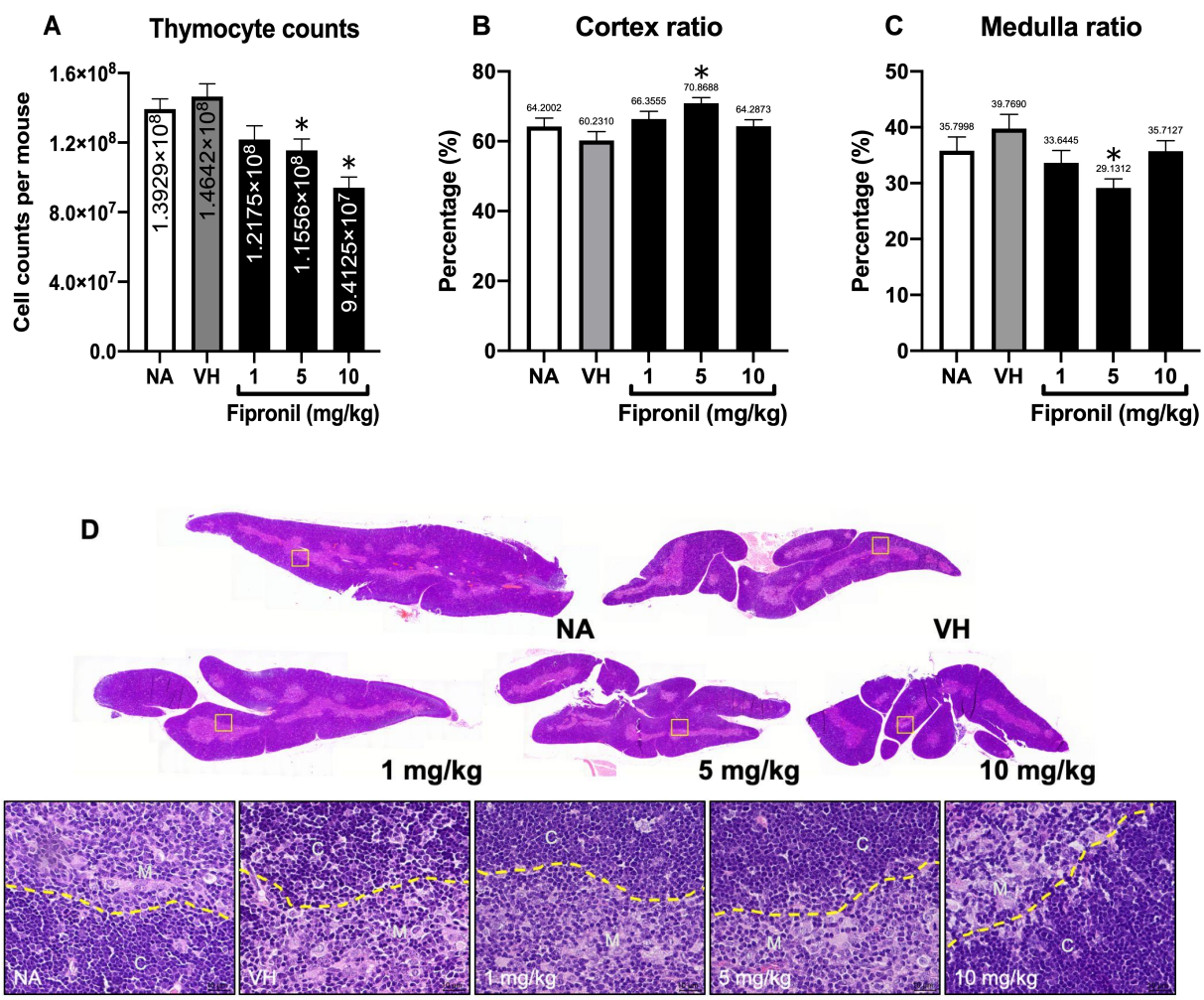

Figure 9. Reduction of thymocyte counts, thymus size, cortex, and medulla ratio by FPN

(A) Total thymocyte counts were isolated from each mouse and expressed as means ± SEM. (B, C) The ratio of cortex and medulla was quantified using ImageJ software as described in Materials and Methods. The cortex and medulla ratio was expressed as the mean ± SEM of thymus sections pooled from four independent experiments. *$p < 0.05$ as compared with the VH group. (D) Representative H&E-stained histological images of thymus sections in each treatment group were shown (spliced from original magnification 100×). The lower panels are enlarged images of the area of the yellow boxes (original magnification, 400×). Areas marked by the dashed line distinguish the cortex or medulla regions. *Adapted from* **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through*

doi:10.6342/NTU202501708

Dysregulation of IL-7-Associated Genes. Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060 © 2024 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence. Reprinted with permission from *Chem. Res. Toxicol.* 2024, 37, 1488−1500. Copyright 2024 American Chemical Society. Reprinted with permission from *Chem. Res. Toxicol.* 2024, 37, 1488−1500. Copyright 2024 American Chemical Society.

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4.2.4 Impact of FPN exposure on IL-7 protein expression

To further investigate the effects of FPN on the thymic microenvironment, I examined the expression of IL-7 protein within the thymus using IHC staining. IL-7 is a crucial cytokine within the thymus, playing an essential role in T cell development and survival.

Our IHC analysis revealed a significant attenuation in the number of IL-7-positive cells in both the cortex and medulla regions of the thymus in the high-dose FPN treatment group (10 mg/kg) (Fig. 10). This reduction in IL-7-producing cells suggests that FPN exposure may suppress IL-7 production within the thymus, potentially impacting thymocyte development and function. This observation is consistent with our previous finding of thymic atrophy in the high-dose FPN group, suggesting that FPN-induced thymic atrophy may, in part, be mediated through a reduction in IL-7 availability. Decreased IL-7 levels could lead to reduced thymocyte survival, impaired T cell development, or both. Further experiments will be necessary to definitively establish the precise mechanism by which FPN affects IL-7 expression and the functional consequences of this reduction in IL-7.

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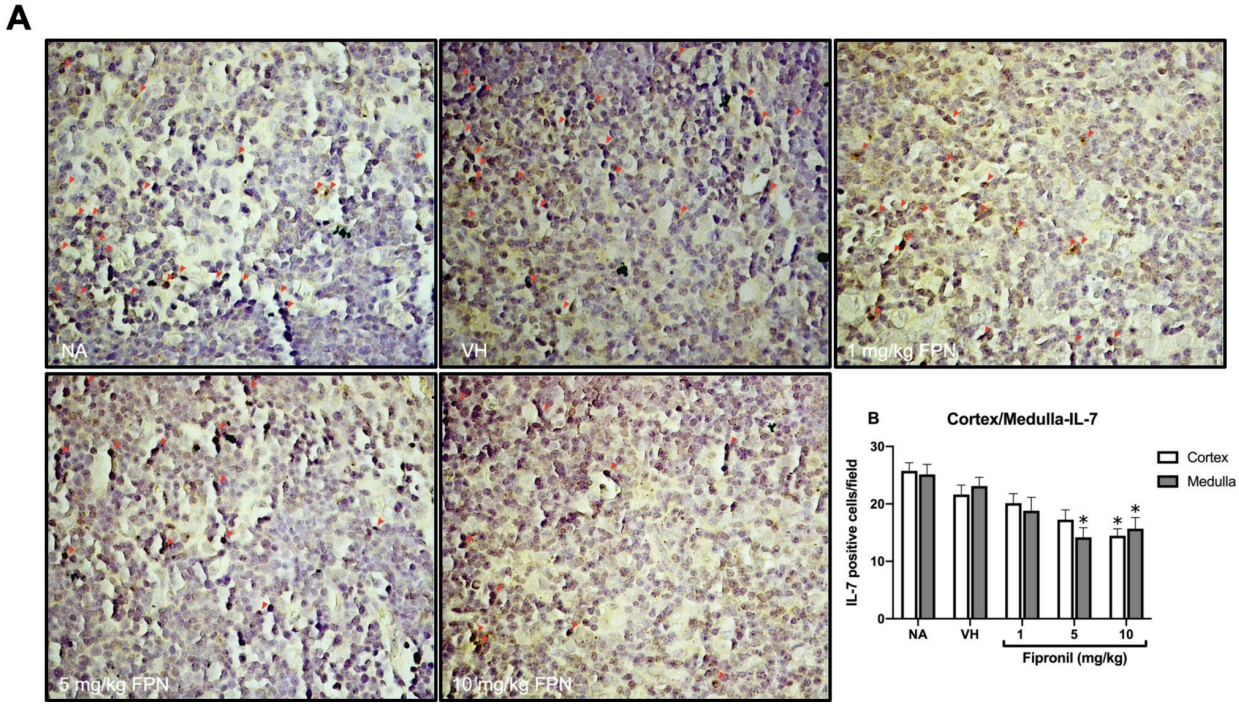

Figure 10. Reduction of IL-7-positive cells in the thymus

(A) Representative immunohistological images of thymus sections in each treatment group were shown (original magnification, 400×). Arrows indicate IL-7^{+} cells with red signals. (B) Quantified data for the number of IL-7 positive cells from the cortex or medulla area were expressed as the mean ± SEM of 20 samples per group (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes.* Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060

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4.2.5 FPN significantly decreases mRNA expression of transcription factors of T-cell lineage and IL-7 signaling in the thymus.

To investigate the molecular mechanisms underlying the observed changes in thymic cellularity, architecture, and IL-7 protein levels, I analyzed the mRNA expression of key genes involved in the IL-7 signaling pathway and T cell development. Total RNA was extracted directly from thymic tissue and subjected to qPCR. Our analysis revealed a significant decrease in the mRNA expression levels of several genes critical for IL-7 signaling, including *Il7*, *Il7r*, *Gabpa*, *Foxo1*, and *Foxo3* (Fig. 11A-E). This coordinated downregulation of multiple components of the IL-7 signaling pathway strongly suggests that FPN exposure significantly impairs IL-7 signaling within the thymus. Specifically, *Il7* mRNA levels were significantly reduced, which likely contributes directly to the observed decrease in IL-7 protein.

In addition to the downregulation of IL-7 signaling genes, I observed significant decreases in the mRNA expression of several key transcription factors essential for T cell maturation and development, including *Scf, Kit, Foxn1*, and *Lyl1* (Fig. 11F-I). The significant decreases in *Scf*, *Kit*, and *Lyl1* further suggest that FPN exposure disrupts the thymic microenvironment and impacts T cell development. These coordinated changes in transcription factor expression suggest that FPN exposure disrupts the expression of genes crucial for thymocyte development. The significant reduction in *Foxn1* mRNA is particularly noteworthy, as *Foxn1* is a critical regulator of TEC development and function, which are essential for supporting thymocyte development and IL-7 production. This decrease in *Foxn1* expression likely contributes to both the observed reduction in IL-7 protein levels and the thymic atrophy. *Sox13* mRNA levels were also assessed but did not show a statistically significant change (Fig. 11J).

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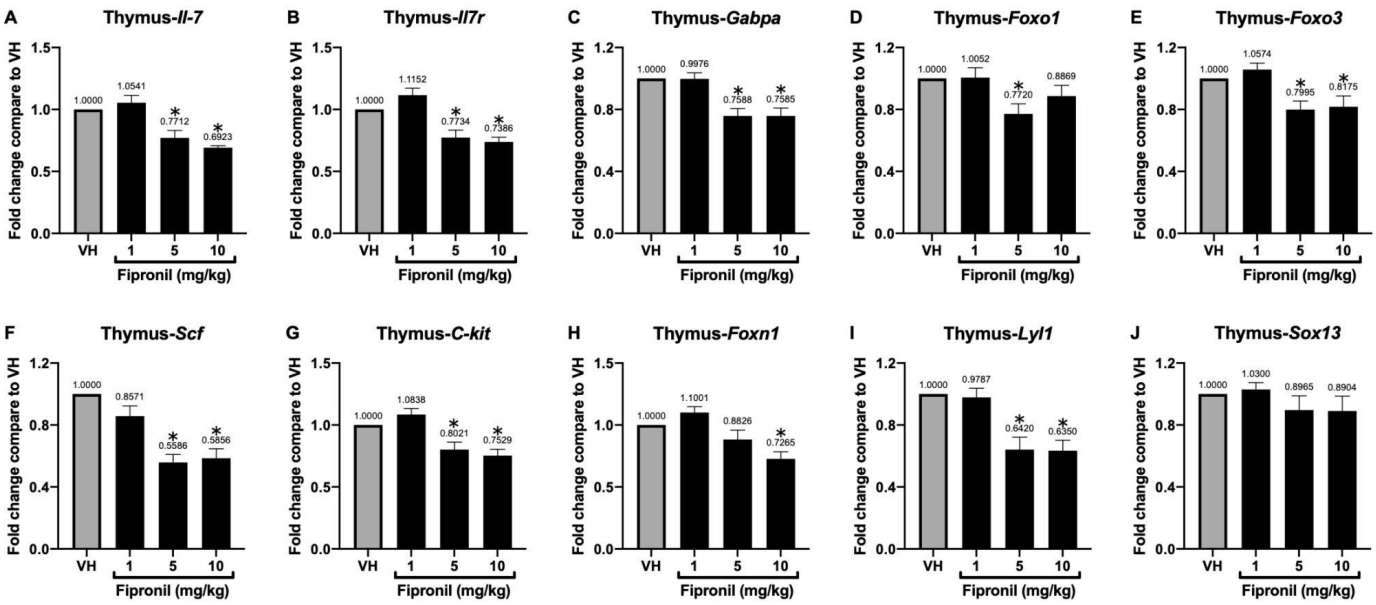

Figure 11. Decreased the mRNA expression of transcription factors of T-cell lineage and IL-7 signaling in the thymus

The total RNA of the thymus harvested from different treatment groups was extracted to detect the mRNA expression of transcription factors of T-cell lineage and IL-7 signaling by qPCR. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes.* Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060

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4.2.6 Reduction of T-Cell lineage transcription factors and IL-7 signaling-associated proteins in the thymus by FPN

To validate the observed changes in gene expression at the protein level, I performed Western blot analysis on thymic tissue lysates from each treatment group. Consistent with the qPCR results, FPN treatment led to a decrease in the protein expression of multiple components of the IL-7 signaling pathway and key transcription factors involved in T cell development (Fig. 12). Relative intensity (RI) values, normalized to β-actin and relative to the VH group, were used for semi-quantification of protein expression.

Specifically, I observed a dose-dependent decrease in IL-7 protein levels, with a marked reduction evident at the 10 mg/kg dose. IL-7R protein levels were also significantly decreased in both the 5 mg/kg and 10 mg/kg groups. GABPA protein levels showed a significant reduction in the 10 mg/kg group, while FOXO3A levels were significantly lower in both the 5 mg/kg and 10 mg/kg groups. Among the transcription factors examined, SCF protein levels were significantly decreased in the 10 mg/kg group. c-KIT protein levels were significantly reduced in the 10 mg/kg group. LYL1 protein levels were also significantly reduced in the 10 mg/kg group. These findings at the protein level strongly corroborate our gene expression data and further support the conclusion that FPN exposure significantly impacts the IL-7 signaling pathway and the expression of key proteins involved in T cell development. To further explore the impact of FPN on T cell development, I next examined the mRNA expression of key genes involved in IL-7 signaling and T cell lineage specification in ConA-stimulated thymocytes.

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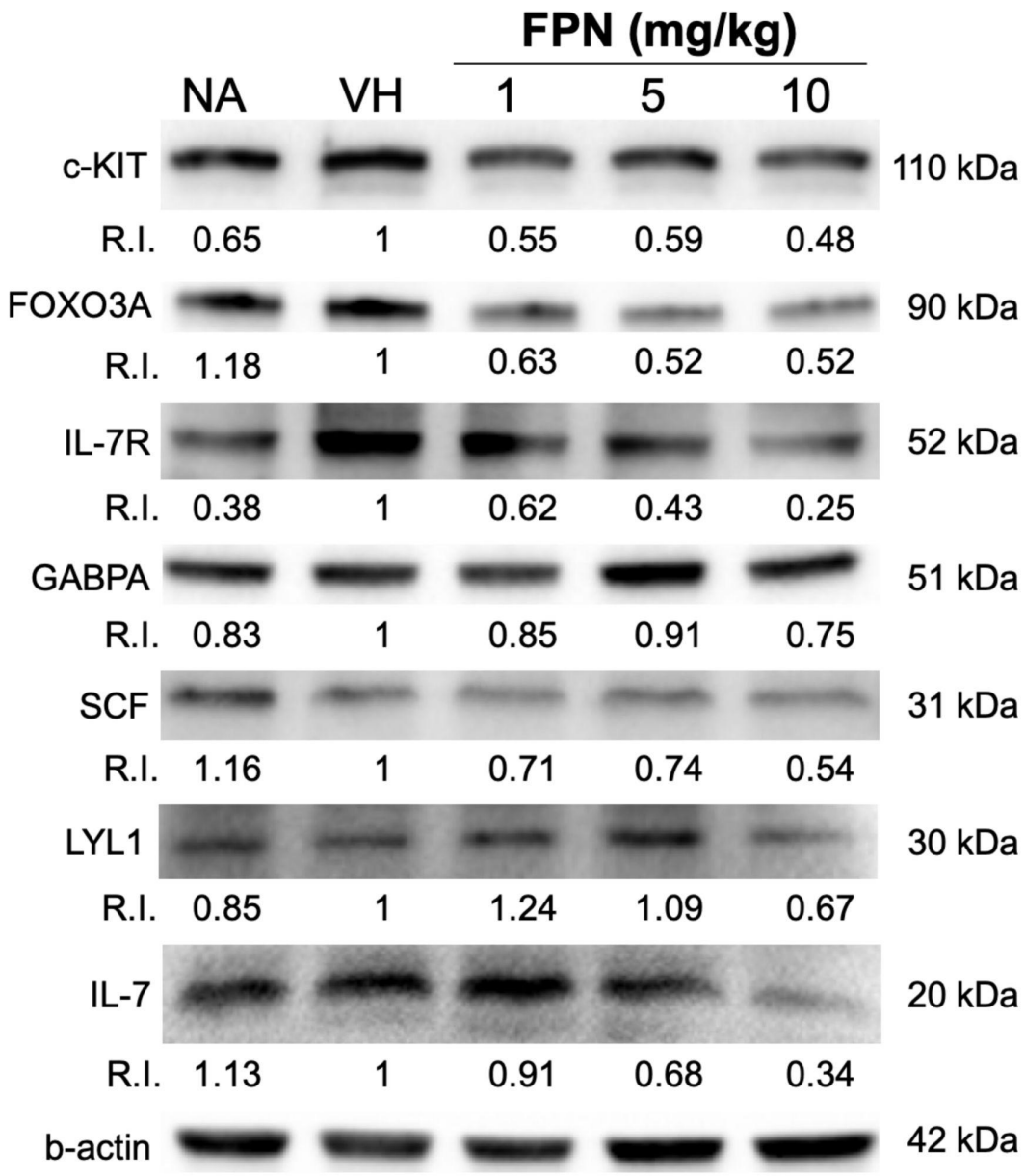

Figure 12. Decreased T-cell lineage transcription factors and IL-7 signaling-associated proteins in the thymus

The total protein of the thymus harvested from different treatment groups was extracted to detect the protein expression of transcription factors of T-cell lineage and IL-7 signaling by Western blotting. The expression level of β-actin was used as the loading control for semiquantification. The different protein/β-actin ratio in the treatment group was divided by the protein/β-actin ratio in VH as the relative intensity (RI). The result was representative of three independent experiments (N=9/ group).

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Adapted from **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes.* Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060

doi:10.6342/NTU202501708

4.2.7 FPN significantly decreased mRNA expression of *Il7r*, *Scf*, *Gabpα*, *Lyl1*, and *Sox13* in ConA-stimulated thymocytes.

To further investigate the impact of FPN on T cell development, primary thymocytes were isolated and stimulated with ConA for 24 hours. ConA, a common T cell activator, mimics antigen-induced T cell activation, triggering a cascade of gene expression changes associated with T cell development and differentiation. This approach allowed us to simulate the effects of FPN on activated T cells *in vitro*, providing insights into its mechanism of action.

I analyze the mRNA expression levels of key genes involved in IL-7 signaling and T-cell lineage transcription factors after ConA stimulation. These genes play critical roles in T cell development and differentiation, and alterations in their expression can directly influence T cell function and immune responses. The results demonstrated a significant downregulation of *Il7r*, *Gabpα*, *Scf*, *Lyl1*, and *Sox13* mRNA expression in thymocytes treated with a high dose of FPN (5 to 10 mg/kg) compared to the VH control (Fig. 13A-B & E-G). These data indicate that FPN significantly suppresses the expression of these critical genes in ConA-stimulated thymocytes.

These findings showed that FPN interferes with IL-7 signaling and the expression of T cell development-related proteins. IL-7 signaling is essential for T cell survival, proliferation, and differentiation, while *Scf*, *Gabpα*, *Lyl1*, and *Sox13* are indispensable transcription factors in T cell lineage development. The inhibitory effect of FPN on these gene expressions may impede T cell development, thereby affecting immune system function.

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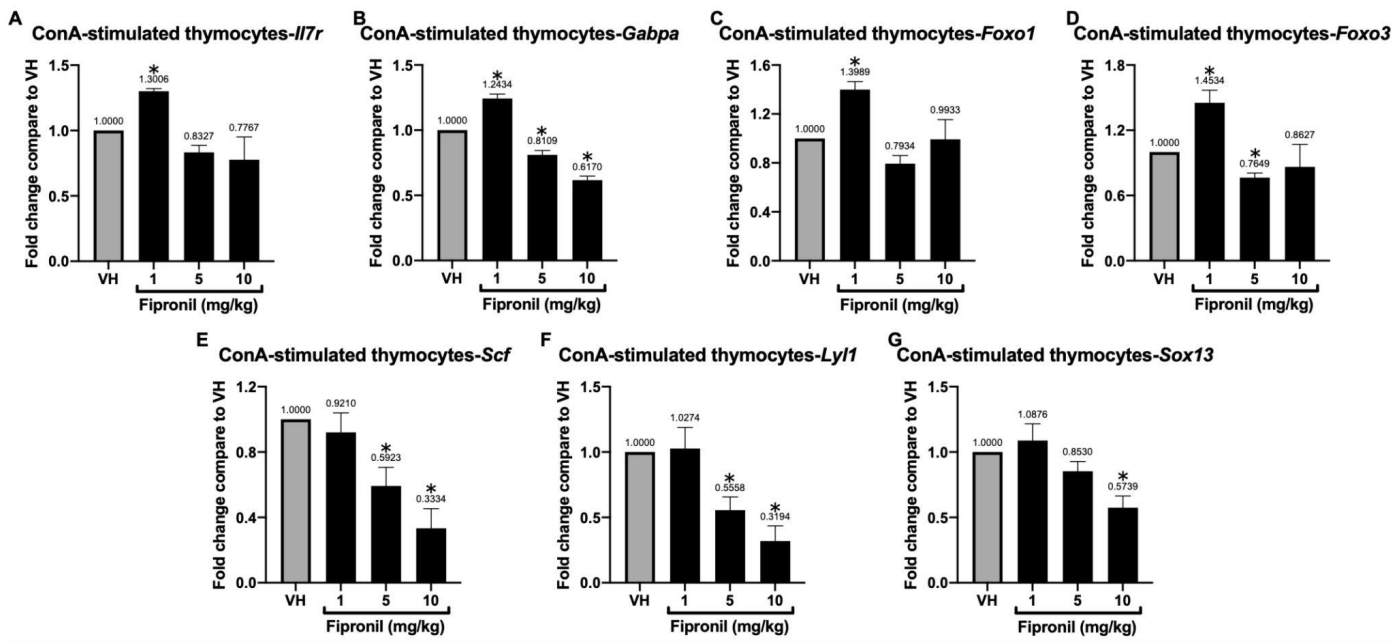

Figure 13. Decreased mRNA expression of *Lyl1*, *Sox13*, *Scf*, *Il-7 receptor*, and *Gabpα* in ConA-stimulated thymocytes

The total RNA of thymocytes (6×10^6 cells/mL) harvested from different treatment groups stimulated by ConA was extracted to detect the mRNA expression of *Lyl1*, *Sox13*, *Scf*, *Il-7 receptor,* and *Gabpα* by qPCR. The expression level of *Hprt* was used as the control for semiquantification. The expression level of HPRT was used as the control for semiquantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes.* Chem. Res. Toxicol. 2024, 37, 1488−1500. doi: https://doi.org/10.1021/acs.chemrestox.4c00060

doi:10.6342/NTU202501708

4.2.8 Differential effects of FPN on the production of IL-2, IL-4, and IFN-γ *ex vivo*.

To explore the potential immunomodulatory effects of FPN, its cytotoxicity against thymocytes was initially assessed using the MTT assay (Fig. 14A).

Subsequently, I investigated the influence of FPN on cytokine secretion by thymocytes activated with ConA, a plant lectin commonly employed as a polyclonal T lymphocyte mitogen. The mechanism of ConA action involves binding to mannose and glucose residues on T-cell surface glycoproteins. This interaction triggers the cross-linking of these receptors, initiating a cascade of downstream intracellular signaling events. Key events include increased calcium influx, leading eventually to the activation of specific transcription factors. Upon activation, these factors translocate to the nucleus to regulate the expression of genes governing cell cycle progression and mitosis, thereby driving T-cell proliferation.

Under these stimulation conditions, our results demonstrated that FPN treatment led to a significant reduction in the secretion of IL-2, a crucial cytokine for T-cell growth and differentiation, by the activated thymocytes. Concurrently, an increase was observed in the production of both IL-4 and IFN-γ (Fig. 14B-D). As these cytokines play distinct regulatory roles in immune responses, this observed shift might suggest a potential for FPN to modulate the balance among functional T-cell subsets.

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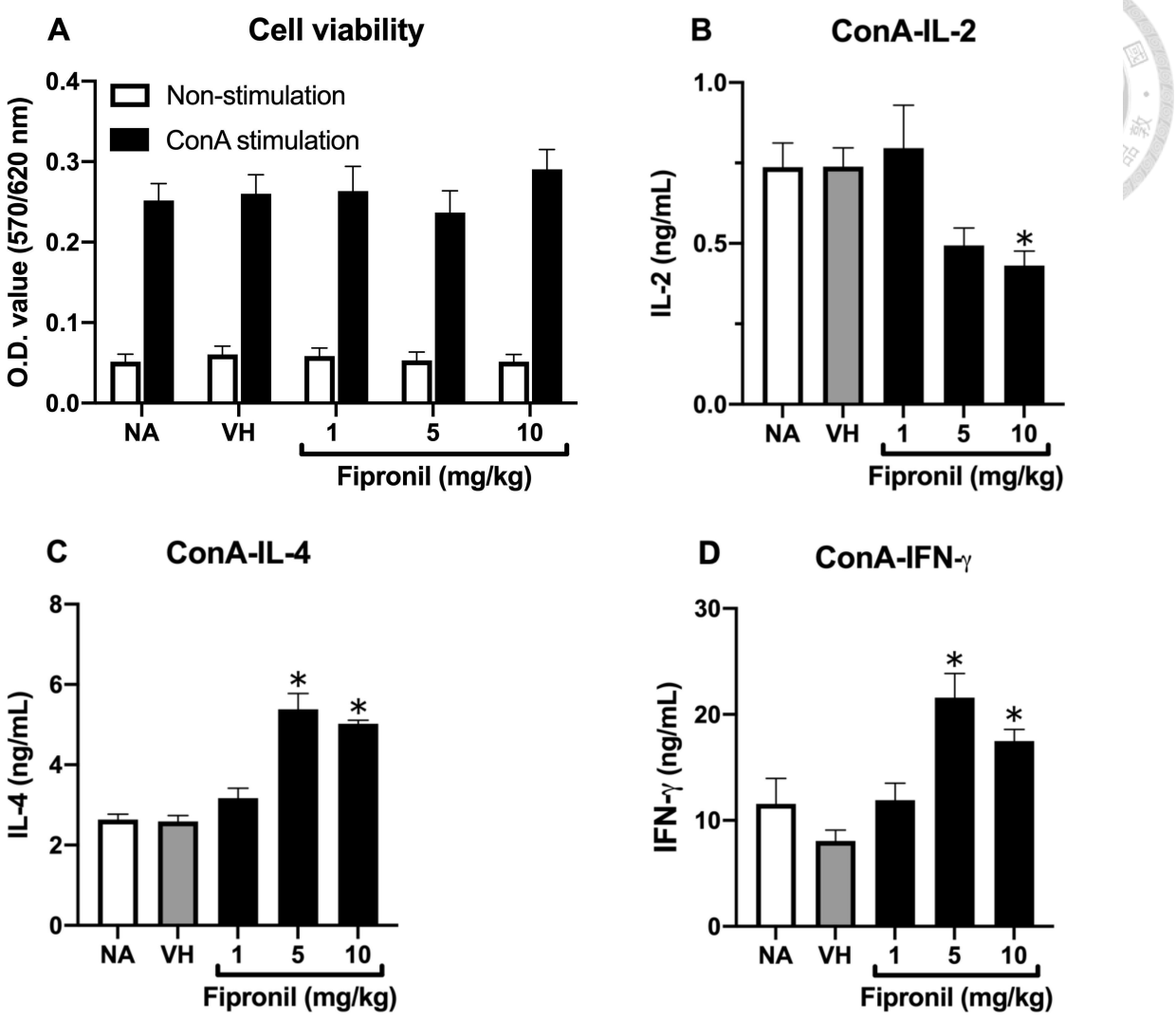

Figure 14. The alternative effects of FPN on IL-2, IL-4, and IFN-γ by ConA-stimulated thymocytes

Thymocytes (6×10^6 cells/mL) were prepared from each group of mice and cultured in the absence or presence of concanavalin A (ConA; 5 μg/mL) for 48 h. The metabolic activity of cells was determined by an MTT assay, and the level of IL-2, IL-4, and IFN-γ in the supernatants was measured by ELISA. Data were expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Tung C-W, Huang W-H, Lin C-S, Wang C-C** (2024) *Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through*

doi:10.6342/NTU202501708

doi:10.6342/NTU202501708

4.3 The potential mechanism of FPN-induced thymic immunotoxicity through reactive oxygen species-driven mitochondrial apoptosis.

Partial reprint from MDPI Toxics

Authors: Jui-Fang Kuo, Yai-Ping Hsiao, Yao-De Wang, Hsin-Pei Weng, and Chia-Chi Wang

Title: Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes (Kuo et al., 2025)

4.3.1 FPN significantly attenuated the mRNA expression of key Bcl-2 family members *in vivo*.

Our preceding research established that 7 doses of exposure to FPN induced a marked thymic atrophy in mice, characterized by a substantial decline in the overall population of thymocytes. To gain a more comprehensive understanding of the molecular mechanisms underpinning these morphological and cellular alterations, I proceeded to investigate the expression profiles of genes critically involved in the intricate regulation of apoptosis and the complex processes of T-cell development. Total mRNA was extracted from thymic tissues across different treatment groups to precisely quantify the expression levels of selected members of the Bcl-2 family, a pivotal group of genes governing apoptotic pathways. This quantitative analysis, performed using qPCR, encompassed key anti-apoptotic members, namely *Bcl-2*, *Bcl-6*, and *Mcl-1*, which are known to promote cell survival by inhibiting the release of pro-apoptotic factors from the mitochondria. Additionally, I examined the expression of the intrinsic pathway-associated pro-apoptotic genes Bim and Bnip3, which play crucial roles in initiating apoptosis in response to cellular stress or developmental signals (Fig. 15A-E).

Following the administration of FPN, a statistically significant downregulation was observed

doi:10.6342/NTU202501708

in the mRNA expression levels of the anti-apoptotic genes *Bcl-2*, *Mcl-1*, and *Bcl-6*. This reduction suggests a compromised cellular defense mechanism against apoptotic stimuli within the thymus, potentially contributing to the observed thymocyte depletion. The decreased expression of these survival-promoting genes could render thymocytes more susceptible to various apoptotic triggers, thereby exacerbating thymic atrophy.

Interestingly, the expression of the pro-apoptotic genes *Bim* and *Bnip3* was also significantly reduced in response to FPN exposure. *Bim* (Bcl-2-like protein 11) is a potent initiator of apoptosis, particularly in response to cytokine deprivation and developmental cues during T-cell selection. *Bnip3* (Bcl-2/adenovirus E1B 19 kDa interacting protein 3) can induce apoptosis under conditions of cellular stress, such as hypoxia. The concurrent downregulation of both pro- and anti-apoptotic genes suggests a complex and potentially multifaceted response of thymocytes to FPN. This observation warrants further investigation to fully elucidate the net effect on thymocyte survival and the specific signaling pathways involved.

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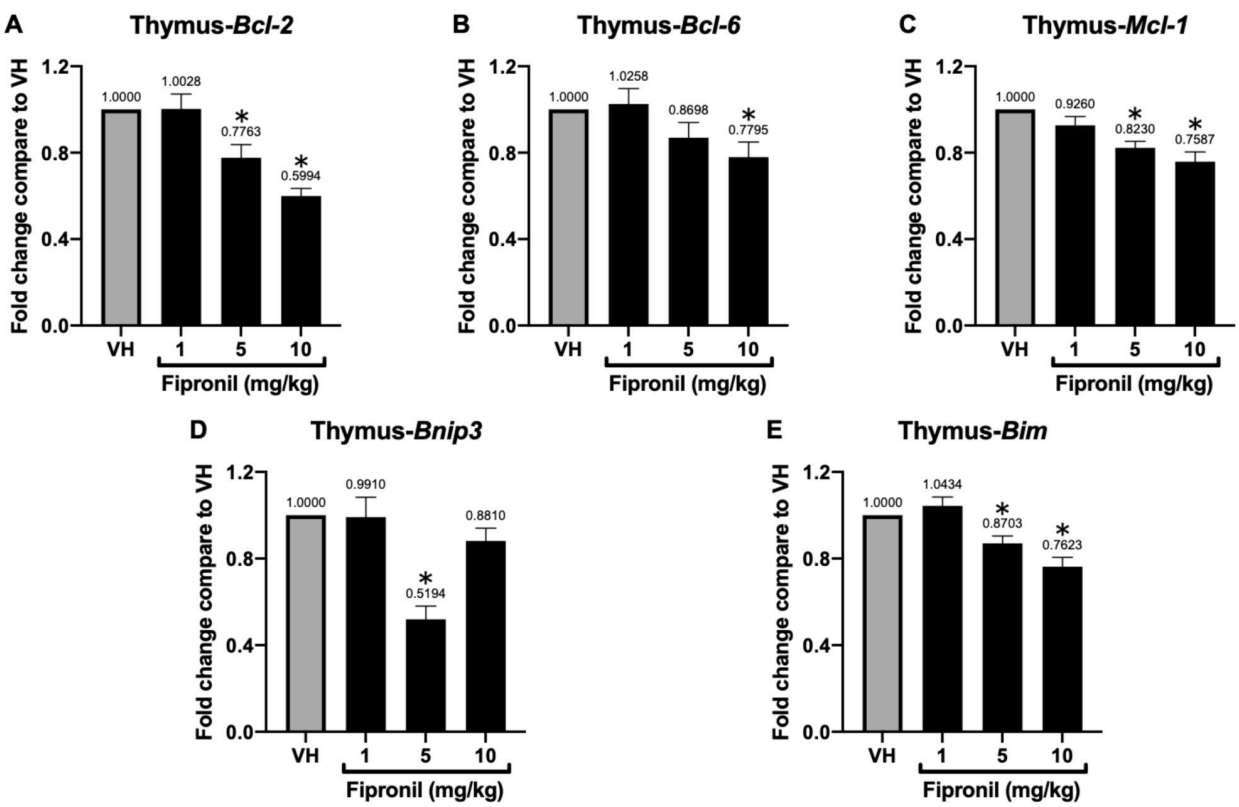

Figure 15. Reduced mRNA expression of the *Bcl-2* family by FPN *in vivo*

The total mRNA harvested from different treatment groups of the thymus was extracted to detect the mRNA expression by qPCR. The expression level of Hprt was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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4.3.2 FPN significantly attenuated Bcl-2 family mRNA expression in ConA-stimulated thymocytes *ex vivo*.

Whole thymic tissue RNA analysis in our previous experiments (as described in section 4.3.1) revealed a significant downregulation of Bcl-2 family target genes following FPN exposure. However, recognizing that whole thymic tissue comprises a heterogeneous population of cell types, I sought to minimize potential confounding effects arising from this cellular heterogeneity and to directly assess the impact of FPN on thymocytes. The thymocytes were subsequently isolated and stimulated *ex vivo* with ConA to evaluate the direct effects of FPN on these cells. Given that *in vivo* FPN exposure might have already influenced thymocyte functions at the time of sampling, I aimed to investigate whether thymocytes responding to ConA stimulation, which mimics T cell activation through TCR crosslinking, exhibit consistent alterations in apoptosis-related gene expression. By analyzing the expression of key apoptotic regulators in these ConA-stimulated thymocytes, I intended to determine if the transcriptional changes observed *in vivo* persist under *ex vivo* activation, potentially indicating a sustained dysregulation of survival pathways following FPN exposure. Consistent with the findings from whole thymic tissue, high-dose FPN treatment significantly reduced the mRNA expression of *Bcl-2*, *Bcl-6*, *Mcl-1*, *Bnip3*, and *Bim* compared to the vehicle control (Fig. 16A-E).

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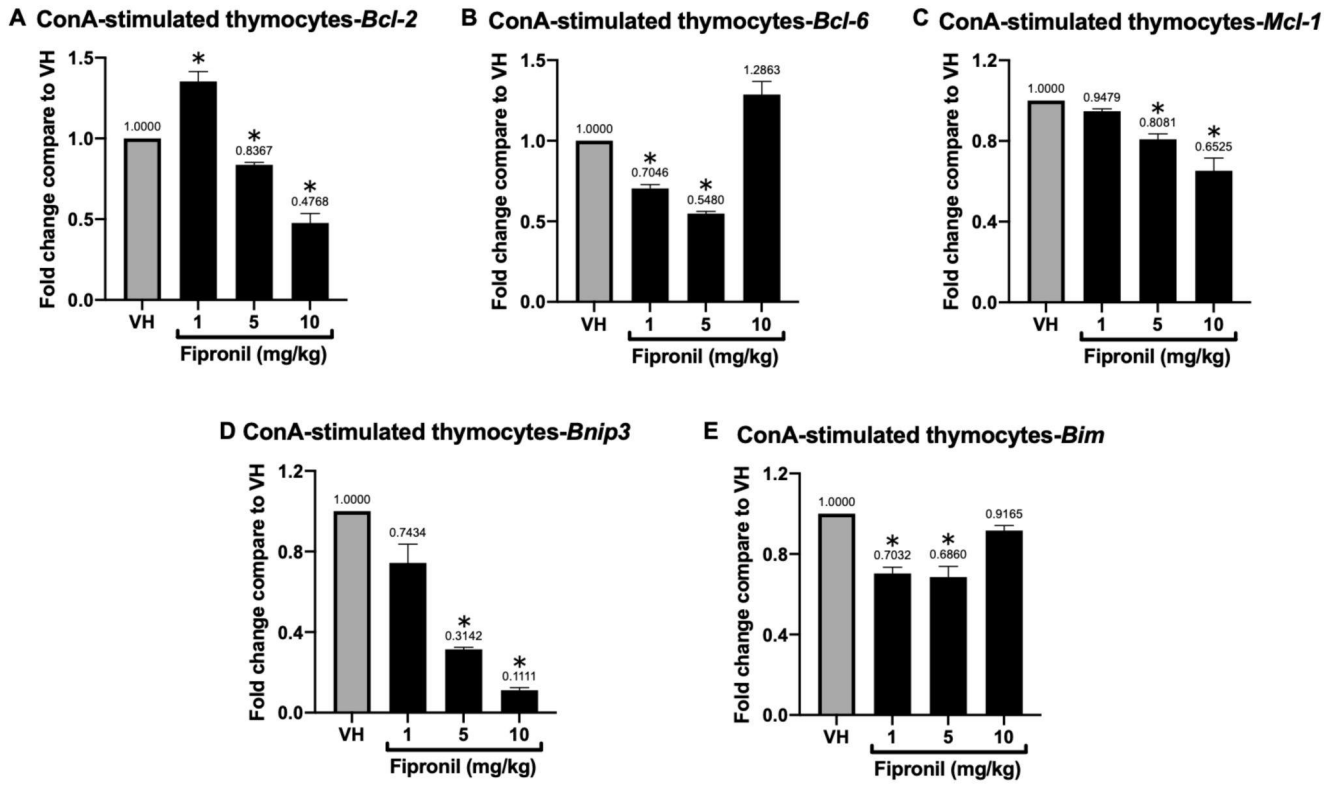

Figure 16. Reduced mRNA expression of the *Bcl-2* family by FPN *ex vivo*

Total RNA was extracted from primary thymocytes following ConA stimulation to quantify mRNA expression by qPCR. The expression level of Hprt was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N=20/group). *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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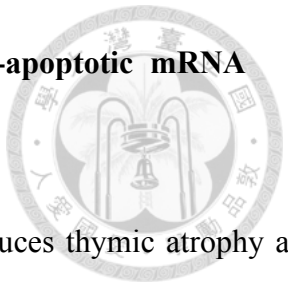

4.3.3 Acute *in vitro* exposure to FPN significantly attenuated anti-apoptotic mRNA expression in primary thymocytes.

Our previous *in vivo* studies demonstrated that FPN exposure induces thymic atrophy and disrupts the expression of genes and proteins essential for T cell development and maturation. To further dissect the underlying cellular and molecular mechanisms, I employed an *in vitro* acute exposure model by treating isolated primary thymocytes with FPN. The anti-apoptotic capacity of these primary thymocytes was evaluated by assessing the mRNA expression of key *Bcl-2* family members. This analysis revealed a significant reduction in the expression of the anti-apoptotic genes *Bcl-2*, *Bcl-6*, and *Mcl-1* following FPN treatment compared to the VH group, mirroring the qPCR results observed in our *in vivo* model. In contrast, the expression of the pro-apoptotic gene *Bim* was significantly upregulated in a dose-dependent manner (Fig. 17A-E). These *in vitro* findings underscore that even acute FPN exposure disrupts multiple apoptotic regulatory mechanisms in thymocytes, not only diminishing their anti-apoptotic potential but also selectively influencing pathways associated with mitochondrial integrity and stress-induced apoptosis.

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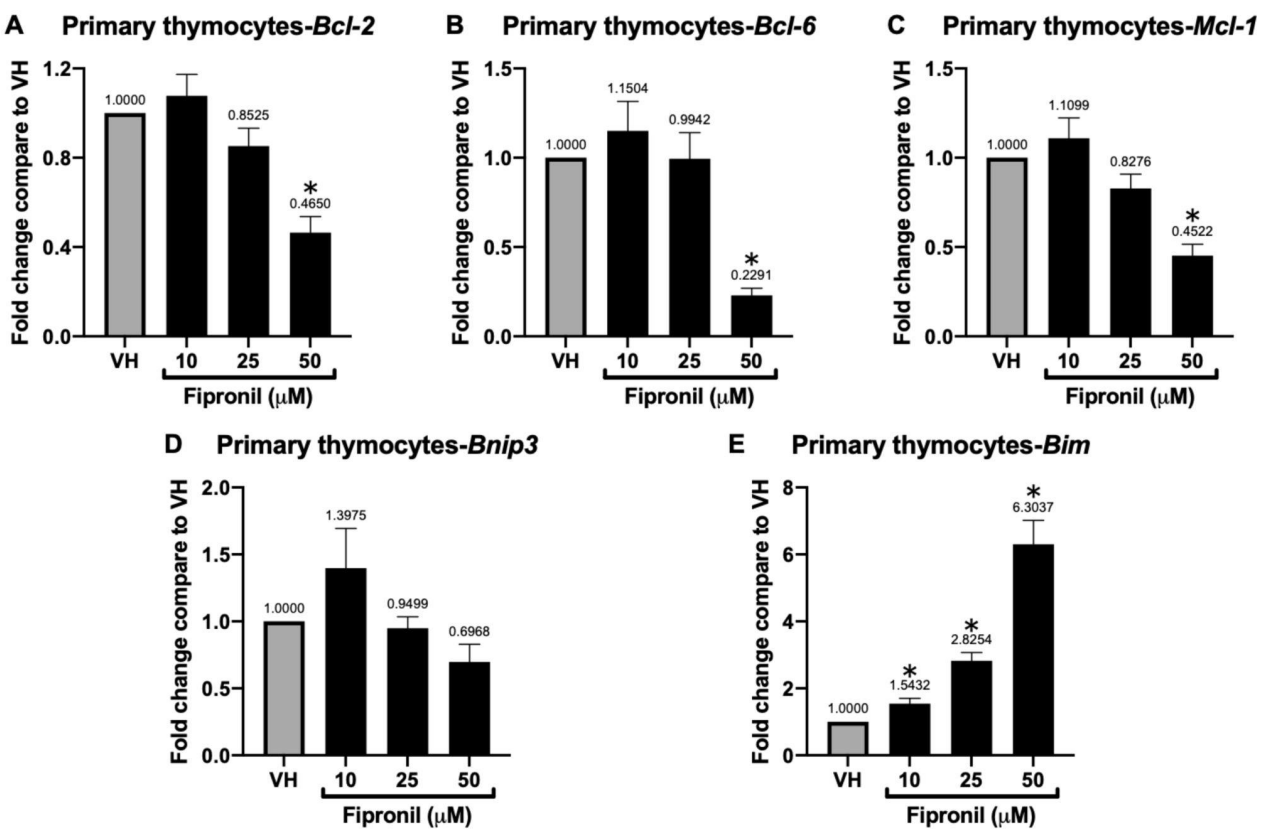

Figure 17. Reduced mRNA expression of the *Bcl-2* family by FPN *in vitro*

The total mRNA harvested from different treatment groups was extracted to detect the mRNA expression by qPCR. The expression level of Hprt was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group. *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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4.3.4 Cytotoxic and immunosuppressive effects of FPN on primary thymocytes

The cytotoxic effect of FPN on primary thymocytes was evaluated using the MTT assay under PMA/Iono stimulation. Ionomycin facilitates the release of intracellular calcium ions (Ca^{2+}) from the endoplasmic reticulum (ER), thereby activating Ca^{2+}-sensitive enzymes and synergistically enhancing protein kinase C (PKC) activation in conjunction with PMA. This combined action effectively induces T cell activation, proliferation, and cytokine production, providing a robust *ex vivo* model for studying T cell function and signaling pathways. Following treatment with FPN at concentrations of 5, 10, 25, and 50 μM, thymocyte viability was significantly reduced in a dose-dependent manner (Figure 18A). Similarly, under PMA/Iono stimulation, high concentrations of FPN (50 μM) significantly decreased IL-2 secretion (Fig. 18C). Notably, supplementation with N-acetylcysteine (NAC, final concentration: 1 mM), a known antioxidant, effectively reversed the toxic effects induced by FPN (Fig. 18B and D).

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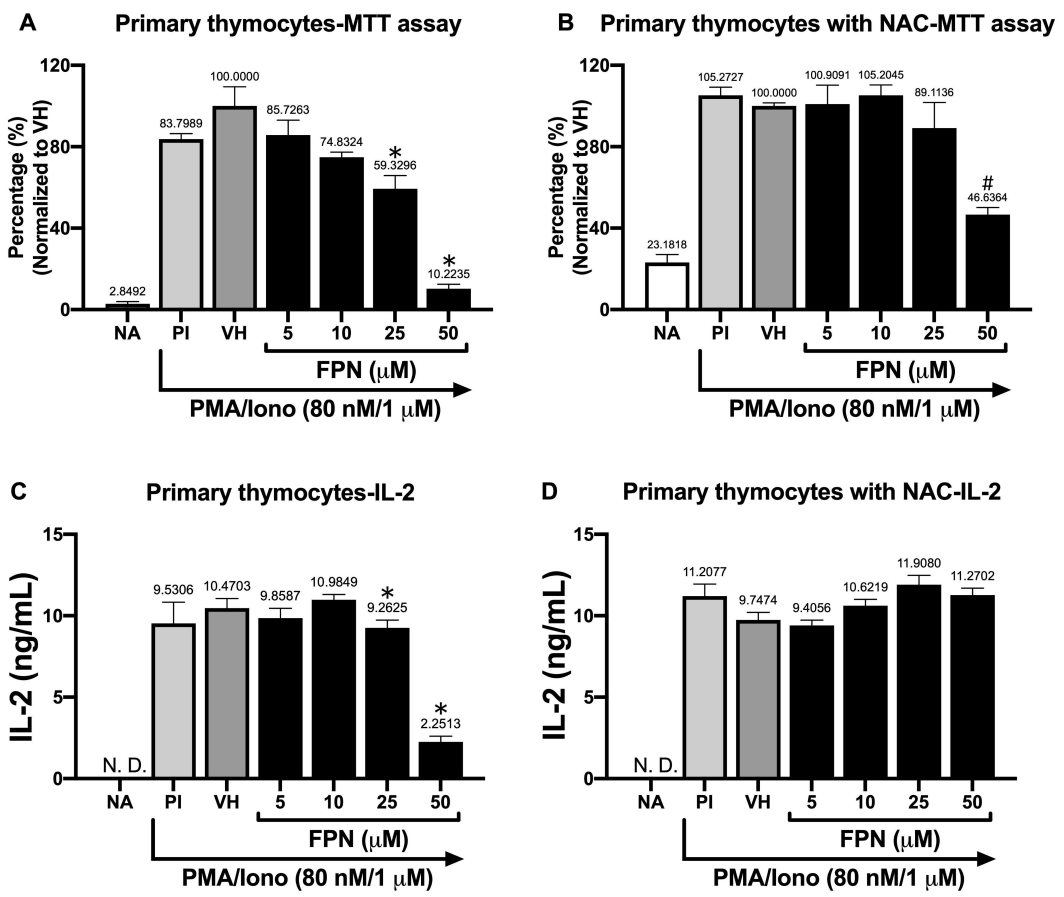

Figure 18. The effects of FPN on cell viability and IL-2 production by PMA/Iono-stimulated primary thymocytes

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations and stimulated with PMA/Iono (PMA/Iono: 80 nM/1 μM) for 24 h. (A) The viability of thymocytes was measured by MTT assay. (B) The level of IL-2 in the supernatants was measured by ELISA. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH without the NAC group. #$p < 0.05$ was significant compared to the VH with the NAC group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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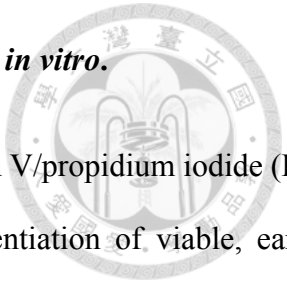

4.3.5 Effects of FPN treatment on apoptosis in the primary thymocytes *in vitro*.

Apoptosis in thymocytes was quantitatively assessed using Annexin V/propidium iodide (PI) double staining, a flow cytometric technique that allows for the differentiation of viable, early apoptotic, and late apoptotic or necrotic cells. As depicted in Figure 19A-C, cells located in the Q4 quadrant (Annexin V^-/PI^-) represent the population of viable thymocytes. These cells exhibit minimal binding of both Annexin V and PI, indicating that they possess intact cell membranes and are not undergoing apoptosis or necrosis. In contrast, cells in the Q2 and Q3 quadrants represent cells with varying degrees of membrane integrity. Annexin V has a high affinity for phosphatidylserine, a phospholipid that is normally located on the inner leaflet of the plasma membrane but translocates to the outer leaflet during the early stages of apoptosis. Therefore, cells in Q3 are considered to be in the early stages of apoptosis, as phosphatidylserine externalization is an early apoptotic event, while the cell membrane is still largely intact and impermeable to PI. Cells in the Q2 quadrant (Annexin V^+/PI^+) are positive for both Annexin V and PI. PI is a fluorescent dye that can only penetrate cells with compromised membrane integrity. Thus, cells in Q2 are considered to be in the late stages of apoptosis or are undergoing necrosis, as they have lost membrane integrity. Importantly, our findings, as summarized in Figure 19, revealed a clear concentration- and time-dependent increase, analyzed by two-way ANOVA, in the proportion of thymocytes undergoing apoptosis following exposure to FPN, suggesting that FPN induces programmed cell death in these cells.

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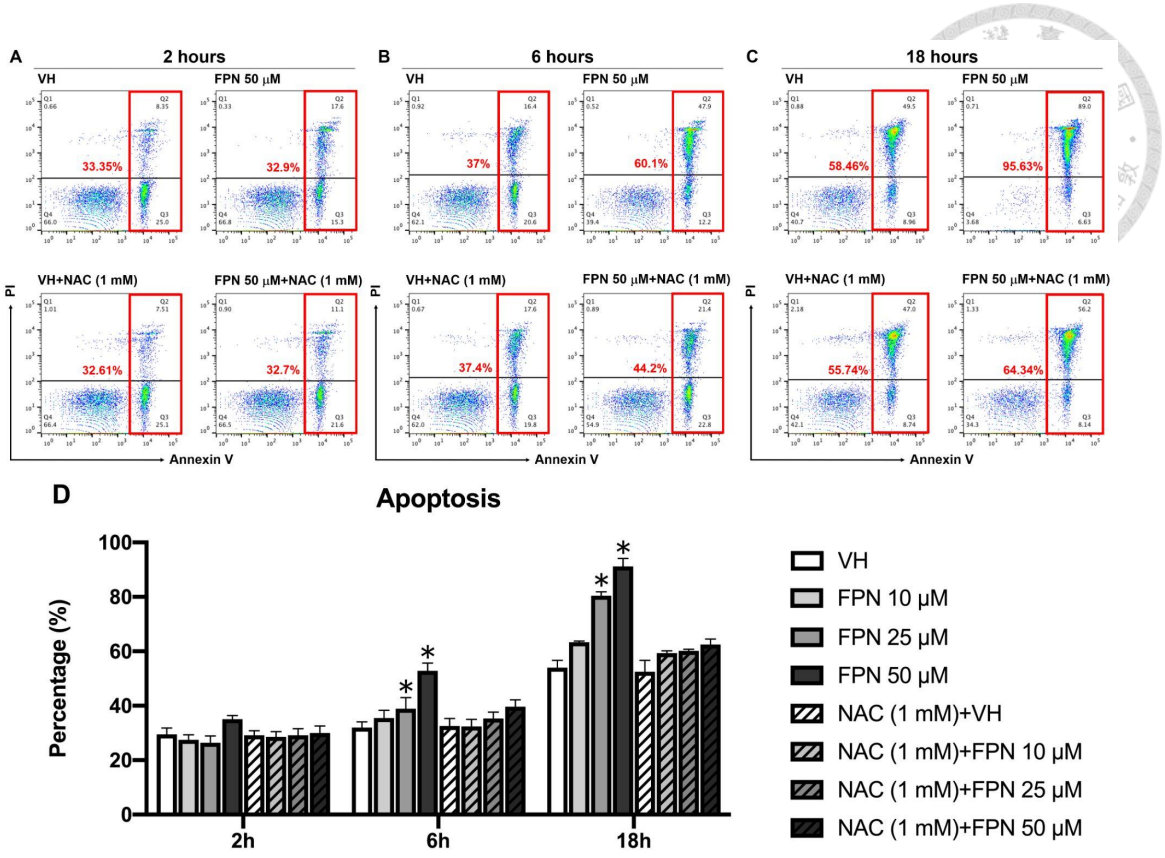

Figure 19. Induction of thymocyte apoptosis by FPN *in vitro*

The apoptosis indicator was measured by Annexin V/PI staining. (A-C) The representative dot plot shows either the VH group and 50 μM FPN with or without NAC treatment at different time points. (D) Statistical data represent the sum of Annexin V^+/PI^+ and Annexin V^+/PI^- populations. The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 2, 6, and 18 h. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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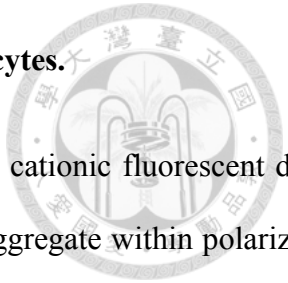

4.3.6 Fipronil induces mitochondrial depolarization in primary thymocytes.

I assessed the mitochondrial membrane potential (Δψm) using the cationic fluorescent dye JC-1. This dye is widely employed to monitor Δψm due to its ability to aggregate within polarized mitochondria, forming J-aggregates that emit red fluorescence. Upon mitochondrial depolarization, the dye exists predominantly as a green fluorescent monomer in the cytoplasm. Consequently, a decrease in the red/green fluorescence ratio indicates a loss of Δψm.

In primary thymocytes exposed to 50 μM FPN, a statistically significant increase in the proportion of cells exhibiting mitochondrial depolarization was observed as early as 6 hours post-treatment. This rapid decline in Δψm at the highest concentration suggests a direct and potent disruption of mitochondrial function. Furthermore, by 18 hours of FPN exposure, a pronounced increase in mitochondrial depolarization was evident across a broader range of concentrations, affecting cells treated with 10, 25, and 50 μM FPN (Fig. 20). This concentration- and time-dependent pattern highlights the progressive impairment of mitochondrial function in thymocytes following FPN exposure, potentially leading to compromised ATP production and contributing to cellular dysfunction and death.

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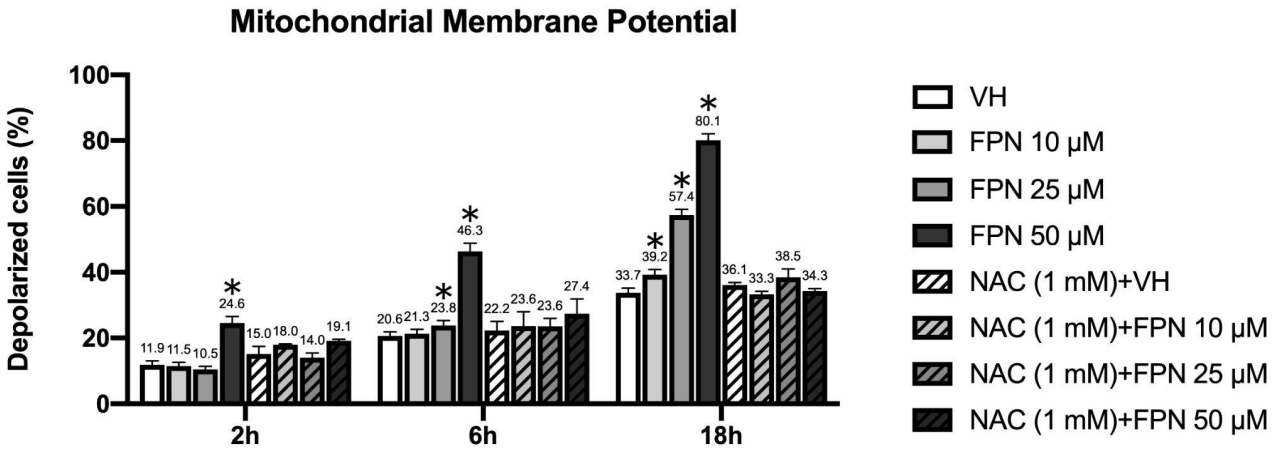

Figure 20. Induced proportion of depolarized primary thymocytes by FPN

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 2, 6, and 18 h. The proportion of depolarized cells was measured by JC-1 staining. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. The supplementation of the antioxidant N-acetylcysteine (NAC, final concentration: 1 mM) can effectively reduce the changes in mitochondrial membrane potential caused by FPN. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204

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4.3.7 Fipronil induces depletion of intracellular calcium in primary thymocytes.

To investigate potential disruptions in intracellular calcium homeostasis resulting from FPN-induced mitochondrial membrane potential loss, intracellular calcium levels (Ca^{2+}) were assessed using the fluorescent calcium indicator Fluo-4 AM. Fluo-4 AM is a cell-permeant dye that, once inside the cell, is cleaved by esterases to release Fluo-4, which exhibits a significant increase in fluorescence upon binding to Ca^{2+}. A decrease in Fluo-4 fluorescence intensity, therefore, indicates a reduction in Ca^{2+}.

In primary thymocytes treated with 50 μM FPN, a significant reduction in Ca^{2+} was observed starting at 6 hours post-treatment. This suggests that FPN exposure impairs the ability of thymocytes to maintain normal intracellular calcium levels. Furthermore, starting from 18 hours, a marked decrease in Ca^{2+} was also evident in cells treated with FPN concentrations ranging from 10 to 50 μM (Fig. 21). Dysregulation of calcium homeostasis is a critical event in cellular signaling and can contribute to various cellular dysfunctions, including apoptosis. The observed calcium depletion may be linked to the disruption of mitochondrial function, as mitochondria play a crucial role in calcium buffering within the cell.

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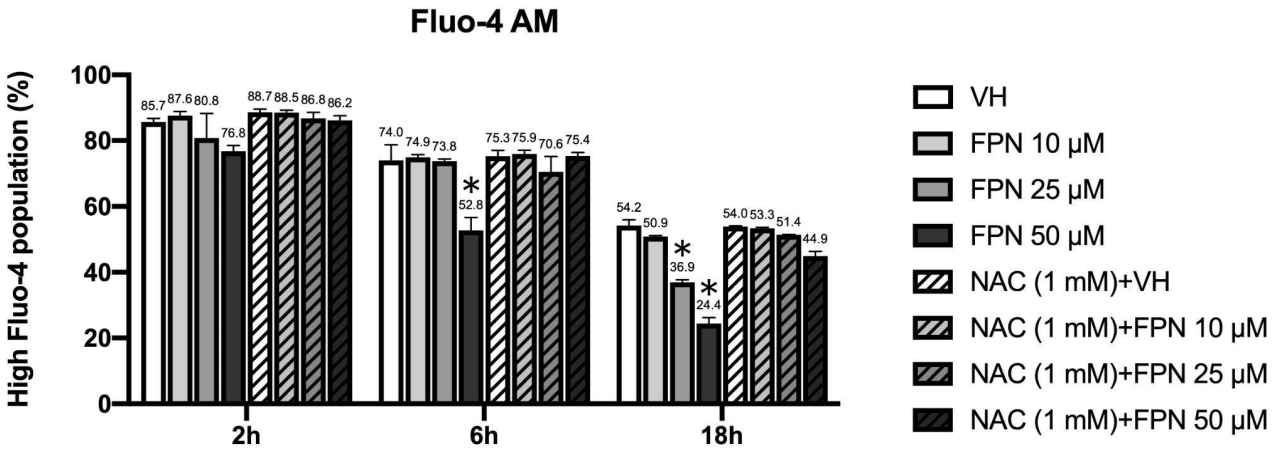

Figure 21. Depletion of intracellular calcium in primary thymocytes by FPN

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 2, 6, and 18 h. The intracellular calcium levels were measured using Fluo-4 AM staining. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. The supplementation of the antioxidant N-acetylcysteine (NAC, final concentration: 1 mM) can effectively reduce the changes in mitochondrial membrane potential caused by FPN. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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4.3.8 Fipronil reduces intracellular glutathione levels in primary thymocytes.

The intracellular levels of the key antioxidant GSH were assessed using CellTracker CMF-DA staining. Results indicated a significant decrease in GSH levels in the 50 μM FPN treatment group over a time course of 2 to 18 hours. Notably, lower doses of FPN (10 and 25 μM) also induced a decrease in GSH levels starting at the 18-hour time point (Fig. 22), which was consistent with the observed increase in apoptosis detected by Annexin V/PI staining in earlier experiments. This reduction in GSH, a critical component of the cellular antioxidant defense system, indicates an increase in oxidative stress within the cells, suggesting that FPN exposure disrupts the delicate redox balance and renders thymocytes more vulnerable to oxidative damage. The observation that the antioxidant NAC (final concentration: 1 mM) effectively mitigated FPN-induced mitochondrial membrane potential changes, calcium depletion, and GSH reduction further supports the role of oxidative stress in FPN's toxicity.

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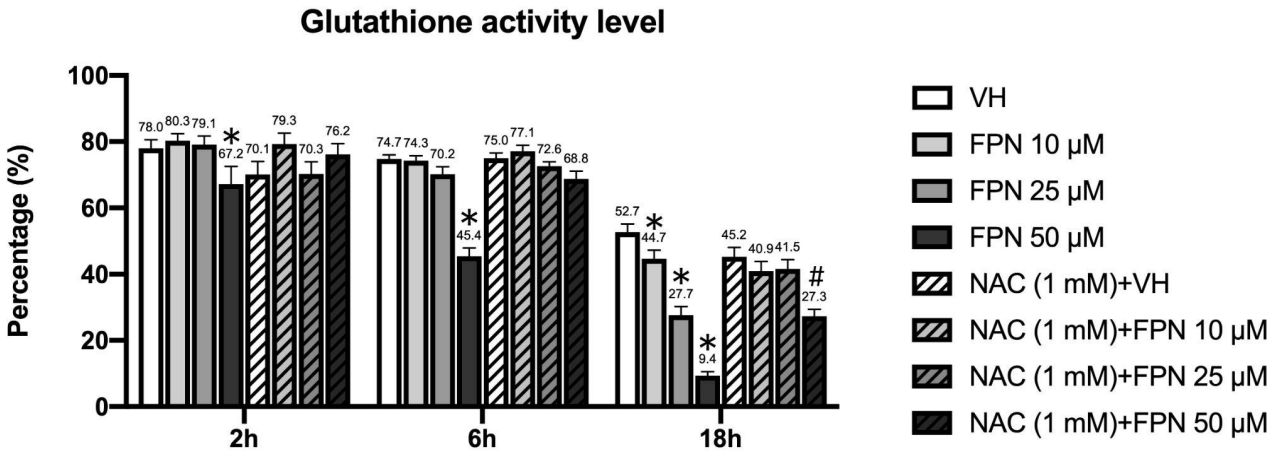

Figure 22. Reduction of GSH level by FPN

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 2, 6, and 18 h. The level of GSH was measured by CMF-DA staining. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. #$p < 0.05$ was significant compared to the VH with the NAC group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204

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4.3.9 Fipronil induces intracellular reactive oxygen species accumulation in primary thymocytes.

The levels of intracellular ROS, specifically hydrogen peroxide (H_2O_2), were determined using the H_2-DCFDA detection kit. Results revealed a marked H_2O_2 accumulation in the 50 μM FPN treatment group at an early time point of 0.5 to 2 hours (Fig. 23), indicating a rapid induction of oxidative stress. Interestingly, after six hours, the effects of increased oxidative damage by FPN were no longer as prominent, suggesting a potential transient ROS burst or the activation of compensatory antioxidant mechanisms at later time points. The observation that treatment with the antioxidant NAC could prevent the oxidative stress induced by FPN further underscores the involvement of ROS in FPN's cellular effects.

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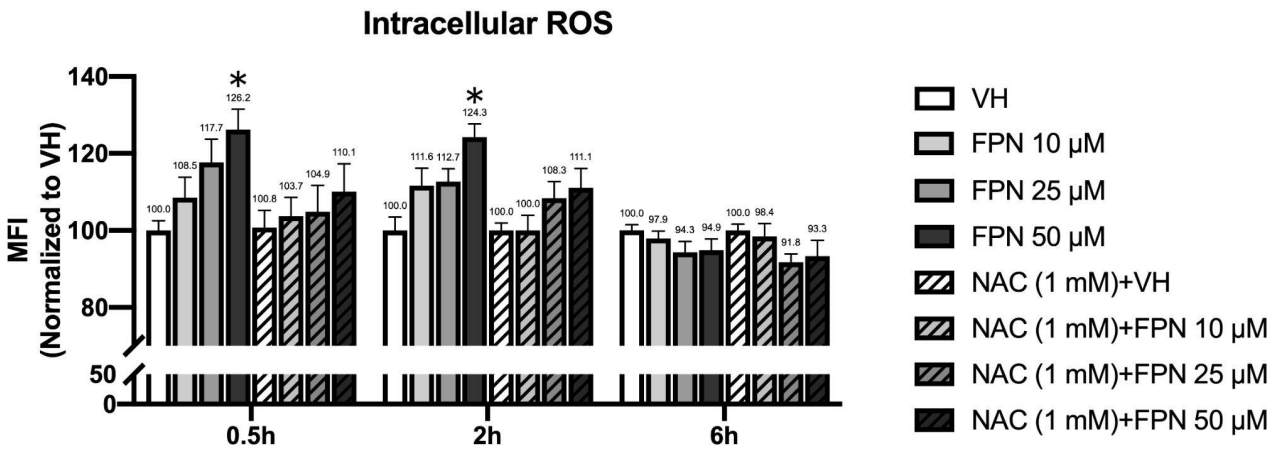

Figure 23. Induction of ROS level by FPN

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 0.5, 2, and 6 h. The level of ROS was measured by H_2-DCFDA staining. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204

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4.3.10 Fipronil exposure elevates lipid peroxidation in primary thymocytes.

To further characterize the oxidative stress induced by FPN, LPO levels in FPN-treated primary thymocytes were quantified using the fluorescent probe C11-Bodipy$^{581/591}$. This sensor specifically detects LPO by undergoing oxidation in the presence of intracellular lipid peroxides. The increase in the green fluorescence signal directly correlates with the level of lipid peroxidation, indicating oxidative damage to cellular membranes. As shown in Fig. 24, treatment with a high concentration of FPN resulted in a corresponding and significant increase in lipid peroxidation levels at an early time point (0.5 hours), confirming the rapid induction of oxidative damage to cellular lipids by FPN.

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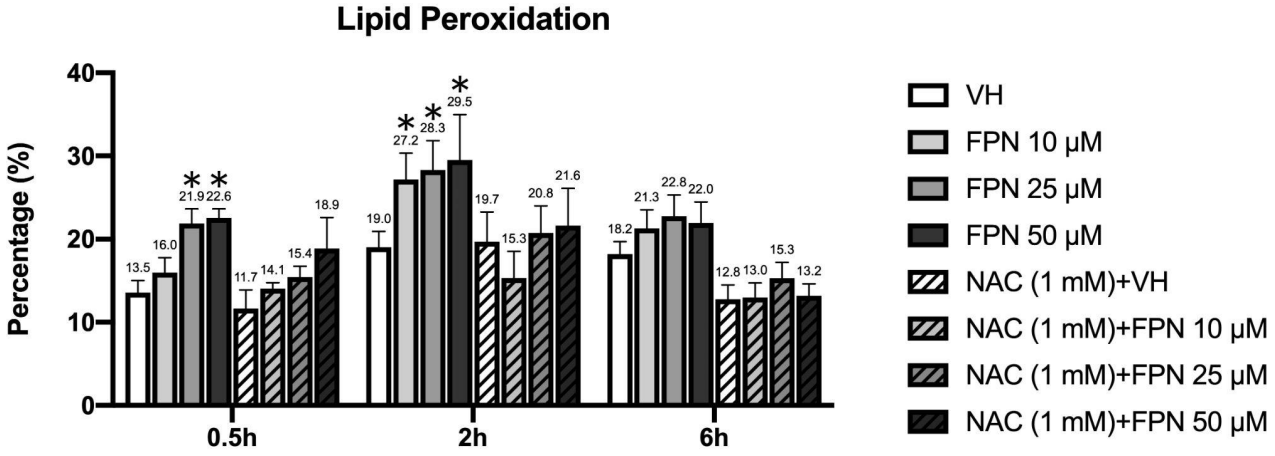

Figure 24. Induction of LPO level by FPN

The primary thymocyte (5×10^6 cells/mL) was treated with 0.05% DMSO (VH) or FPN in different concentrations for 0.5, 2, and 6 h. The lipid peroxidation was determined by C11-Bodipy$^{581/591}$ staining. Data were expressed as the mean ± SEM of quadruplicate cultures and were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group. *Adapted from* **Kuo J-F, Hsiao Y-P, Wang Y-D, Weng H-P, Wang C-C** (2025) *Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes.* Toxics 2025, 13, 204. doi: https://doi.org/10.3390/toxics13030204 © 2025 The Authors. Distributed under the terms of the Creative Commons Attribution 4.0 International Licence.

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4.4 Bioinformatic analysis of the gene-network and inferring diseases by FPN-altered genes

To explore the biological significance of differentially expressed genes and proteins affected by FPN exposure in T cells, I performed integrative bioinformatic analysis using DAVID Bioinformatics Resources and the Comparative Toxicogenomics Database (CTD), which is a comprehensive resource that integrates information on chemical–gene interactions and their links to diseases, curated from the scientific literature. These platforms can systematically annotate gene functions based on public databases and perform enrichment analysis of biological pathways.

A list of 17 genes altered in this study was uploaded to DAVID, of which 15 were successfully mapped to known pathways in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Fig. 25A and B). Pathway enrichment analysis revealed that several key genes, including *Il-7*, *Il-7r*, *Foxo*, *Bcl-2*, and *Mcl-1*, are involved in regulating the PI3K-Akt signaling pathway (Fig. 26). This pathway plays a central role in promoting T cell survival, proliferation, and metabolism during immune activation. Additionally, *Il-7*, *Il-7r* (*Cd127*), *Scf*, and *C-kit* (*Cd117*) were identified as regulators of the hematopoietic cell lineage pathway, which is essential for early lymphoid lineage commitment and thymopoiesis (Fig. 27). These results indicate that FPN may alter T cell development and immune function by disrupting core signaling networks in early hematopoietic and lymphoid stages.

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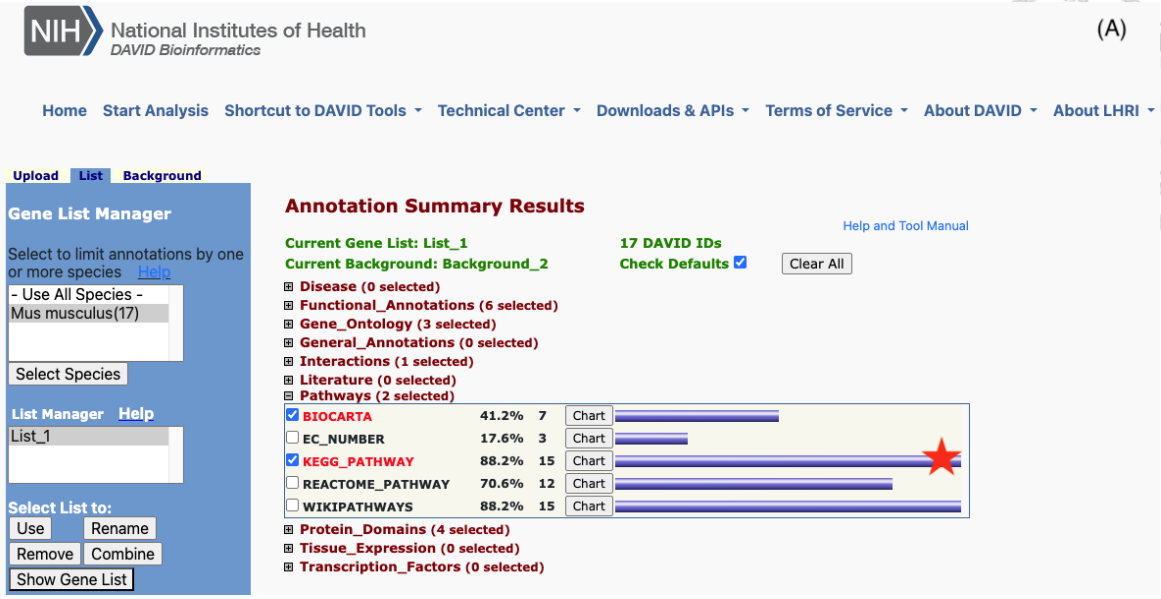

Functional Annotation Table

Help and Manual

Current Gene List: List_1
Current Background: Background_2
17 DAVID IDs

(B)

15 record(s)

Download File

P10417	B cell leukemia/lymphoma 2(Bcl2)	Related Genes	Mus musculus
KEGG_PATHWAY	EGFR tyrosine kinase inhibitor resistance, Endocrine resistance, Platinum drug resistance, NF-kappa B signaling pathway, HIF-1 signaling pathway, Sphingolipid signaling pathway, p53 signaling pathway, Autophagy - animal, Protein processing in endoplasmic reticulum, PI3K-Akt signaling pathway, Apoptosis, Apoptosis - multiple species, Necroptosis, Adrenergic signaling in cardiomyocytes, Hedgehog signaling pathway, Focal adhesion, NOD-like receptor signaling pathway, JAK-STAT signaling pathway, Neurotrophin signaling pathway, Cholinergic synapse, Estrogen signaling pathway, Parathyroid hormone synthesis, secretion and action, AGE-RAGE signaling pathway in diabetic complications, Amyotrophic lateral sclerosis, Pathways of neurodegeneration - multiple diseases, Salmonella infection, Toxoplasmosis, Tuberculosis, Hepatitis B, Measles, Herpes simplex virus 1 infection, Epstein-Barr virus infection, Human immunodeficiency virus 1 infection, Pathways in cancer, MicroRNAs in cancer, Chemical carcinogenesis - receptor activation, Colorectal cancer, Prostate cancer, Small cell lung cancer, Gastric cancer, Lipid and atherosclerosis, Fluid shear stress and atherosclerosis,		
P41183	B cell leukemia/lymphoma 6(Bcl6)	Related Genes	Mus musculus
KEGG_PATHWAY	FoxO signaling pathway, Transcriptional misregulation in cancer, Chemical carcinogenesis - receptor activation,		
O54918	BCL2 like 11(Bcl2l11)	Related Genes	Mus musculus
KEGG_PATHWAY	EGFR tyrosine kinase inhibitor resistance, FoxO signaling pathway, PI3K-Akt signaling pathway, Apoptosis, Apoptosis - multiple species, Non-alcoholic fatty liver disease, Epstein-Barr virus infection, Pathways in cancer, MicroRNAs in cancer, Colorectal cancer,		
O55003	BCL2/adenovirus E1B interacting protein 3(Bnip3)	Related Genes	Mus musculus
KEGG_PATHWAY	FoxO signaling pathway, Mitophagy - animal, Autophagy - animal, Legionellosis,		
P05532	KIT proto-oncogene receptor tyrosine kinase(Kit)	Related Genes	Mus musculus
KEGG_PATHWAY	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Phospholipase D signaling pathway, PI3K-Akt signaling pathway, Hematopoietic cell lineage, Melanogenesis, Pathways in cancer, Acute myeloid leukemia, Breast cancer, Central carbon metabolism in cancer,		
Q9R1E0	forkhead box O1(Foxo1)	Related Genes	Mus musculus
KEGG_PATHWAY	FoxO signaling pathway, AMPK signaling pathway, Longevity regulating pathway, Longevity regulating pathway - multiple species, Cellular senescence, Insulin signaling pathway, Thyroid hormone signaling pathway, Glucagon signaling pathway, Insulin resistance, AGE-RAGE signaling pathway in diabetic complications, Alcoholic liver disease, Human papillomavirus infection, Pathways in cancer, Transcriptional misregulation in cancer, Prostate cancer,		
Q9WVH4	forkhead box O3(Foxo3)	Related Genes	Mus musculus
KEGG_PATHWAY	EGFR tyrosine kinase inhibitor resistance, Chemokine signaling pathway, FoxO signaling pathway, Mitophagy - animal, PI3K-Akt signaling pathway, AMPK signaling pathway, Longevity regulating pathway, Longevity regulating pathway - multiple species, Cellular senescence, Neurotrophin signaling pathway, Prolactin signaling pathway, Alcoholic liver disease, Chemical carcinogenesis - reactive oxygen species, Endometrial cancer, Non-small cell lung cancer,		
P48318	glutamate decarboxylase 1(Gad1)	Related Genes	Mus musculus
KEGG_PATHWAY	Alanine, aspartate and glutamate metabolism, beta-Alanine metabolism, Taurine and hypotaurine metabolism, Butanoate metabolism, Metabolic pathways, GABAergic synapse, Type I diabetes mellitus,		
P48320	glutamic acid decarboxylase 2(Gad2)	Related Genes	Mus musculus
KEGG_PATHWAY	Alanine, aspartate and glutamate metabolism, beta-Alanine metabolism, Taurine and hypotaurine metabolism, Butanoate metabolism, Metabolic pathways, GABAergic synapse, Type I diabetes mellitus,		
P16872	interleukin 7 receptor(Il7r)	Related Genes	Mus musculus
KEGG_PATHWAY	Cytokine-cytokine receptor interaction, FoxO signaling pathway, PI3K-Akt signaling pathway, JAK-STAT signaling pathway, Hematopoietic cell lineage, Pathways in cancer, Primary immunodeficiency,		
P10168	interleukin 7(Il7)	Related Genes	Mus musculus
KEGG_PATHWAY	Cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, JAK-STAT signaling pathway, Hematopoietic cell lineage, Pathways in cancer,		
P20826	kit ligand(Kitl)	Related Genes	Mus musculus
KEGG_PATHWAY	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Phospholipase D signaling pathway, PI3K-Akt signaling pathway, Hematopoietic cell lineage, Melanogenesis, Pathways in cancer,		
P27792	lymphoblastomic leukemia 1(Lyl1)	Related Genes	Mus musculus
KEGG_PATHWAY	Transcriptional misregulation in cancer,		
P97287	myeloid cell leukemia sequence 1(Mcl1)	Related Genes	Mus musculus
KEGG_PATHWAY	PI3K-Akt signaling pathway, Apoptosis, JAK-STAT signaling pathway, MicroRNAs in cancer,		
P31648	solute carrier family 6 (neurotransmitter transporter, GABA), member 1(Slc6a1)	Related Genes	Mus musculus
KEGG_PATHWAY	Synaptic vesicle cycle, GABAergic synapse,		

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Figure 25. KEGG pathway analysis for the genes of IL-7 signaling, transcriptional factors, and the Bcl-2 family

(A) Input a total of 17 genes identified in this study to DAVID Bioinformatics Resources. Fifteen genes were successfully mapped to known pathways in the KEGG database. (B) IL-7 interacts with multiple genes and participates in important regulatory pathways for thymus development. This screenshot and workflow were obtained from the official DAVID platform.

doi:10.6342/NTU202501708

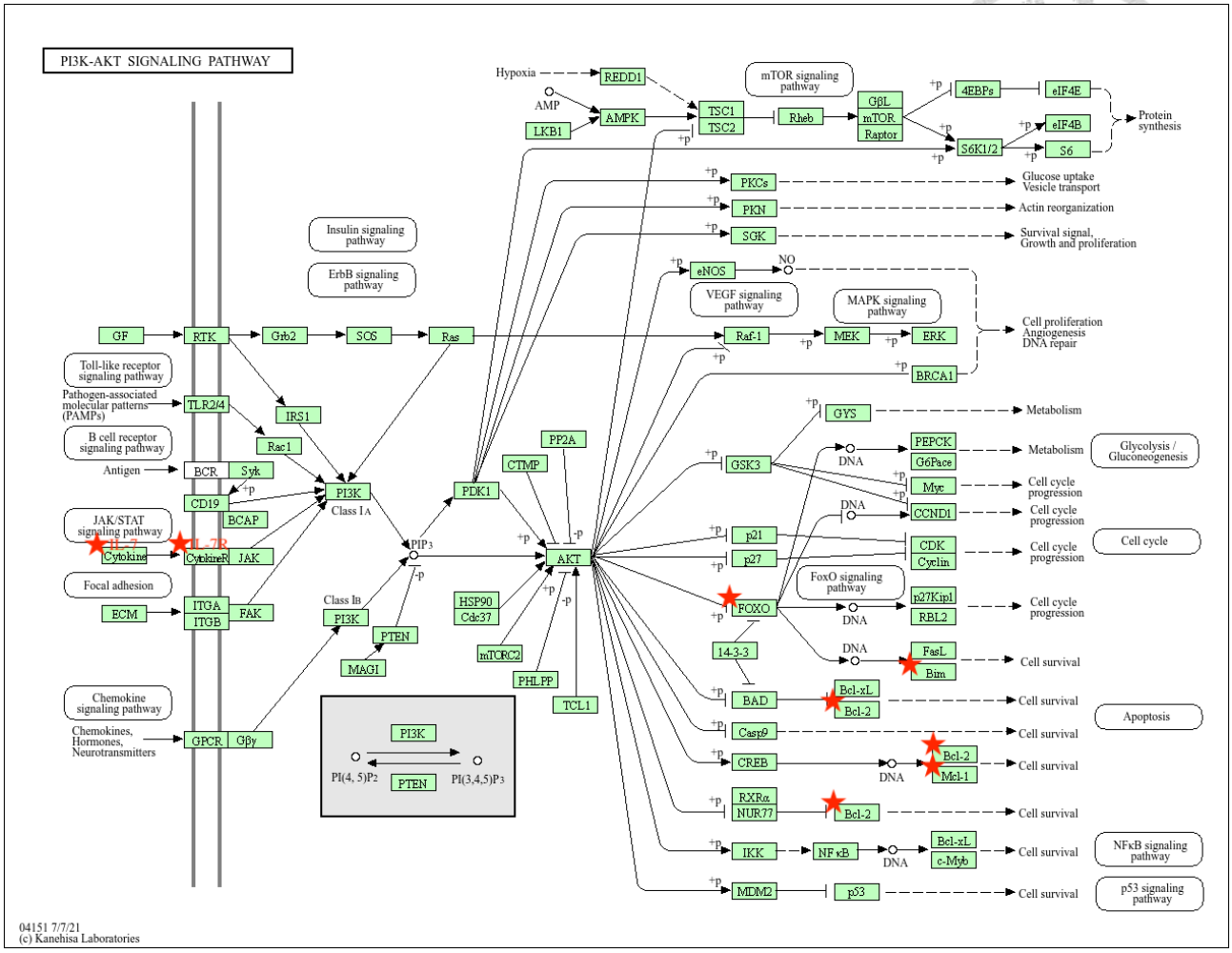
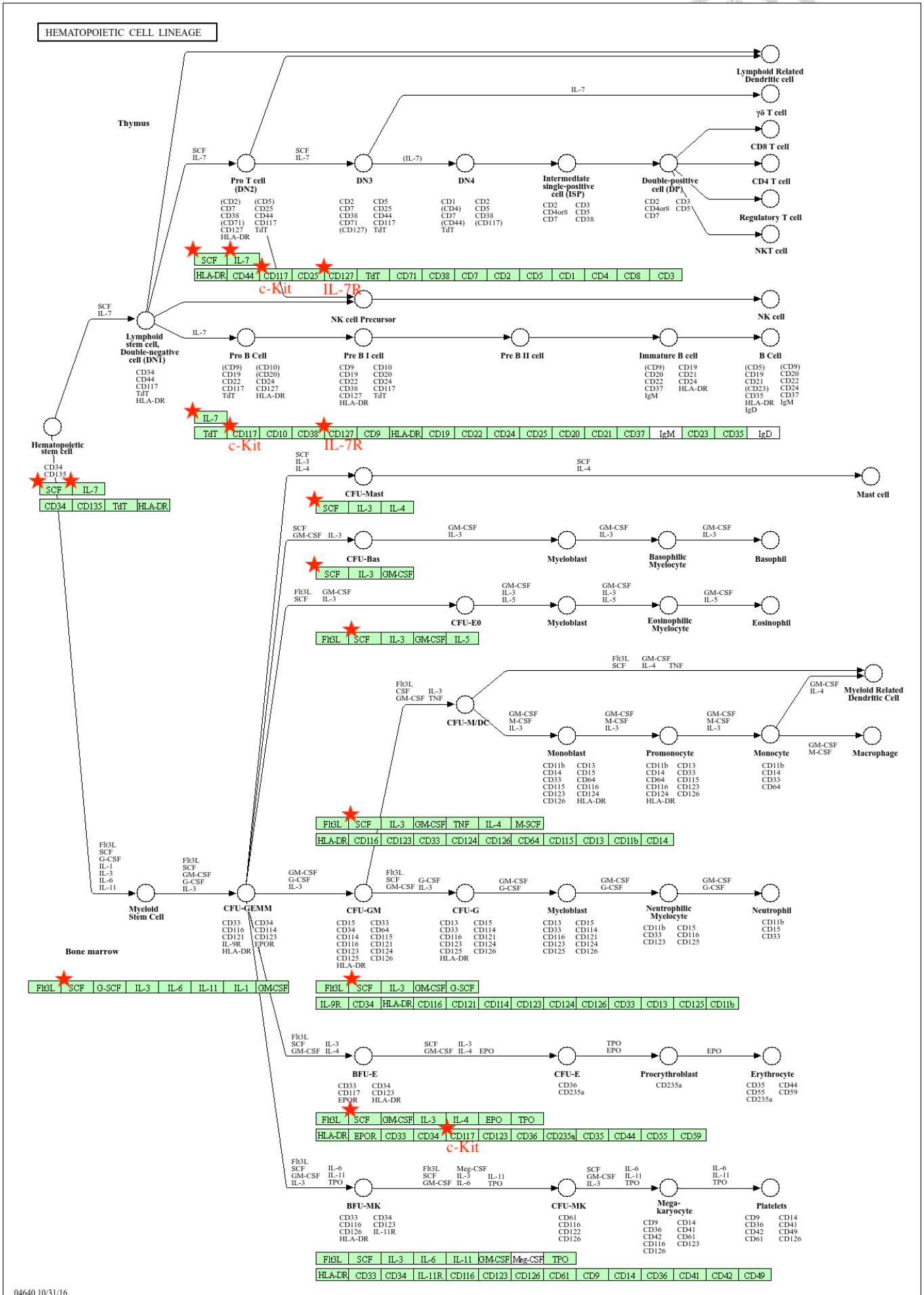


Figure 26. The interaction of IL-7 signaling and the Bcl-2 family gene in the PI3K-Akt signaling pathway

The genes of *Il-7*, *Il-7r*, *Foxo*, *Bcl-2*, and *Mcl-1* are involved in regulating the PI3K-Akt signaling pathway. Asterisks represent genes significantly affected by FPN in this study. This screenshot and workflow were obtained from the official DAVID platform.

doi:10.6342/NTU202501708



doi:10.6342/NTU202301708

Figure 27. The interaction of IL-7 signaling and the thymic microenvironment transcriptional factor gene in the hematopoietic cell lineage pathway

The genes of *Il-7*, *Il-7r*, *Scf*, and *C-kit* are involved in regulating the PI3K-Akt signaling pathway. Asterisks represent genes significantly affected by FPN. *Cd127* represents *Il-7r,* and *Cd117* represents *C-kit*. This screenshot and workflow were obtained from the official DAVID platform.

doi:10.6342/NTU202501708

To further elucidate the potential health effects of FPN, inferring diseases analysis was conducted using CTD by analyzing the FPN-altered genes in this study and all the FPN-altered genes curated from the literature in the CTD database. CTD enables the prediction of disease risks based on chemical-induced gene expression profiles (Fig. 28). Analysis of the 11 FPN-regulated genes in CTD revealed that 7 genes (*Il-7*, *Il-7r*, *Kit*, *Foxn1*, *Foxo3*, *Bcl-2*, and *Mcl-1*) are strongly associated with immune system diseases, highlighting the potential immunopathological risks of FPN exposure (Fig. 29). Interestingly, 6 of these genes (*Il-7*, *Il-7r*, *Slc6a1* (*Gat1*), *Gad1* (*Gad67*), *Foxo3*, and *Bcl-2*) were also classified as highly relevant to nervous system diseases, suggesting possible involvement in neurotoxic mechanisms (Fig. 30).

To broaden the scope of this enrichment analysis, I also queried “fipronil” as a keyword in CTD to retrieve all curated FPN-related gene interactions from existing literature and datasets. This search identified a total of 2,940 genes associated with FPN. I then cross-referenced these with our 11 experimentally identified FPN-regulated genes and performed an integrated enrichment analysis using the CTD tool. Results from this expanded analysis further confirmed that FPN-responsive genes are predominantly enriched in immune-related disease categories. In particular, these genes showed strong associations with immune system diseases, including autoimmune disorders, immunoproliferative conditions, and lymphoproliferative syndromes (Fig. 31).

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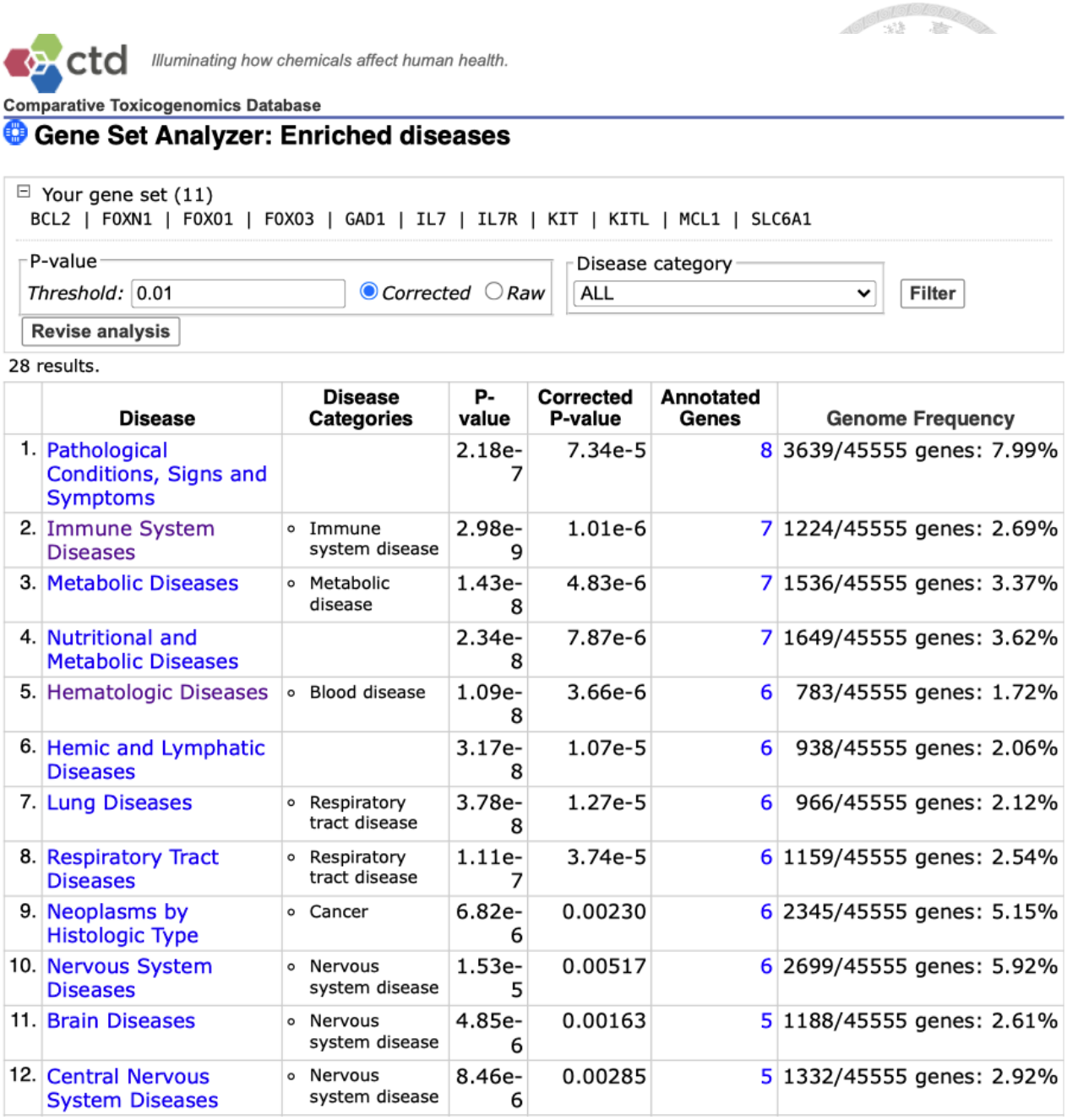

ctd Illuminating how chemicals affect human health.

Comparative Toxicogenomics Database

Gene Set Analyzer: Enriched diseases

Your gene set (11)

BCL2 | FOXN1 | FOXO1 | FOXO3 | GAD1 | IL7 | IL7R | KIT | KITL | MCL1 | SLC6A1

P-value Threshold: 0.01 Corrected Raw

Disease category ALL Filter

Revise analysis

28 results.

	Disease	Disease Categories	P-value	Corrected P-value	Annotated Genes	Genome Frequency
1.	Pathological Conditions, Signs and Symptoms		2.18e-7	7.34e-5	8	3639/45555 genes: 7.99%
2.	Immune System Diseases	◦ Immune system disease	2.98e-9	1.01e-6	7	1224/45555 genes: 2.69%
3.	Metabolic Diseases	◦ Metabolic disease	1.43e-8	4.83e-6	7	1536/45555 genes: 3.37%
4.	Nutritional and Metabolic Diseases		2.34e-8	7.87e-6	7	1649/45555 genes: 3.62%
5.	Hematologic Diseases	◦ Blood disease	1.09e-8	3.66e-6	6	783/45555 genes: 1.72%
6.	Hemic and Lymphatic Diseases		3.17e-8	1.07e-5	6	938/45555 genes: 2.06%
7.	Lung Diseases	◦ Respiratory tract disease	3.78e-8	1.27e-5	6	966/45555 genes: 2.12%
8.	Respiratory Tract Diseases	◦ Respiratory tract disease	1.11e-7	3.74e-5	6	1159/45555 genes: 2.54%
9.	Neoplasms by Histologic Type	◦ Cancer	6.82e-6	0.00230	6	2345/45555 genes: 5.15%
10.	Nervous System Diseases	◦ Nervous system disease	1.53e-5	0.00517	6	2699/45555 genes: 5.92%
11.	Brain Diseases	◦ Nervous system disease	4.85e-6	0.00163	5	1188/45555 genes: 2.61%
12.	Central Nervous System Diseases	◦ Nervous system disease	8.46e-6	0.00285	5	1332/45555 genes: 2.92%

Figure 28. CTD database prediction for the FPN-affected genes in this research

Input the genes affected by FPN to analyze the chemical–gene interactions and their links to diseases.

This screenshot and workflow were obtained from the official CTD platform.

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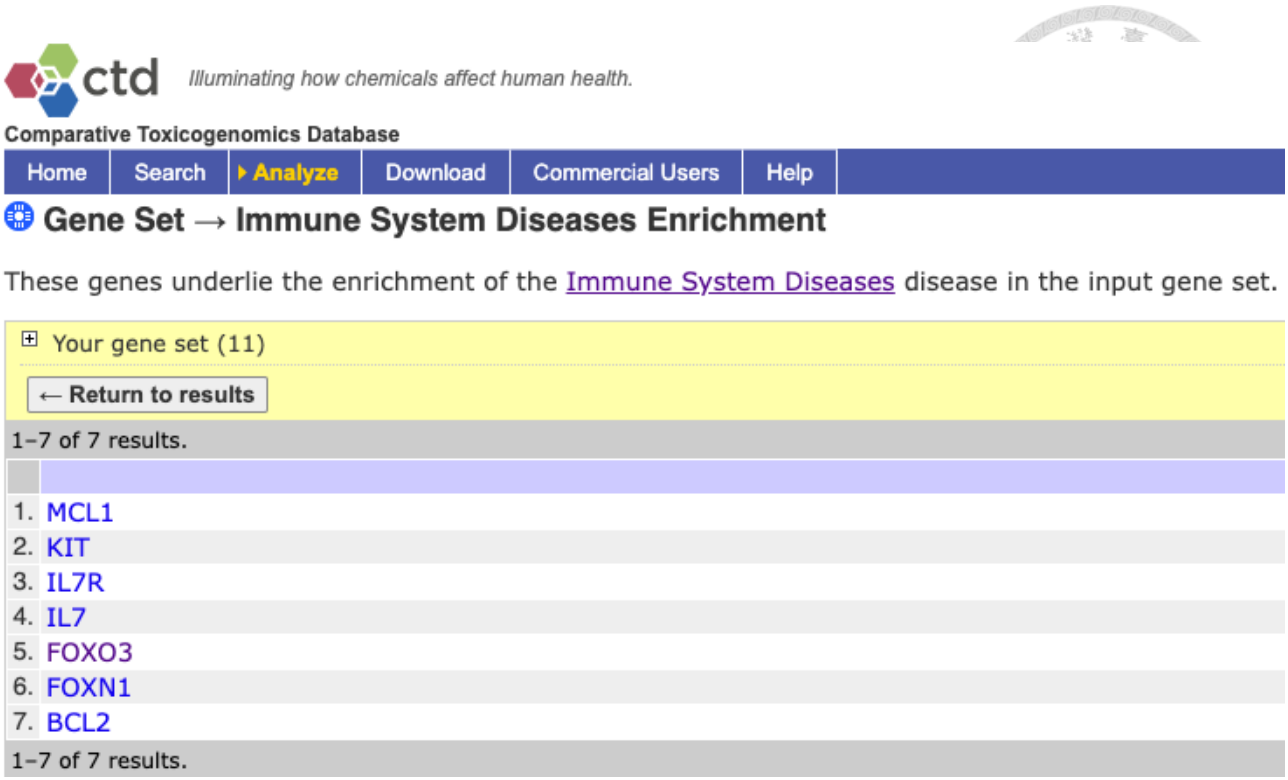

Figure 29. IL-7 signaling and the Bcl-2 family genes are predicted to play a regulatory role in immune system diseases.

A total of 7 genes (*Il-7*, *Il-7r*, *Kit*, *Foxn1*, *Foxo3*, *Bcl-2*, and *Mcl-1*) are related to immune system diseases. This screenshot and workflow were obtained from the official CTD platform.

doi:10.6342/NTU202501708

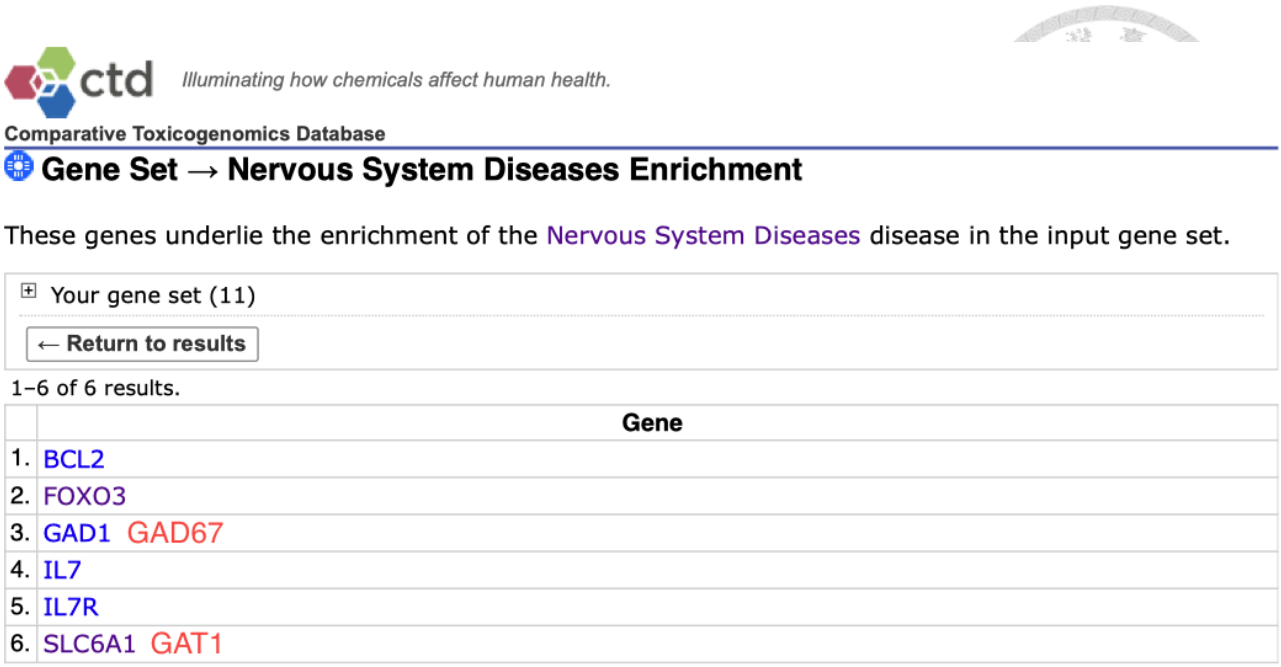

Figure 30. IL-7 signaling, GABAergic, and the Bcl-2 genes are predicted to play a regulatory role in nervous system diseases.

A total of 6 genes (*Il-7*, *Il-7r*, *Slc6a1* (*Gat1*), *Gad1* (*Gad67*), *Foxo3*, and *Bcl-2*) are enriched to nervous system diseases. *Slc6a1* represents *Gat1* and *Gad1* represents *Gad67*. This screenshot and workflow were obtained from the official CTD platform.

doi:10.6342/NTU202501708

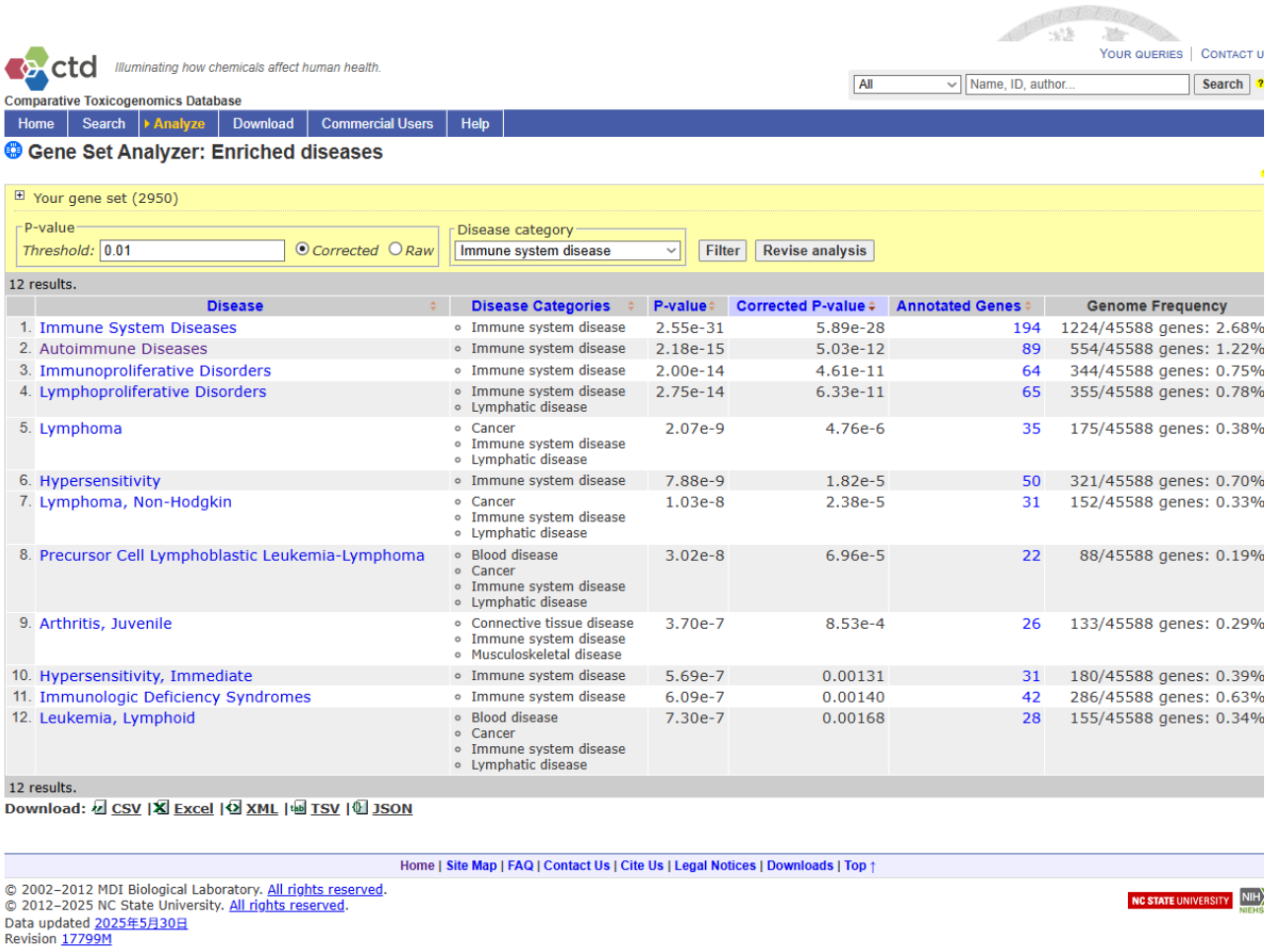

Figure 31. Enriched-immune system disease prediction of 2950 altered genes in the literature and in this research conducted by CTD.

This screenshot and workflow were obtained from the official CTD platform.

doi:10.6342/NTU202501708

Chapter 5. Discussion

5.1 Off-target toxicity of FPN

The systemic effects of FPN extend beyond a single organ, impacting crucial regulators of homeostasis such as the liver, thyroid, kidneys, and central nervous system (Awad et al., 2021; Bano and Mohanty, 2020a; Vidau et al., 2011). These multi-organ disruptions can have cascading consequences, indirectly contributing to the observed immunotoxicity. For instance, hepatic metabolism of FPN via cytochrome P450 (CYP) enzymes produces fipronil sulfone, a more persistent and biologically active metabolite. This bioactivation process can generate oxidative stress and impair hepatic detoxification pathways, thereby influencing systemic immune responses (Caballero et al., 2015; Das et al., 2006). Furthermore, FPN has been shown to disrupt thyroid hormone homeostasis by modulating thyroid-stimulating hormone (TSH) and thyroid peroxidase activity (Bano and Mohanty, 2020a). Given the critical role of thyroid hormones in T cell maturation and differentiation, FPN-induced alterations in thyroid levels could indirectly contribute to immune dysregulation. Additionally, FPN's well-documented antagonistic action on $GABA_A$ receptors disrupts neuronal signaling, leading to neurotoxicity (Awad et al., 2021; Vidau et al., 2011). Considering the intricate interplay between the immune and nervous systems, mediated by shared molecular pathways, the neurotoxic effects of FPN could further exacerbate immune dysregulation.

doi:10.6342/NTU202501708

5.2 Fipronil induced both Th1 and Th2 responses by dysregulation of GABAergic signaling

The present study provides novel insights into the immunotoxic effects of FPN on antigen-specific immune responses *in vivo*. To our knowledge, this study represents the first report specifically addressing the immunotoxic effects of orally administered FPN on antigen-specific immunity.

5.2.1 Immunostimulatory effects of FPN on adaptive immune responses and the complexities of Th1/Th2 balance

Previous studies have clearly established that dysregulated Th1/Th2 cytokine production has the potential to contribute significantly to the development of autoimmune disorders and allergic conditions (Dardalhon et al., 2008; Wan and Flavell, 2009). Our study demonstrates that FPN exerts a robust immunostimulatory effect on adaptive immune responses. I observed a significant increase in serum levels of OVA-specific IgG_1 and IgG_{2a} in FPN-treated mice, strongly suggesting FPN interfered with T cell-dependent antibody production, potentially influencing B cell activation (Fig. 5). *Ex vivo* experiments further confirmed that FPN promoted the viability and proliferation of antigen-specific T cells in response to OVA stimulation. This augmented response was accompanied by elevated levels of IL-2, IL-4, and IFN-γ, suggesting broad activation of both Th1 and Th2 responses (Fig. 6). Collectively, these results unequivocally demonstrate the broad immunostimulatory effects of FPN on adaptive immune responses.

Interestingly, despite this clear increase in cytokine production, the mRNA expression of Th1/Th2 cytokines and their respective upstream transcription factors in the thymus remained unchanged or even slightly decreased (Fig. 7). This divergence between protein and mRNA levels suggests that FPN's immunostimulatory effects may not be mediated through direct transcriptional

doi:10.6342/NTU202501708

regulation of Th1/Th2 differentiation or cytokine gene expression. Instead, it implies that FPN's immunomodulatory effects might involve more intricate mechanisms, such as influences on cytokine secretion pathways, alterations in T cell activation thresholds, or other non-transcriptional regulatory processes. This observation warrants further in-depth investigation. Notably, similar immunostimulatory effects of FPN have been reported in other cellular models, such as its ability to induce pro-inflammatory cytokines in human nasal epithelial cells through MAPK and NF-κB pathways (Kwak et al., 2022).

5.2.2 The pivotal role of GABAergic signaling in immune regulation

To further elucidate the mechanisms underlying FPN-mediated immunomodulatory effects *in vivo*, I investigated the regulation of GABAergic genes by FPN, given its known antagonistic action on GABA receptors (Fig. 8). A growing body of evidence highlights the critical regulatory roles of GABAergic signaling within the immune system. *In vitro* studies have shown that GABA dose-dependently inhibits antigen-specific T cell proliferation and T cell responses to both foreign and self-antigens (Tian et al., 1999). Furthermore, GABAergic agents have been demonstrated to inhibit antigen-specific T-cell immune responses. In peripheral macrophages, GABA treatment has been shown to reduce levels of inflammatory cytokines (Bhat et al., 2010). In a non-obese diabetic mouse model of type 1 diabetes, GABA and/or $GABA_A$ receptor agonists attenuated inflammatory responses, antigen-specific cytotoxic immune responses, and antigen-primed delayed-type hypersensitivity reactions (Bhat et al., 2010; Tian et al., 2004, 1999). Collectively, these lines of evidence underscore the significant regulatory roles of GABAergic signaling in tempering over-reactive adaptive immune responses.

5.2.3 FPN's potential influence on GABA synthesis and transport pathways

Glutamate decarboxylase (GAD) is a pivotal enzyme that catalyzes the decarboxylation of

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glutamate to GABA. Notably, GABA secretion has been observed in stimulated T cells when cultured in a conditioned medium (Bhat et al., 2010). Additionally, dendritic cells and macrophages express GAD65 transcripts to synthesize GABA. Although the main GAD isoform in stimulated T cells has not been extensively studied, the bulk secretion of GABA by T cells may be influenced by GABA reuptake, storage, and secretion mechanisms (Bhat et al., 2010). In the present study, the expression of GAD67, an enzyme known to be expressed in B cells (Zhang et al., 2021), was significantly reduced in antigen-stimulated splenocytes from FPN-treated mice in a dose-dependent manner (Fig. 8B). This data strongly indicates that FPN might interfere with the synthesis of GABA through the downregulation of GAD67 in splenocytes.

Furthermore, GABA transporter type 1 (GAT1) is primarily involved in GABA binding and transport from the cytoplasm to the extracellular space (reverse mode) and back into the cytoplasm (forward mode). Dysfunctional GAT1 can lead to delayed communication with post-synaptic GABA receptors, contributing to various neurological diseases (Zafar and Jabeen, 2018, p. 1). Previous literature has shown that $CD4^+$ T cells isolated from $GAT1^{-/-}$ mice exhibit higher IL-2 and IFN-γ secretion under conditional stimulation, thereby promoting T cell activation and survival through PKC-dependent signaling pathways (Ren et al., 2017). These findings from prior studies are consistent with our cytokine data, where a significant increase in the secretion of IL-2 and IFN-γ was observed in the FPN-treated groups. Additionally, a slight decrease in the expression of GAT1 mRNA was observed in our study, suggesting that impairment of GABA recycling may also be involved in the immunostimulatory effects of FPN, albeit to a lesser extent than the changes seen in GAD67 (Fig. 8C).

5.2.4 Impact of FPN on $GABA_A$ receptor expression and potential compensatory mechanisms

Human and mouse immune cells express $GABA_A$ receptors, with mouse T cells

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predominantly exhibiting α5, β2, β3, and δ subunits (Sparrow et al., 2021). Although T cells can assemble various $GABA_A$ receptor subtypes, their exact composition and functional roles remain unclear due to differences in subunit affinity and pharmacological properties. Functional $GABA_A$ receptors, rather than $GABA_B$ receptors, have been shown to suppress antigen-stimulated T cell proliferation when exposed to high levels of GABA, suggesting an immunosuppressive effect (Tian et al., 1999).

Our study provides compelling evidence that FPN exposure significantly alters the expression of GABA receptor genes in splenocytes. Specifically, I observed a notable increase in the mRNA expression of the β2 and δ subunits, with a slight induction of α5 and β3 transcripts in the 1 and 5 mg/kg FPN treatment groups (Fig. 8D-G). Given that FPN is known to be a competitive antagonist of GABA receptors, this upregulation of receptor subunits could be interpreted as a cellular compensatory mechanism. In the presence of an antagonist, cells might attempt to maintain responsiveness to GABA by increasing the number of available receptors on their surface, thereby trying to overcome the FPN-induced blockade and restore GABAergic signaling.

Our data, showing alterations in GABAergic genes without obvious changes in spleen composition, are concordant with the FPN-mediated abnormal immunostimulatory effects. This strongly suggests that the dysregulation of GABAergic genes plays a significant role in the immunotoxicity of FPN.

In summary, this study proposes a model whereby FPN, by potentially inhibiting GABA synthesis and/or affecting $GABA_A$ receptor expression, may attenuate the inhibitory effects of GABAergic signaling on immune responses. This, in turn, leads to increased antigen-specific T cell activation and cytokine production, resulting in the observed immunostimulatory effects. Future research should further investigate the more intricate effects of FPN on GABAergic signaling pathways in different immune cell types, including GABA synthesis, release, transport, and receptor

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subtype-specific functions. Moreover, delving into the molecular links between FPN-mediated GABAergic dysregulation and Th1/Th2 imbalance will be crucial for a comprehensive understanding of FPN's role in immunomodulation and for identifying novel therapeutic targets for immune-imbalance-related disorders.

doi:10.6342/NTU202501708

5.3 Impact of FPN on thymic development and its influence on IL-7 signaling and critical transcription factors

The thymus, a primary lymphoid organ, is indispensable for generating a diverse repertoire of self-tolerant and immunocompetent T lymphocytes through a highly orchestrated process known as thymopoiesis. This intricate developmental cascade, involving the maturation and differentiation of immature T cells into functional T-cell subsets, is meticulously regulated by a complex interplay of signaling pathways, lineage-specific transcription factors, and crucial interactions with thymic stromal cells (Shanker, 2004; Shanley et al., 2009). While thymic size naturally declines with age, a process termed thymic involution, its fundamental capacity for T cell production persists, especially critical during periods of hematopoietic stress. Notably, murine thymic involution typically peaks around 4 weeks of age, preceding human involution, which begins around 1 year of age (Sutherland et al., 2005). The functional consequences of thymic atrophy are profound, contributing to reduced pathogen defense, increased susceptibility to autoimmune responses, and compromised immunosurveillance, particularly in clinical conditions marked by severe peripheral T cell depletion (Liang et al., 2022; Velardi et al., 2021). This study presents the first investigation into the immunotoxic effects of FPN on thymic development, specifically highlighting its disruption of essential developmental transcription factors and IL-7-associated pathways following short-term oral exposure.

5.3.1 FPN-induced thymic atrophy and developmental blocks

Our investigation reveals that FPN exposure, even at low doses (1 to 10 mg/kg), leads to significant alterations in both thymic index and thymocyte cellularity (Table 3). A consistent and

doi:10.6342/NTU202501708

dose-dependent decrease in the total number of thymocytes was observed across all FPN treatment groups, mirroring a reduction in the thymus index. Histological examination further elucidated these macroscopic changes, demonstrating a reduction in the medulla-cortex ratio, indicative of atrophy predominantly within the medullary region of the thymus (Fig. 9). The most severe reductions, approximately 20% in overall thymic size and a substantial 35% in total thymocyte numbers, were observed at the highest dose. This collective evidence points to a generalized thymic atrophy induced by FPN.

Beyond mere size reduction, FPN exposure profoundly disrupted the intricate balance of CD4 and CD8 thymocyte populations (Table 3). I observed a clear imbalance in the ratio of these subsets, reflecting a phenotypic confusion also evident in the absolute numbers of each cell population. Critically, there was a pronounced block in the maturation of immature thymocytes at the $CD4^-/CD8^-$ DN stage, leading to a consequent deficiency in the $CD4^+/CD8^+$ DP T-cell population. This strongly suggests that FPN exerts its toxic effects early in thymocyte development, impeding the successful transition from the DN to the DP stage. Interestingly, despite this early developmental arrest, the proportion of mature $CD4^+$ and $CD8^+$ SP thymocytes remained largely unchanged or even slightly increased. I speculate that this apparent stability in SP populations could represent a compensatory response to the FPN-induced disruptions in earlier developmental stages, whereby the remaining thymocytes might undergo accelerated maturation to sustain the peripheral T cell pool. Collectively, these findings underscore FPN's potent immunotoxic effects on the thymus, characterized by induced atrophy and a critical impediment to DP thymocyte expansion (Gruver and Sempowski, 2008). The impairment of early thymocyte transitions suggests disruption of TCR beta and pre-TCR signaling, which are critical checkpoints for DP development and downstream selection processes (Carpenter and Bosselut, 2010; Cui et al., 2022).

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5.3.2 Dysregulation of IL-7 signaling

The dynamic regulation of IL-7 signaling is a fundamental requirement for lymphocyte development, profoundly influencing thymopoiesis and T-cell lineage commitment (Corcoran et al., 1998; Durum et al., 1998; Maki et al., 1996; Muegge et al., 1993). IL-7 is essential for T-cell survival and proliferation, especially during the DN stages of thymocyte development (Vicente et al., 2010). Its receptor, IL-7Rα, begins expression upon entry into the DN2 stage and initiates critical signaling pathways, including the STAT5 pathway, to support thymopoiesis (Durum et al., 1998; Maki et al., 1996; Muegge et al., 1993; Yu et al., 2004). The indispensable nature of IL-7 in thymopoiesis is well-established. Experimental depletion of IL-7 or its receptor *in vivo* leads to a drastic reduction in overall thymocyte numbers, often exceeding 99% (Bhatia et al., 1995; Grabstei et al., 1993; Sudo et al., 1993). Similarly, genetic deletions of IL-7, IL-7R, or their proximal signaling molecules result in severe thymopoiesis defects and a developmental block at the DN3 stage (Bhatia et al., 1995; Freeden-Jeffery et al., 1995; Peschon et al., 1994; Sudo et al., 1993).

Our results compellingly demonstrate that FPN exposure significantly reduced the expression of a series of genes and proteins associated with IL-7 signaling. This widespread downregulation strongly suggests that the FPN-mediated immunodeficiency observed in this study is closely associated with the dysregulation of the IL-7 signaling pathway. This finding aligns with previous research where other chemical stressors, such as oral administration of tributyltin acetate, similarly downregulated IL-7 mRNA in TECs, leading to decreased CD4 and CD8 SP T-cell populations and a block in thymocyte differentiation (da Silva et al., 2023; Im et al., 2015). Conversely, during severe thymic atrophy induced by dexamethasone or irradiation, thymus regeneration was notably facilitated by the upregulation of IL-7 expression (Zubkova et al., 2005). Collectively, these data underscore the critical role of IL-7 regulation in mediating the immune system's response to various chemical stimuli. Thus, FPN's interference with IL-7 signaling likely disrupts the vital crosstalk between TECs and thymocytes, leading to impaired T cell development and a reduction in the mature T-cell pool.

doi:10.6342/NTU202501708

5.3.3 Alteration of transcription factors associated with thymus development by FPN

Beyond IL-7 signaling, FPN exposure significantly downregulated the expression of several crucial transcription factors indispensable for proper thymic development. These included GABPα, FOXO1, FOXO3, FOXN1, LYL1, SCF, and c-KIT. This broad suppression of a critical transcriptional network suggests a comprehensive disruption of thymopoiesis.

Each of these factors plays a distinct yet interconnected role. GABPα and FOXO family members (FOXO1, FOXO3) are intricately linked to IL-7Rα expression and are vital for directing normal thymocyte development (Bupp et al., 2009; Kerdiles et al., 2009; Lin et al., 2004; Ouyang et al., 2012; Xue et al., 2004). FOXN1 is critical for the growth and differentiation of TECs and promotes genes essential for thymus organogenesis. Its absence leads to severe primary T-cell immunodeficiency (Amorosi et al., 2008; Blackburn et al., 1996; Cunningham-Rundles and Ponda, 2005; Frank et al., 1999; Müller et al., 2001, p. 121; Nehls et al., 1996; Romano et al., 2013). LYL1 orchestrates lymphoid specification in bone marrow progenitors and is vital for the survival and expansion of thymic cell progenitors, particularly during pro-T-cell expansion (Zohren et al., 2012a, 2012b). Furthermore, SCF produced by stromal cells, interacting with its ligand c-Kit expressed by DN thymocytes, forms a crucial axis with IL-7 signaling for optimal thymopoiesis (Chung et al., 2011; Politikos et al., 2015).

Immunohistochemical staining and Western blot analyses consistently confirmed reductions in these key proteins, reinforcing the gene expression findings (Fig. 10-12). Notably, GABPα was consistently reduced across FPN doses, suggesting a higher sensitivity to FPN toxicity. The broad suppression of these transcriptional regulators, deeply involved in T-cell progenitor differentiation, survival, and expansion, likely impairs thymocyte progression and stability. This ultimately contributes to the observed CD4 and CD8 ratio imbalances and overall immunodeficiency (Liang et al., 2022; Velardi et al., 2021). Given FPN's documented persistent bioaccumulation (Aldayel et al.,

doi:10.6342/NTU202501708

2021; Cam et al., 2018; Romero et al., 2016; Song et al., 2021), sustained exposure poses a significant threat to thymic functionality and homeostasis.

Taken together, our results identify IL-7 signaling and its regulatory transcription factors as central targets of FPN-induced immunotoxicity. The disruption of these pathways hinders thymopoiesis at multiple levels, from early precursor survival to lineage commitment, which compromises overall T-cell development.

5.3.4 FPN alters cytokine secretion in thymocytes

Ex vivo ConA stimulation revealed a notable decrease in IL-2 production, alongside elevated IL-4 and IFN-γ levels (Fig. 14). IL-2 is crucial for T-cell proliferation and maintenance of immune tolerance, and its deficiency has been linked to autoimmunity and lymphoproliferative disorders (Sadlack et al., 1993; Tentori et al., 1988). Overexpression of IL-4 and IFN-γ has been previously associated with skewed thymocyte maturation and elevated $CD8^+$ SP populations (Carty et al., 2014; Smiley and Grusby, 1998; Young et al., 1997). The increased IFN-γ observed in our study may result from the downregulation of FOXO3a, which normally represses excessive inflammatory cytokine production (Kerdiles et al., 2010). Collectively, these changes suggest functional perturbation of maturing thymocytes.

5.3.5 Potential involvement of glucocorticoid pathways in FPN-induced thymic atrophy

Beyond direct cellular effects, the thymus's exquisite sensitivity to glucocorticoids (GCs) represents another potential avenue for FPN's immunotoxicity (Salehzadeh and Soma, 2021). High GC levels are known to induce T-cell apoptosis and exert immunosuppressive effects, affecting T-cell selection and ultimately causing thymic atrophy (Ashwell et al., 2000; Deobagkar-Lele et al., 2013; Roggero et al., 2006; Yan et al., n.d.). Pertinently, previous research showed that Wistar rats treated with 1/20 LD50 of FPN for 6 weeks exhibited significantly higher serum corticosterone levels

doi:10.6342/NTU202501708

(approximately 141.31 pg/mL) (Mahmoud et al., 2021). Corticosterone is a major stress hormone, tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The immunotoxic mechanism of GCs is often linked to oxidative damage (Gallo-Payet and Battista, 2014; Mahmoud et al., 2021; Prevatto et al., 2017). GCs exert their effects by binding to the glucocorticoid receptor (GR), which is expressed by all thymocytes at various developmental stages, albeit at different levels across CD4/CD8 subsets (Gallo-Payet and Battista, 2014; Mahmoud et al., 2021). Importantly, while $CD4^+/CD8^+$ DP cells possess the lowest GR levels, they paradoxically exhibit the highest sensitivity to GC-induced apoptosis (Kerdiles et al., 2010; Young et al., 1997). Given that FPN can induce elevated serum corticosterone, these increased cortisol effects on the thymus represent a compelling potential mechanism contributing to FPN-induced thymic atrophy.

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5.4 Oxidative stress as a central mechanism in FPN-induced immunotoxicity and apoptosis

While the capacity of FPN to induce oxidative stress, and disrupt antioxidant defenses has been documented across various organ systems (Caballero et al., 2015; Das et al., 2006), its direct implications for immune cells, particularly the highly dynamic thymocytes, have remained largely unexplored. Unlike more metabolically stable cell types such as hepatocytes or neurons, thymocytes exhibit unique susceptibility to oxidative stress due to their rapid turnover rate and the critical role of apoptosis in T cell selection and immune repertoire shaping. Dysregulation of thymocyte apoptosis carries profound consequences for immune function, potentially increasing susceptibility to infections, compromising immune tolerance, and predisposing individuals to autoimmune disorders. Our findings reveal that even acute FPN exposure rapidly triggers oxidative stress-mediated apoptosis in thymocytes, a mechanism that likely contributes to the observed persistent immune dysfunction.

It is important to note our choice of 4-week-old mice for the thymic development *in vivo* study. This developmental stage is characterized by peak thymic function and active T cell development, making it particularly relevant for assessing potential immunotoxic effects. Moreover, age-related differences in CYP enzyme activity can significantly influence xenobiotic metabolism and toxicity. Juvenile mice generally exhibit lower basal CYP activity compared to adults (Caballero et al., 2015), which could lead to slower FPN metabolism and consequently prolonged systemic exposure. This distinction is especially pertinent given the established link between CYP activity, oxidative stress, and apoptosis. Enhanced CYP-mediated metabolism can generate excessive ROS, potentially overwhelming redox homeostasis and triggering apoptotic pathways.

5.4.1 Apoptotic dysregulation in the thymus

Our investigation into the core molecular machinery of apoptosis revealed that short-term *in*

doi:10.6342/NTU202501708

vivo FPN administration significantly downregulated the mRNA expression of key anti-apoptotic genes, specifically *Bcl2*, *Bcl-6*, and *Mcl-1* (Fig. 15 and 16). These genes are critical for maintaining thymic homeostasis and ensuring the survival of developing T cells. Notably, BCL-2 and MCL-1 are indispensable downstream mediators of IL-7 signaling, which provides crucial survival signals for lymphocyte precursors (Akashi et al., 1997; Maraskovsky et al., 1997; Opferman et al., 2003). The concurrent downregulation of these anti-apoptotic genes indicates a critical disruption of intrinsic survival mechanisms, likely contributing to compromised thymocyte maturation and increased apoptosis.

Paradoxically, our *in vivo* study also observed a reduction in the expression of specific pro-apoptotic genes, *Bim* and *Bnip3*. *Bim* is a pivotal mediator of negative selection in the thymus, essential for eliminating autoreactive or dysfunctional thymocytes (Wang et al., 2017). *Bnip3*, on the other hand, plays a dual role in mitochondrial dynamics and T-cell homeostasis, contributing to the removal of damaged mitochondria (Gustafsson, 2011; Rikka et al., 2011; Wan et al., 2003). The suppression of these pro-apoptotic genes in the thymic microenvironment suggests a complex and nuanced interplay, possibly reflecting compensatory mechanisms aimed at preserving thymic cellularity under prolonged FPN-induced stress. However, such a compensatory response, while potentially mitigating acute cell loss, could come at the cost of efficient immune selection, potentially allowing the persistence of compromised cells.

Adding another layer of complexity, a noteworthy divergence emerged when comparing *in vivo* and *in vitro Bim* expression. Contrary to the *in vivo* reduction of *Bim* in the thymus, acute *in vitro* FPN exposure directly induced Bim mRNA expression in a dose-dependent manner (Fig. 15E). As a central pro-apoptotic BH3-only protein (Fischer et al., 2007; Mérino et al., 2009), *Bim*'s upregulation *in vitro* likely reflects rapid cytotoxic stress, mitochondrial damage, and elevated ROS levels induced by FPN, leading to immediate apoptotic signaling activation (Fig. 17E). This stark contrast between *in vivo* suppression and *in vitro* induction of Bim underscores the profound influence of the complex

doi:10.6342/NTU202501708

thymic microenvironment and suggests that in the short-term *in vivo* model, thymocytes may have undergone selection pressure, favoring the survival of apoptosis-resistant cells that downregulate Bim expression as an adaptive response to limit excessive cell loss. These findings highlight the dynamic interplay between intrinsic thymocyte responses and extrinsic regulatory signals in maintaining immune homeostasis under FPN exposure.

5.4.2 Mitochondrial dysfunction and oxidative stress

Under physiological conditions, mitochondria generate a small but steady amount of ROS, typically converting 1–2% of consumed oxygen into superoxide anions (Shaki et al., 2012). However, excessive ROS production, often triggered by toxicant exposure or cellular dysfunction, is a potent inducer of intrinsic apoptosis. Consequently, mitochondria serve as the primary intracellular source of ROS generation.

Our investigation precisely characterized the mechanisms of FPN-induced mitochondrial toxicity in primary thymocytes by assessing key indicators of mitochondrial health, including MMP, intracellular calcium, and ROS. Two-way ANOVA revealed a significant main effect of time on MMP depolarization, intracellular calcium concentration reduction, and GSH depletion. Further analysis confirmed these alterations were all time-dependent, indicating that prolonged exposure progressively exacerbates mitochondrial dysfunction and oxidative stress. FPN exposure consistently led to MMP depolarization (Fig. 20), a critical early event in the apoptotic process (Ly et al., 2003; Wang and Youle, 2009). This mitochondrial dysfunction was further evidenced by a decrease in intracellular calcium levels (Fig. 21). While mitochondria typically sequester calcium via the mitochondrial calcium uniporter (MCU) (Bhosale et al., 2015), excessive MMP elevation can impair this uptake, leading to reduced mitochondrial calcium sequestration and consequently lower cytosolic calcium levels (Pathak and Trebak, 2018). This calcium dysregulation further compromises ATP production and exacerbates oxidative stress, amplifying the apoptotic cascade.

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Concurrently, FPN triggered a rapid and dose-dependent increase in ROS levels (Fig. 23) in primary thymocytes. This early surge in ROS suggests its pivotal role as an initiating signal for intracellular stress responses, which then cascade into mitochondrial dysfunction. The broad non-target toxicity of FPN is largely attributed to oxidative stress, stemming from an imbalance between ROS generation and antioxidant capacity (Aboul-Enein et al., 2003; Khan et al., 2015; Ki et al., 2012; Park et al., 2016; Romero et al., 2016; Wang et al., 2016). Indeed, our findings unequivocally demonstrated that FPN exposure disrupted intracellular oxidative balance, as evidenced by a decline in intracellular GSH levels (Fig. 22) and a concomitant increase in lipid peroxidation (Fig. 24). GSH is the predominant intracellular antioxidant crucial for neutralizing ROS and protecting against oxidative damage (Aydin, 2011; Wang et al., 2013; Yadav and Mishra, 2012). Its rapid depletion, coupled with increased lipid peroxidation, caused by oxidative degradation of membrane fatty acids (Paal et al., 2015; Yang et al., 2020; Zheng et al., 2024), confirms that FPN-induced ROS rapidly overwhelmed cellular redox homeostasis, creating a self-reinforcing loop of mitochondrial damage and oxidative stress leading to thymocyte death.

5.4.3 Antioxidant intervention with N-acetylcysteine

To definitively confirm the central role of oxidative stress in FPN-induced thymocyte apoptosis, I performed co-treatment experiments with the antioxidant NAC. Despite the inherent sensitivity of primary thymocytes to *in vitro* culture conditions, which can induce baseline apoptosis and redox imbalance, the distinct and significant deleterious effects observed in FPN-treated cells, and their reversal by NAC, unequivocally demonstrate a direct toxic effect of FPN.

Consistent with its dual role as a cysteine donor and a direct ROS scavenger, NAC replenished intracellular GSH, blunted the FPN-elicited surge in reactive oxygen species, and curtailed lipid peroxidation (Pedre et al., 2021). Furthermore, NAC preserved mitochondrial membrane potential, as evidenced by JC-1 staining, and significantly decreased the proportion of Annexin V^+/PI^+ cells.

doi:10.6342/NTU202501708

These protective effects align with previous reports in pesticide-exposed hepatocytes and lymphocytes, where NAC improved mitochondrial integrity and suppressed caspase-dependent apoptosis (Singh et al., 2025).

The robust protective effects of NAC unequivocally underscore the central and causative role of oxidative stress in FPN-mediated immunotoxicity. This provides a compelling mechanistic explanation for the observed reductions in thymic gene expression and dysregulation of apoptosis. Importantly, these results also highlight the therapeutic potential of antioxidant interventions in mitigating pesticide-induced immune dysfunction and preserving thymic health and immune competence under chemical stress conditions.

doi:10.6342/NTU202501708

5.5 Potential off-target toxicities and disease risks associated with FPN-induced gene dysregulation

Beyond its immunotoxic effects on T cell activation and thymic development, our pathway and disease enrichment analyses suggest that FPN may exert broader biological consequences involving multiple organ systems. Several key genes significantly altered by FPN, including *Il-7*, *Il-7r*, *Bcl-2*, *Foxo*, and *Kit*, are not only critical for immune regulation but also participate in pathways essential for neural development, hematopoiesis, and respiratory tract epithelial tissue homeostasis.

Importantly, data derived from the CTD database indicated that these genes are associated with nervous system diseases. This finding implies that FPN-induced dysregulation may contribute to neurotoxic outcomes (Fig. 30). The alteration of *Gad1* (*Gad67*) and *Slc6a1* (*Gat1*), both of which are central to GABAergic signaling, reinforces this possibility. Previous research has shown that FPN exposure can affect neurotransmitter balance and induce neurological symptoms such as seizures in rodent models. These observations raise concern about the potential for FPN to disrupt neurodevelopmental processes, especially during sensitive stages of central nervous system formation.

In addition to nervous system involvement, our results also revealed predicted associations between FPN-responsive genes and diseases of the respiratory tract, including lung disorders (Fig. 28). Moreover, CTD predictions identified links between several of these genes and hemic and lymphatic diseases, which include disorders affecting blood cell production and lymphatic homeostasis. This highlights the potential for FPN to disturb hematopoietic integrity and lymphoid function, possibly compromising both systemic immunity and tissue-level defense. I further uploaded the affected genes by FP, both from curated data in CTD and this study, to perform disease prediction inference by CTD. There are 194 genes associated with immune system disease (Fig. 31). In addition, autoimmune disease, immunoproliferative disorders, and lymphoproliferative disorders have been inferred to be the toxicity of FPN, indicating that this study successfully verified the immunotoxicity

doi:10.6342/NTU202501708

of FPN and provided more scientific evidence.

Taken together, the evidence supports the view that FPN exposure leads to the dysregulation of gene networks involved in multiple physiological systems. The overlap of affected genes in immune, neural, pulmonary, and hematologic contexts underscores the systemic nature of FPN's biological impacts. This highlights the urgent need to reassess the safety of FPN by considering not only its intended insecticidal effects but also its unintended influence on molecular pathways that are fundamental to vertebrate health.

doi:10.6342/NTU202501708

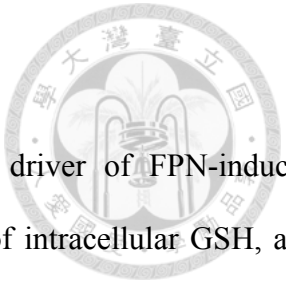
Chapter 6. Summary and Conclusion

This study provides novel insights into the immunotoxic mechanisms of FPN, revealing how it perturbs both mature and immature T-cell populations through dysregulation of GABAergic signaling, cytokine imbalance, and oxidative damage. Using integrated *in vivo* and *in vitro* models, I demonstrate that FPN exerts its effects by first altering mature T-cell responses in the periphery, followed by targeting thymic development and early T-cell differentiation at their source.

Our findings reveal that FPN disrupts immune regulatory homeostasis by interfering with GABAergic signaling, a pathway increasingly recognized for its immunomodulatory functions. FPN exposure resulted in the downregulation of *Gad67*, the key enzyme responsible for GABA synthesis, and a slight suppression of *Gat1*, the main GABA transporter in immune cells. These disruptions likely weaken inhibitory GABAergic tone in splenocytes, promoting excessive T-cell activation. Consistently, I observed increased production of Th1- and Th2-associated cytokines (IL-2, IL-4, IFN-γ) and elevated antigen-specific antibody responses. Interestingly, FPN also induced compensatory upregulation of $GABA_A$ receptor subunits, particularly *β2* and *δ*, potentially as an attempt to restore inhibitory signaling in the face of receptor antagonism. Together, these findings suggest that FPN promotes peripheral immune hyperactivity through the dysregulation of GABA metabolism and signaling.

To understand the upstream origins of these immune perturbations, I examined the effects of FPN on thymic development. Our results clearly show that FPN exposure impairs the thymic architecture and blocks the developmental transition of thymocytes at the DN stage, resulting in a reduction of DP T cells and altered CD4/CD8 ratios. These disruptions are mechanistically linked to the downregulation of IL-7 and IL-7 receptor expression, which are essential for early thymocyte survival and proliferation. In addition, transcription factors critical for thymopoiesis, including FOXO1, FOXO3, GABPα, FOXN1, and LYL1, were significantly suppressed in thymic tissues,

doi:10.6342/NTU202501708



further impairing lineage progression and thymic epithelial cell support.

At the molecular level, oxidative stress emerged as a central driver of FPN-induced immunotoxicity. FPN exposure caused a rapid surge in ROS, depletion of intracellular GSH, and increased lipid peroxidation, all of which are key markers of redox imbalance. Mitochondrial dysfunction, indicated by membrane potential depolarization and reduced calcium homeostasis, contributed to intrinsic apoptotic signaling in thymocytes. Both anti-apoptotic genes (*Bcl-2*, *Mcl-1*) and pro-apoptotic regulators (*Bim*, *Bnip3*) were dysregulated, underscoring the collapse of apoptotic control. Importantly, antioxidant intervention with NAC significantly ameliorated mitochondrial damage and cell death, reinforcing the role of oxidative stress in FPN-mediated toxicity.

Taken together, our findings suggest that FPN compromises immune function through a two-tiered mechanism. First, by stimulating peripheral T-cell hyperactivation via disruption of GABAergic inhibitory signaling. Second, by impairing thymic T-cell development through the suppression of IL-7 signaling, transcriptional regulators, and redox balance. These immunotoxic effects highlight the vulnerability of both mature and developing T-cell compartments to pesticide exposure. The important findings of this study are summarized in a schematic diagram (Fig. 32).

This work underscores the need for more stringent safety evaluations of pesticides like FPN, particularly in developmental animals. Future research should investigate the long-term immune consequences of early-life FPN exposure and explore therapeutic strategies aimed at preserving immune resilience, such as antioxidant support or modulation of GABAergic pathways.

doi:10.6342/NTU202501708

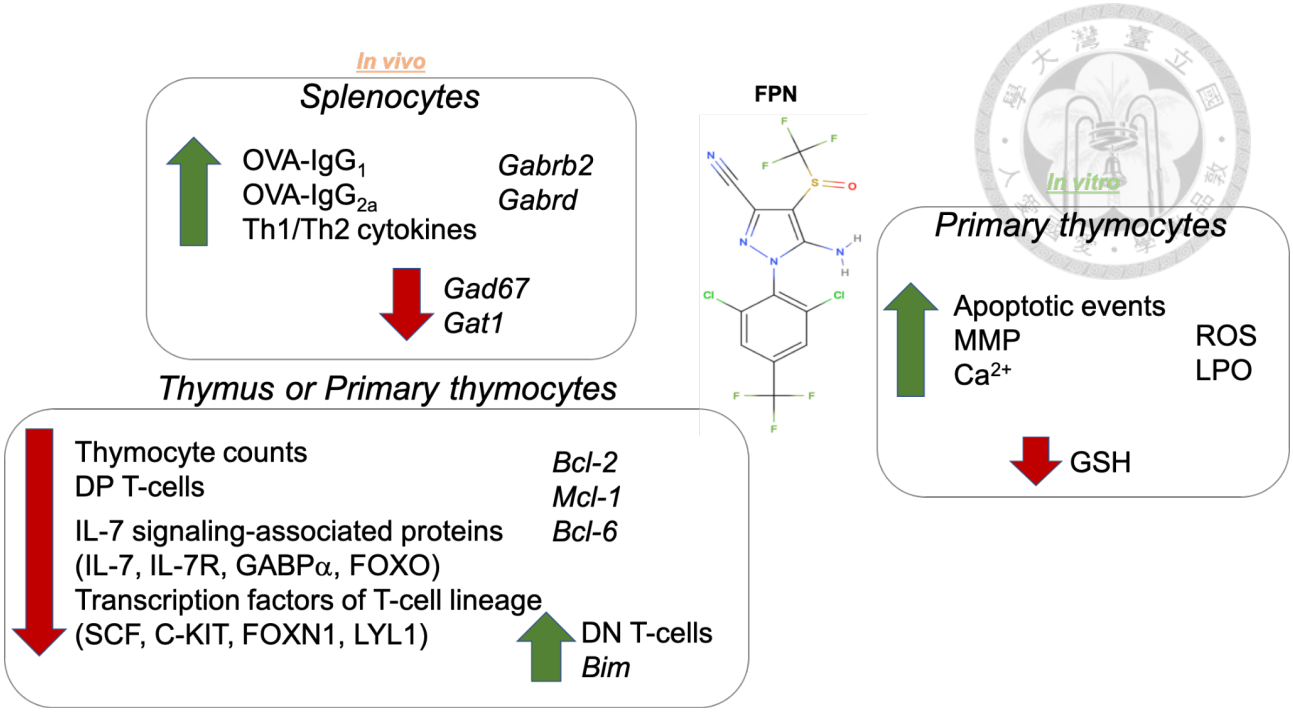


Figure 32. Summary of the main findings in this study.

doi:10.6342/NTU202501708

Chapter 7. Future perspectives

While the current study sheds light on FPN-induced immunotoxicity in T-cell compartments, it also opens important avenues for future research regarding FPN's effects on the central nervous system (CNS), particularly in the context of neuroinflammation and glial vulnerability.

Growing environmental exposure to neurotoxic chemicals has been paralleled by an alarming rise in neurodevelopmental disorders (Grandjean and Landrigan, 2014). It is estimated that approximately 25 percent of industrial chemicals in use are neurotoxic (Boyes, 2012), and pesticides have been strongly implicated in neuronal death (Ki et al., 2012). The widespread agricultural, domestic, and veterinary use of FPN has led to increased human exposure. FPN is known to accumulate in mammalian tissues, including the brain and adipose compartments (Cravedi et al., 2013), and has been associated with transient behavioral and neurological effects in rats and humans, including seizures and vomiting (Mohamed et al., 2004; Szegedi et al., 2005). In rodents, FPN exposure reduces locomotor activity and increases fear-associated behaviors (Gibbons et al., 2015; Terçariol and Godinho, 2011). Even if acute symptoms are reversible, chronic and repeated exposures may contribute to long-term neurodegenerative diseases such as Parkinson's or Alzheimer's. *In vitro*, FPN disrupts neuronal development and reduces neurite outgrowth in SH-SY5Y cells (Ruangjaroon et al., 2017) and induces neurotoxicity in PC12 cells lacking $GABA_A$ receptors, suggesting additional, receptor-independent pathways (Lassiter et al., 2009).

Within the CNS, glial cells represent approximately 90 percent of all cellular populations and include oligodendrocytes, astrocytes, ependymal cells, and microglia. These cells are essential for neuronal support, immune defense, and synaptic regulation. Oligodendrocytes are responsible for myelin formation and maintaining efficient signal conduction. Myelin sheaths reduce ion leakage and membrane capacitance to enable rapid action potential propagation. Damage to oligodendrocytes is closely associated with diseases like multiple sclerosis, where demyelination disrupts signal

doi:10.6342/NTU202501708

transmission and induces motor and sensory deficits ("Multiple Sclerosis Information Page: National Institute of Neurological Disorders and Stroke (NINDS)," n.d.). Oligodendrocyte development follows a complex progression from neural stem cells to mature myelinating forms. This lineage is highly sensitive to redox imbalance. OPCs, in particular, are vulnerable due to high metabolic demands and low antioxidant enzyme expression. Oxidative stress not only impairs OPC differentiation but also initiates apoptosis, contributing to progressive demyelination (French et al., 2009).

Studies using chlorpyrifos, another insecticide, have shown increased ROS production in CG-4 oligodendrocyte progenitor cells, and vitamin E co-treatment mitigates this damage, indicating a redox-based mechanism (Saulsbury et al., 2009). Active multiple sclerosis lesions are characterized by oxidative damage and mitochondrial dysfunction in myelin and oligodendrocytes (Aboul-Enein et al., 2003). Initial lesions often involve loss of myelin-associated glycoprotein and subsequent oligodendrocyte apoptosis (Barnett and Prineas, 2004). High lipid content in myelin, age-related iron accumulation, and inherently low antioxidant levels make these cells especially prone to peroxidative injury (Connor and Menzies, 1995; Juurlink et al., 1998).

Oxidative stress occurs when free radical production outpaces antioxidant defenses. This involves ROS such as hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O2^{\bullet-}$), and reactive nitrogen species like nitric oxide (NO) (Wang et al., 2016). Insecticide exposure significantly elevates oxidative stress markers (DiCiero Miranda et al., 2000). FPN induces ROS generation and disrupts antioxidant systems, leading to lipid, DNA, and protein damage both *in vitro* and *in vivo* (Clasen et al., 2012; Khan et al., 2015; Weidinger and Kozlov, 2015). ROS also influences apoptosis, cell cycle regulation, and signal transduction (Chtourou et al., 2015; Valvassori et al., 2015). FPN treatment in SH-SY5Y cells results in ROS accumulation, caspase-3 activation, and cytochrome c release, indicative of mitochondrial apoptosis, while NAC co-treatment reverses these effects (Ki et al., 2012; J. E. Lee et al., 2011; Park et al., 2016, 2013). In PC12 cells, FPN exposure increases MDA levels, a

doi:10.6342/NTU202501708

marker of lipid peroxidation (Lassiter et al., 2009; Slotkin and Seidler, 2010). In rat brain tissue, FPN depletes glutathione and suppresses key antioxidant enzymes such as SOD and CAT (Khalaf et al., 2019). However, the majority of oxidative stress studies on FPN focus on neurons, with little attention paid to glial cells. Given the similarities in oxidative vulnerability, it is crucial to investigate whether FPN similarly affects oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes.

In addition to experimental findings, I also conducted a disease association analysis using the Comparative Toxicogenomics Database (CTD) ("The Comparative Toxicogenomics Database | CTD," n.d.). This approach enabled us to evaluate whether the genes regulated in our study were enriched in any disease categories. The analysis revealed that several key genes affected by FPN, including BCL2, FOXO3, GAD1, IL7, IL7R, and SLC6A1 (GAT1), are significantly associated with nervous system diseases. These genes are involved in immune regulation, mitochondrial homeostasis, and neurotransmission. Their dysregulation suggests that FPN may exert dual toxicity by disrupting both immune function and neurological integrity. Therefore, our results point to FPN as a potential environmental risk factor not only for immunodeficiency but also for the development of neurodegenerative or neurodevelopmental disorders.

Future studies should examine the effects of FPN on glial subtypes, especially OPCs, by evaluating oxidative stress-induced impairments in differentiation, myelination, and mitochondrial function. Clarifying the interaction between neuroinflammation and oxidative injury in glial cells could help define the broader neurotoxic profile of FPN. These investigations are particularly relevant for understanding pesticide contributions to chronic neurological diseases, including multiple sclerosis, Parkinson's disease, and Alzheimer's disease.

doi:10.6342/NTU202501708

Reference

1. A review of Animal Safety Studies for Fipronil in the dog and cat [WWW Document], n.d. URL https://www.apvma.gov.au/sites/default/files/publication/15186-fipronil-prf-vol2-animal-safety-studies.pdf (accessed 4.22.25).
2. Aboul-Enein, F., Rauschka, H., Kornek, B., Stadelmann, C., Stefferl, A., Brück, W., Lucchinetti, C., Schmidbauer, M., Jellinger, K., Lassmann, H., 2003. Preferential Loss of Myelin-Associated Glycoprotein Reflects Hypoxia-Like White Matter Damage in Stroke and Inflammatory Brain Diseases. J Neuropathol Exp Neurol 62, 25–33. https://doi.org/10.1093/jnen/62.1.25
3. Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R., Weissman, I.L., 1997. Bcl-2 Rescues T Lymphopoiesis in Interleukin-7 Receptor–Deficient Mice. Cell 89, 1033–1041. https://doi.org/10.1016/S0092-8674(00)80291-3
4. Alam, S., Laughton, D.L., Walding, A., Wolstenholme, A.J., 2006. Human peripheral blood mononuclear cells express GABAA receptor subunits. Molecular Immunology 43, 1432–1442. https://doi.org/10.1016/j.molimm.2005.07.025
5. Aldayel, T.S., Abdel-Rahman, H.G., Gad EL-Hak, H.N., Abdelrazek, H.M.A., Mohamed, R.M., El-Sayed, R.M., 2021. Assessment of modulatory activity of Uncaria tomentosa extract against fipronil immunotoxicity in male rats. Ecotoxicology and Environmental Safety 224, 112674. https://doi.org/10.1016/j.ecoenv.2021.112674
6. Amorosi, S., D'Armiento, M., Calcagno, G., Russo, I., Adriani, M., Christiano, A., Weiner, L., Brissette, J., Pignata, C., 2008. FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus. Clinical Genetics 73, 380–384. https://doi.org/10.1111/j.1399-0004.2008.00977.x
7. Ansar Ahmed, S., 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology 150, 191–206. https://doi.org/10.1016/S0300-483X(00)00259-6
8. Ashwell, J.D., Lu, F.W.M., Vacchio, M.S., 2000. Glucocorticoids in T Cell Development and Function. Annual Review of Immunology 18, 309–345. https://doi.org/10.1146/annurev.immunol.18.1.309
9. Authority (EFSA), E.F.S., 2006. Conclusion regarding the peer review of the pesticide risk assessment of the active substance fipronil. EFSA Journal 4, 65r. https://doi.org/10.2903/j.efsa.2006.65r
10. Awad, M.A., Ahmed, Z.S.O., AbuBakr, H.O., Elbargeesy, G.A.E.-F.H., Moussa, M.H.G., 2022. Oxidative stress, apoptosis and histopathological alterations in brain stem and diencephalon induced by subacute exposure to fipronil in albino rats. Environ Sci Pollut Res 29, 936–948. https://doi.org/10.1007/s11356-021-15537-3
11. Awad, M.A., Ahmed, Z.S.O., AbuBakr, H.O., Elbargeesy, G.A.E.-F.H., Moussa, M.H.G., 2021. Fipronil induced oxidative stress in neural tissue of albino rat with subsequent apoptosis and tissue reactivity. Acta Histochemica 123, 151764. https://doi.org/10.1016/j.acthis.2021.151764
12. Aydin, B., 2011. Effects of thiacloprid, deltamethrin and their combination on oxidative stress in lymphoid organs, polymorphonuclear leukocytes and plasma of rats. Pesticide Biochemistry and Physiology 100, 165–171. https://doi.org/10.1016/j.pestbp.2011.03.006
13. Badgujar, P.C., Chandratre, G.A., Pawar, N.N., Telang, A.G., Kurade, N.P., 2016. Fipronil induced oxidative stress involves alterations in SOD1 and catalase gene expression in male mice liver: Protection by vitamins E and C. Environmental Toxicology 31, 1147–1158. https://doi.org/10.1002/tox.22125
14. Badgujar, P.C., Pawar, N.N., Chandratre, G.A., Telang, A.G., Sharma, A.K., 2015. Fipronil

doi:10.6342/NTU202501708

induced oxidative stress in kidney and brain of mice: Protective effect of vitamin E and vitamin C. Pesticide Biochemistry and Physiology 118, 10–18. https://doi.org/10.1016/j.pestbp.2014.10.013
15. Bae, J.-W., Kwon, W.-S., 2024. Proteomic analysis of fipronil-induced molecular defects in spermatozoa. Sci Rep 14, 7668. https://doi.org/10.1038/s41598-024-57876-4
16. Bae, J.-W., Kwon, W.-S., 2020. Investigating the effects of fipronil on male fertility: Insight into the mechanism of capacitation. Reproductive Toxicology 94, 1–7. https://doi.org/10.1016/j.reprotox.2020.04.002
17. Bano, F., Mohanty, B., 2020a. Thyroid disrupting pesticides mancozeb and fipronil in mixture caused oxidative damage and genotoxicity in lymphoid organs of mice. Environmental Toxicology and Pharmacology 79, 103408. https://doi.org/10.1016/j.etap.2020.103408
18. Bano, F., Mohanty, B., 2020b. Thyroxine modulation of immune toxicity induced by mixture pesticides mancozeb and fipronil in mice. Life Sciences 240, 117078. https://doi.org/10.1016/j.lfs.2019.117078
19. Barnett, M.H., Prineas, J.W., 2004. Relapsing and remitting multiple sclerosis: Pathology of the newly forming lesion. Annals of Neurology 55, 458–468. https://doi.org/10.1002/ana.20016
20. Barragan, A., Weidner, J.M., Jin, Z., Korpi, E.R., Birnir, B., 2015. GABAergic signalling in the immune system. Acta Physiologica 213, 819–827. https://doi.org/10.1111/apha.12467
21. Bergeret, M., Khrestchatisky, M., Tremblay, E., Bernard, A., Gregoire, A., Chany, C., 1998. GABA modulates cytotoxicity of immunocompetent cells expressing GABAA receptor subunits. Biomedicine & Pharmacotherapy 52, 214–219. https://doi.org/10.1016/S0753-3322(98)80019-X
22. Bhandage, A.K., Barragan, A., 2021. GABAergic signaling by cells of the immune system: more the rule than the exception. Cell. Mol. Life Sci. 78, 5667–5679. https://doi.org/10.1007/s00018-021-03881-z
23. Bhandage, A.K., Hellgren, C., Jin, Z., Olafsson, E.B., Sundström-Poromaa, I., Birnir, B., 2015. Expression of GABA receptors subunits in peripheral blood mononuclear cells is gender dependent, altered in pregnancy and modified by mental health. Acta Physiologica 213, 575–585. https://doi.org/10.1111/apha.12440
24. Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R.W., Steinman, L., 2010. Inhibitory role for GABA in autoimmune inflammation. Proceedings of the National Academy of Sciences 107, 2580–2585. https://doi.org/10.1073/pnas.0915139107
25. Bhatia, S.K., Tygrett, L.T., Grabstein, J.H., Waldschmidt, T.J., 1995. The effect of in vivo IL-7 deprivation on T cell maturation. J Exp Med 181, 1399–1409.
26. Bhatt, P., Gangola, S., Ramola, S., Bilal, M., Bhatt, K., Huang, Y., Zhou, Z., Chen, S., 2023. Insights into the toxicity and biodegradation of fipronil in contaminated environment. Microbiological Research 266, 127247. https://doi.org/10.1016/j.micres.2022.127247
27. Bhosale, G., Sharpe, J.A., Sundier, S.Y., Duchen, M.R., 2015. Calcium signaling as a mediator of cell energy demand and a trigger to cell death. Ann N Y Acad Sci 1350, 107–116. https://doi.org/10.1111/nyas.12885
28. Bjurström, H., Wang, J., Ericsson, I., Bengtsson, M., Liu, Y., Kumar-Mendu, S., Issazadeh-Navikas, S., Birnir, B., 2008. GABA, a natural immunomodulator of T lymphocytes. Journal of Neuroimmunology 205, 44–50. https://doi.org/10.1016/j.jneuroim.2008.08.017
29. Blackburn, C.C., Augustine, C.L., Li, R., Harvey, R.P., Malin, M.A., Boyd, R.L., Miller, J.F., Morahan, G., 1996. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. Proceedings of the National Academy of Sciences 93, 5742–5746. https://doi.org/10.1073/pnas.93.12.5742
30. Bloomquist, J.R., 2001. GABA and Glutamate Receptors as Biochemical Sites for Insecticide Action, in: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer,

doi:10.6342/NTU202501708

Berlin, Heidelberg, pp. 17–41. https://doi.org/10.1007/978-3-642-59549-3_2
31. Bonneau, S., Reymond, N., Gupta, S., Navarro, C., 2015. Efficacy of a fixed combination of permethrin 54.5% and fipronil 6.1% (Effitix®) in dogs experimentally infested with Ixodes ricinus. Parasites & Vectors 8, 204. https://doi.org/10.1186/s13071-015-0805-6
32. Boyes, W.K., 2012. Neurotoxicology and Behavior, in: Patty's Toxicology. American Cancer Society, pp. 35–74. https://doi.org/10.1002/0471435139.tox025.pub2
33. Brigelius-Flohé, R., Traber, M.G., 1999. Vitamin E: function and metabolism. The FASEB Journal 13, 1145–1155. https://doi.org/10.1096/fasebj.13.10.1145
34. Bugelski, P.J., Kim, C., 2007. T-Dependent Antigen Response (TDAR) Tests: Meta-Analysis of Results Generated Across Multiple Laboratories. Journal of Immunotoxicology 4, 159–164. https://doi.org/10.1080/15476910701337126
35. Bullenkamp, J., Mengoni, V., Kaur, S., Chhetri, I., Dimou, P., Astroulakis, Z.M.J., Kaski, J.C., Dumitriu, I.E., 2021. Interleukin-7 and interleukin-15 drive CD4+CD28null T lymphocyte expansion and function in patients with acute coronary syndrome. Cardiovascular Research 117, 1935–1948. https://doi.org/10.1093/cvr/cvaa202
36. Bupp, M.G., Edwards, B., Guo, C., Wei, D., Chen, G., Wong, B., Masteller, E., Peng, S.L., 2009. T cells require Foxo1 to populate the peripheral lymphoid organs. Eur J Immunol 39, 2991–2999. https://doi.org/10.1002/eji.200939427
37. Caballero, M.V., Ares, I., Martínez, M., Martínez-Larrañaga, M.R., Anadón, A., Martínez, M.A., 2015. Fipronil induces CYP isoforms in rats. Food and Chemical Toxicology 83, 215–221. https://doi.org/10.1016/j.fct.2015.06.019
38. Cam, M., Durieu, E., Bodin, M., Manousopoulou, A., Koslowski, S., Vasylieva, N., Barnych, B., Hammock, B.D., Bohl, B., Koch, P., Omori, C., Yamamoto, K., Hata, S., Suzuki, T., Karg, F., Gizzi, P., Erakovic Haber, V., Bencetic Mihaljevic, V., Tavcar, B., Portelius, E., Pannee, J., Blennow, K., Zetterberg, H., Garbis, S.D., Auvray, P., Gerber, H., Fraering, J., Fraering, P.C., Meijer, L., 2018. Induction of Amyloid-β42 Production by Fipronil and Other Pyrazole Insecticides. J Alzheimers Dis 62, 1663–1681. https://doi.org/10.3233/JAD-170875
39. Carpenter, A.C., Bosselut, R., 2010. Decision checkpoints in the thymus. Nat Immunol 11, 666–673. https://doi.org/10.1038/ni.1887
40. Carty, S.A., Koretzky, G.A., Jordan, M.S., 2014. Interleukin-4 Regulates Eomesodermin in CD8+ T Cell Development and Differentiation. PLoS One 9, e106659. https://doi.org/10.1371/journal.pone.0106659
41. Cenini, G., Lloret, A., Cascella, R., 2019. Oxidative Stress in Neurodegenerative Diseases: From a Mitochondrial Point of View. Oxidative Medicine and Cellular Longevity 2019, 2105607. https://doi.org/10.1155/2019/2105607
42. Chen, D., Li, J., Zhao, Y., Wu, Y., 2022. Human Exposure of Fipronil Insecticide and the Associated Health Risk. J. Agric. Food Chem. 70, 63–71. https://doi.org/10.1021/acs.jafc.1c05694
43. Chtourou, Y., Aouey, B., Kebieche, M., Fetoui, H., 2015. Protective role of naringin against cisplatin induced oxidative stress, inflammatory response and apoptosis in rat striatum via suppressing ROS-mediated NF-κB and P53 signaling pathways. Chemico-Biological Interactions 239, 76–86. https://doi.org/10.1016/j.cbi.2015.06.036
44. Chung, B., Min, D., Joo, L.W., Krampf, M.R., Huang, J., Yang, Y., Shashidhar, S., Brown, J., Dudl, E.P., Weinberg, K.I., 2011. Combined Effects of Interleukin-7 and Stem Cell Factor Administration on Lymphopoiesis after Murine Bone Marrow Transplantation. Biology of Blood and Marrow Transplantation 17, 48–60. https://doi.org/10.1016/j.bbmt.2010.07.027
45. Clasen, B., Loro, V.L., Cattaneo, R., Moraes, B., Lópes, T., de Avila, L.A., Zanella, R., Reimche, G.B., Baldisserotto, B., 2012. Effects of the commercial formulation containing fipronil on the non-target organism Cyprinus carpio: Implications for rice−fish cultivation. Ecotoxicology and Environmental Safety 77, 45–51.

doi:10.6342/NTU202501708

https://doi.org/10.1016/j.ecoenv.2011.10.001
46. Cochran, R.C., Yu ,Liu, Krieger ,R. I., and Ross, J.H., 2015. Postapplication Fipronil Exposure Following Use on Pets. Journal of Toxicology and Environmental Health, Part A 78, 1217–1226. https://doi.org/10.1080/15287394.2015.1076363
47. Connor, J.R., Menzies, S.L., 1995. Cellular management of iron in the brain. Journal of the Neurological Sciences 134, 33–44. https://doi.org/10.1016/0022-510X(95)00206-H
48. Corcoran, A.E., Riddell, A., Krooshoop, D., Venkitaraman, A.R., 1998. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. Nature 391, 904–907. https://doi.org/10.1038/36122
49. Cordeiro, F., Bratinova, S., Karasek, L., Buttinger, G., Stroka, J., Emteborg, H., Seghers, J., Robouch, P., Emons, H., 2019. Can official control laboratories quantify reliably fipronil in eggs? Evidence from a proficiency testing round. Food Additives & Contaminants: Part A 36, 1–7. https://doi.org/10.1080/19440049.2019.1602885
50. Cravedi, J.P., Delous, G., Zalko, D., Viguié, C., Debrauwer, L., 2013. Disposition of fipronil in rats. Chemosphere 93, 2276–2283. https://doi.org/10.1016/j.chemosphere.2013.07.083
51. Cui, Z., Zhao, F., Chen, X., Li, J., Jin, X., Han, Y., Wang, L., Zhou, Y., Lu, L., 2022. NPAT Supports CD8+ Immature Single-Positive Thymocyte Proliferation and Thymic Development. The Journal of Immunology 209, 916–925. https://doi.org/10.4049/jimmunol.2200214
52. Cunningham-Rundles, C., Ponda, P.P., 2005. Molecular defects in T- and B-cell primary immunodeficiency diseases. Nat Rev Immunol 5, 880–892. https://doi.org/10.1038/nri1713
53. D., kson, C. B., C., B., L., K., B., D., S., 2009. Fipronil General Fact Sheet [WWW Document]. URL https://secure.livechatinc.com/customer/action/open_chat?license_id=18981025&group=0&embedded=1&widget_version=3&unique_groups=0&organizationId=ae087525-9322-4fda-ac7c-7f902f4380dc&use_parent_storage=1&x-region=us-south1 (accessed 7.8.25).
54. da Silva, R.C., Teixeira, M.P., de Paiva, L.S., Miranda-Alves, L., 2023. Environmental Health and Toxicology: Immunomodulation Promoted by Endocrine-Disrupting Chemical Tributyltin. Toxics 11, 696. https://doi.org/10.3390/toxics11080696
55. Dardalhon, V., Korn, T., Kuchroo, V.K., Anderson, A.C., 2008. Role of Th1 and Th17 cells in organ-specific autoimmunity. J Autoimmun 31, 252–256. https://doi.org/10.1016/j.jaut.2008.04.017
56. Das, P.C., Cao, Y., Cherrington, N., Hodgson, E., Rose, R.L., 2006. Fipronil induces CYP isoforms and cytotoxicity in human hepatocytes. Chemico-Biological Interactions 164, 200–214. https://doi.org/10.1016/j.cbi.2006.09.013
57. de Groote, L., Linthorst, A.C.E., 2007. Exposure to novelty and forced swimming evoke stressor-dependent changes in extracellular GABA in the rat hippocampus. Neuroscience 148, 794–805. https://doi.org/10.1016/j.neuroscience.2007.06.030
58. De la Fuente, M., Hernanz, A., Viniegra, S., Miquel, J., 2011. Sulfur-containing antioxidants increase in vitro several functions of lymphocytes from mice. International Immunopharmacology 11, 661–669. https://doi.org/10.1016/j.intimp.2011.01.008
59. De Oliveira, P.R., Bechara, G.H., Denardi, S.E., Oliveira, R.J., Mathias, M.I.C., 2012. Cytotoxicity of fipronil on mice liver cells. Microscopy Research and Technique 75, 28–35. https://doi.org/10.1002/jemt.21018
60. Deobagkar-Lele, M., Chacko, S.K., Victor, E.S., Kadthur, J.C., Nandi, D., 2013. Interferon-γ- and glucocorticoid-mediated pathways synergize to enhance death of CD4+ CD8+ thymocytes during Salmonella enterica serovar Typhimurium infection. Immunology 138, 307–321. https://doi.org/10.1111/imm.12047
61. DiCiero Miranda, M., de Bruin, V.M.S., Vale, M.R., Viana, G.S.B., 2000. Lipid Peroxidation and Nitrite plus Nitrate Levels in Brain Tissue from Patients with Alzheimer's Disease. Gerontology 46, 179–184. https://doi.org/10.1159/000022156

doi:10.6342/NTU202501708

62. Dionisio, L., De Rosa, M.J., Bouzat, C., Esandi, M. del C., 2011. An intrinsic GABAergic system in human lymphocytes. Neuropharmacology 60, 513–519. https://doi.org/10.1016/j.neuropharm.2010.11.007
63. Drela, N., 2006. Xenobiotic-induced alterations in thymocyte development. APMIS 114, 399–419. https://doi.org/10.1111/j.1600-0463.2006.apm_343.x
64. Dumont, P., Liebenberg, J., Beugnet, F., Fankhauser, B., 2015. Repellency and acaricidal efficacy of a new combination of fipronil and permethrin against Ixodes ricinus and Rhipicephalus sanguineus ticks on dogs. Parasites & Vectors 8, 531. https://doi.org/10.1186/s13071-015-1150-5
65. Durum, S.K., Candèias, S., Nakajima, H., Leonard, W.J., Baird, A.M., Berg, L.J., Muegge, K., 1998. Interleukin 7 Receptor Control of T Cell Receptor γ Gene Rearrangement: Role of Receptor-associated Chains and Locus Accessibility. J Exp Med 188, 2233–2241.
66. Environmental Protection Agency, 2007. Fipronil; Pesticide Tolerances [WWW Document]. Federal Register. URL https://www.federalregister.gov/documents/2007/08/22/E7-16621/fipronil-pesticide-tolerances (accessed 7.9.25).
67. European Medicines Agency, 2010. Guideline on Repeated Dose Toxicity Corr.
68. Ezeriņa, D., Takano, Y., Hanaoka, K., Urano, Y., Dick, T.P., 2018. N-Acetyl Cysteine Functions as a Fast-Acting Antioxidant by Triggering Intracellular H2S and Sulfane Sulfur Production. Cell Chemical Biology 25, 447-459.e4. https://doi.org/10.1016/j.chembiol.2018.01.011
69. Fenninger, F., Han, J., Stanwood, S.R., Nohara, L.L., Arora, H., Choi, K.B., Munro, L., Pfeifer, C.G., Shanina, I., Horwitz, M.S., Jefferies, W.A., 2019. Mutation of an L-Type Calcium Channel Gene Leads to T Lymphocyte Dysfunction. Front. Immunol. 10. https://doi.org/10.3389/fimmu.2019.02473
70. Fipronil Risk Characterization Document [WWW Document], n.d. URL https://www.cdpr.ca.gov/wp-content/uploads/2024/10/fipronil_rcd.pdf (accessed 5.20.25).
71. Fischer, S.F., Bouillet, P., O'Donnell, K., Light, A., Tarlinton, D.M., Strasser, A., 2007. Proapoptotic BH3-only protein Bim is essential for developmentally programmed death of germinal center-derived memory B cells and antibody-forming cells. Blood 110, 3978–3984. https://doi.org/10.1182/blood-2007-05-091306
72. Frank, J., Pignata, C., Panteleyev, A.A., Prowse, D.M., Baden, H., Weiner, L., Gaetaniello, L., Ahmad, W., Pozzi, N., Cserhalmi-Friedman, P.B., Aita, V.M., Uyttendaele, H., Gordon, D., Ott, J., Brissette, J.L., Christiano, A.M., 1999. Exposing the human nude phenotype. Nature 398, 473–474. https://doi.org/10.1038/18997
73. Freeden-Jeffery, U. con, Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E.G., Murray, R., 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181, 1519–1526.
74. French, H.M., Reid, M., Mamontov, P., Simmons, R.A., Grinspan, J.B., 2009. Oxidative Stress Disrupts Oligodendrocyte Maturation. J Neurosci Res 87, 3076–3087. https://doi.org/10.1002/jnr.22139
75. Frumento, G., Zuo, J., Verma, K., Croft, W., Ramagiri, P., Chen, F.E., Moss, P., 2019. CD117 (c-Kit) Is Expressed During CD8+ T Cell Priming and Stratifies Sensitivity to Apoptosis According to Strength of TCR Engagement. Front. Immunol. 10. https://doi.org/10.3389/fimmu.2019.00468
76. FSS_Fipronil_QA [WWW Document], 2017. URL https://www.foodstandards.gov.scot/downloads/FSS_Fipronil_QA_-_17_August_2017.pdf (accessed 5.25.25).
77. Fu, Z., Tindall, D.J., 2008. FOXOs, cancer and regulation of apoptosis. Oncogene 27, 2312–2319. https://doi.org/10.1038/onc.2008.24
78. Fuks, J.M., Arrighi, R.B.G., Weidner, J.M., Mendu, S.K., Jin, Z., Wallin, R.P.A., Rethi, B.,

doi:10.6342/NTU202501708

Birnir, B., Barragan, A., 2012. GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by Toxoplasma gondii. PLOS Pathogens 8, e1003051. https://doi.org/10.1371/journal.ppat.1003051
79. Gallo-Payet, N., Battista, M.-C., 2014. Steroidogenesis—Adrenal Cell Signal Transduction, in: Comprehensive Physiology. John Wiley & Sons, Ltd, pp. 889–964. https://doi.org/10.1002/cphy.c130050
80. Germain, R.N., 2002. T-cell development and the CD4–CD8 lineage decision. Nat Rev Immunol 2, 309–322. https://doi.org/10.1038/nri798
81. Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ Sci Pollut Res Int 22, 103–118. https://doi.org/10.1007/s11356-014-3180-5
82. Gill, K.K., Dumka, V.K., 2016. Antioxidant status in oral subchronic toxicity of fipronil and fluoride co-exposure in buffalo calves. Toxicol Ind Health 32, 251–259. https://doi.org/10.1177/0748233713500376
83. Godfrey, D.I., Kennedy, J., Suda, T., Zlotnik, A., 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol 150, 4244–4252.
84. Gondhalekar, A.D., Scharf, M.E., 2012. Mechanisms Underlying Fipronil Resistance in a Multiresistant Field Strain of the German Cockroach (Blattodea: Blattellidae). Journal of Medical Entomology 49, 122–131. https://doi.org/10.1603/ME11106
85. Grabstei, K.H., Waldschmidt, T.J., Finkelman, F.D., Hess, B.W., Alpert, A.R., Boiani, N.E., Namen, A.E., Morrissey, P.J., 1993. Inhibition of murine B and T lymphopoiesis in vivo by an anti- interleukin 7 monoclonal antibody. J Exp Med 178, 257–264.
86. Grandjean, P., Landrigan, P.J., 2014. Neurobehavioural effects of developmental toxicity. The Lancet Neurology 13, 330–338. https://doi.org/10.1016/S1474-4422(13)70278-3
87. Gruver, A.L., Sempowski, G.D., 2008. Cytokines, leptin, and stress-induced thymic atrophy. J Leukoc Biol 84, 915–923. https://doi.org/10.1189/jlb.0108025
88. Guelfi, M., Maioli, M.A., Tavares, M.A., Mingatto, F.E., 2015. Citotoxicity of Fipronil on Hepatocytes Isolated from Rat and Effects of Its Biotransformation. Braz. arch. biol. technol. 58, 843–853. https://doi.org/10.1590/S1516-89132015060298
89. Gülow, K., Tümen, D., Heumann, P., Schmid, S., Kandulski, A., Müller, M., Kunst, C., 2024. Unraveling the Role of Reactive Oxygen Species in T Lymphocyte Signaling. Int J Mol Sci 25, 6114. https://doi.org/10.3390/ijms25116114
90. Guo, Q., Zhao, S., Zhang, J., Qi, K., Du, Z., Shao, B., 2018. Determination of fipronil and its metabolites in chicken egg, muscle and cake by a modified QuEChERS method coupled with LC-MS/MS. Food Additives & Contaminants: Part A 35. https://doi.org/10.1080/19440049.2018.1472395
91. Gupta, R.C., Doss, R.B., 2024. Phenylpyrazole (Fipronil) Toxicosis in Animals - Toxicology [WWW Document]. MSD Veterinary Manual. URL https://www.msdvetmanual.com/toxicology/insecticide-and-acaricide-organic-toxicity/phenylpyrazole-fipronil-toxicosis-in-animals (accessed 7.7.25).
92. Gustafsson, Å.B., 2011. Bnip3 as a Dual Regulator of Mitochondrial Turnover and Cell Death in the Myocardium. Pediatr Cardiol 32, 267–274. https://doi.org/10.1007/s00246-010-9876-5
93. Hainzl, D., Casida, J.E., 1996. Fipronil insecticide: Novel photochemical desulfinylation with retention of neurotoxicity. Proceedings of the National Academy of Sciences 93, 12764–12767. https://doi.org/10.1073/pnas.93.23.12764
94. Hainzl, D., Cole, L.M., Casida, J.E., 1998. Mechanisms for Selective Toxicity of Fipronil Insecticide and Its Sulfone Metabolite and Desulfinyl Photoproduct. Chem. Res. Toxicol. 11, 1529–1535. https://doi.org/10.1021/tx980157t
95. Herin, F., Boutet-Robinet, E., Levant, A., Dulaurent, S., Manika, M., Galatry-Bouju, F., Caron,

doi:10.6342/NTU202501708

P., Soulat, J.-M., 2011. Thyroid Function Tests in Persons with Occupational Exposure to Fipronil. Thyroid® 21, 701–706. https://doi.org/10.1089/thy.2010.0449
96. Ibrahim, S.A., Henderson, G., Fei, H., 2003. Toxicity, Repellency, and Horizontal Transmission of Fipronil in the Formosan Subterranean Termite (Isoptera: Rhinotermitidae). Journal of Economic Entomology 96, 461–467. https://doi.org/10.1093/jee/96.2.461
97. Im, E., Kim, H., Kim, J., Lee, H., Yang, H., 2015. Tributyltin acetate-induced immunotoxicity is related to inhibition of T cell development in the mouse thymus. Molecular & Cellular Toxicology 11, 231–239. https://doi.org/10.1007/s13273-015-0022-6
98. Jackson, D., Cornell, C.B., Luukinen, B., Buhl, K., Stone, D., 2009. Fipronil Technical Fact Sheet [WWW Document]. URL http://npic.orst.edu/factsheets/archive/fiptech.html (accessed 6.17.24).
99. Jennings, K., Canerdy, T., Keller, R., Atieh, B., Doss, R., Gupta, R., 2002. Human exposure to fipronil from dogs treated with Frontline. Veterinary and human toxicology 44, 301–3.
100. Jha, M.K., Badou, A., Meissner, M., McRory, J.E., Freichel, M., Flockerzi, V., Flavell, R.A., 2009. Defective survival of naive CD8+ T lymphocytes in the absence of the β3 regulatory subunit of voltage-gated calcium channels. Nat Immunol 10, 1275–1282. https://doi.org/10.1038/ni.1793
101. Jiang, W., Soeprono, A., Rust, M.K., Gan, J., 2014. Ant control efficacy of pyrethroids and fipronil on outdoor concrete surfaces. Pest Management Science 70, 271–277. https://doi.org/10.1002/ps.3555
102. Jin, Z., Mendu, S.K., Birnir, B., 2013. GABA is an effective immunomodulatory molecule. Amino Acids 45, 87–94. https://doi.org/10.1007/s00726-011-1193-7
103. Juurlink, B.H.J., Thorburne, S.K., Hertz, L., 1998. Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. Glia 22, 371–378. https://doi.org/10.1002/(SICI)1098-1136(199804)22:4<371::AID-GLIA6>3.0.CO;2-6
104. Kartheek, R.M., David, M., 2018. Assessment of fipronil toxicity on wistar rats: A hepatotoxic perspective. Toxicology Reports 5, 448–456. https://doi.org/10.1016/j.toxrep.2018.02.019
105. Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., Hedrick, S.M., 2009. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat Immunol 10, 176–184. https://doi.org/10.1038/ni.1689
106. Kerdiles, Y.M., Stone, E.L., Beisner, D.L., McGargill, M.A., Ch'en, I.L., Stockmann, C., Katayama, C.D., Hedrick, S.M., 2010. Foxo transcription factors control regulatory T cell development and function. Immunity 33, 890–904. https://doi.org/10.1016/j.immuni.2010.12.002
107. Khalaf, A.A., Galal, M.K., Ibrahim, M.A., Allah, A.A.A., Afify, M.M., Refaat, R., 2019. The Terminalia laxiflora modulates the neurotoxicity induced by fipronil in male albino rats. Biosci Rep 39. https://doi.org/10.1042/BSR20181363
108. Khan, S., Jan, M.H., Kumar, D., Telang, A.G., 2015. Firpronil induced spermotoxicity is associated with oxidative stress, DNA damage and apoptosis in male rats. Pesticide Biochemistry and Physiology 124, 8–14. https://doi.org/10.1016/j.pestbp.2015.03.010
109. Ki, Y.-W., Lee, J.E., Park, J.H., Shin, I.C., Koh, H.C., 2012. Reactive oxygen species and mitogen-activated protein kinase induce apoptotic death of SH-SY5Y cells in response to fipronil. Toxicology Letters 211, 18–28. https://doi.org/10.1016/j.toxlet.2012.02.022
110. Kim, J.K., Kim, Y.S., Lee, H.-M., Jin, H.S., Neupane, C., Kim, S., Lee, S.-H., Min, J.-J., Sasai, M., Jeong, J.-H., Choe, S.-K., Kim, J.-M., Yamamoto, M., Choy, H.E., Park, J.B., Jo, E.-K., 2018. GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. Nat Commun 9, 4184. https://doi.org/10.1038/s41467-018-06487-5
111. Kim, Y.A., Yoon, Y.S., Kim, H.S., Jeon, S.J., Cole, E., Lee, J., Kho, Y., Cho, Y.H., 2019. Distribution of fipronil in humans, and adverse health outcomes of in utero fipronil sulfone

doi:10.6342/NTU202501708

exposure in newborns. International Journal of Hygiene and Environmental Health 222, 524–532. https://doi.org/10.1016/j.ijheh.2019.01.009
112. Köchl, R., Thelen, F., Vanes, L., Brazão, T.F., Fountain, K., Xie, J., Huang, C.-L., Lyck, R., Stein, J.V., Tybulewicz, V.L.J., 2016. WNK1 kinase balances T cell adhesion versus migration in vivo. Nat Immunol 17, 1075–1083. https://doi.org/10.1038/ni.3495
113. Koslowski, S., Latapy, C., Auvray, P., Blondel, M., Meijer, L., 2020. Long-Term Fipronil Treatment Induces Hyperactivity in Female Mice. International Journal of Environmental Research and Public Health 17, 1579. https://doi.org/10.3390/ijerph17051579
114. Kuo, J.-F., Cheng, Y.-H., Tung, C.-W., Wang, C.-C., 2024a. Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in the ovalbumin-immunized BALB/c mice. BMC Veterinary Research 20, 30. https://doi.org/10.1186/s12917-024-03878-3
115. Kuo, J.-F., Hsiao, Y.-P., Wang, Y.-D., Weng, H.-P., Wang, C.-C., 2025. Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes. Toxics 13, 204. https://doi.org/10.3390/toxics13030204
116. Kuo, J.-F., Wu, H.-Y., Tung, C.-W., Huang, W.-H., Lin, C.-S., Wang, C.-C., 2024b. Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes. Chem. Res. Toxicol. 37, 1488–1500. https://doi.org/10.1021/acs.chemrestox.4c00060
117. Kuo, T.C., Schlissel, M.S., 2009. Mechanisms controlling expression of the RAG locus during lymphocyte development. Curr Opin Immunol 21, 173–178. https://doi.org/10.1016/j.coi.2009.03.008
118. Kwak, S., Cho, Y.S., Na, H.G., Bae, C.H., Song, S.-Y., Kim, Y.-D., 2022. Fipronil upregulates inflammatory cytokines and MUC5AC expression in human nasal epithelial cells. Rhinology 58, 66–73. https://doi.org/10.4193/Rhin19.172
119. Ladi, E., Yin, X., Chtanova, T., Robey, E.A., 2006. Thymic microenvironments for T cell differentiation and selection. Nat Immunol 7, 338–343. https://doi.org/10.1038/ni1323
120. Lassiter, T.L., MacKillop, E.A., Ryde, I.T., Seidler, F.J., Slotkin, T.A., 2009. Is fipronil safer than chlorpyrifos? Comparative developmental neurotoxicity modeled in PC12 cells. Brain Research Bulletin 78, 313–322. https://doi.org/10.1016/j.brainresbull.2008.09.020
121. Lee, J.E., Kang, J.S., Ki, Y.-W., Lee, S.-H., Lee, S.-J., Lee, K.S., Koh, H.C., 2011. Akt/GSK3β signaling is involved in fipronil-induced apoptotic cell death of human neuroblastoma SH-SY5Y cells. Toxicology Letters 202, 133–141. https://doi.org/10.1016/j.toxlet.2011.01.030
122. Lee, M., Schwab, C., Mcgeer, P.L., 2011. Astrocytes are GABAergic cells that modulate microglial activity. Glia 59, 152–165. https://doi.org/10.1002/glia.21087
123. Lee, S.-J., Kim ,Hong-Pyo, Jin ,Yang, Choi ,Augustine M.K., and Ryter, S.W., 2011. Beclin 1 deficiency is associated with increased hypoxia-induced angiogenesis. Autophagy 7, 829–839. https://doi.org/10.4161/auto.7.8.15598
124. Lee, S.-J., Mulay, P., Diebolt-Brown, B., Lackovic, M.J., Mehler, L.N., Beckman, J., Waltz, J., Prado, J.B., Mitchell, Y.A., Higgins, S.A., Schwartz, A., Calvert, G.M., 2010. Acute illnesses associated with exposure to fipronil—surveillance data from 11 states in the United States, 2001–2007. Clinical Toxicology 48, 737–744. https://doi.org/10.3109/15563650.2010.507548
125. Leghait, J., Gayrard, V., Picard-Hagen, N., Camp, M., Perdu, E., Toutain, P.-L., Viguié, C., 2009. Fipronil-induced disruption of thyroid function in rats is mediated by increased total and free thyroxine clearances concomitantly to increased activity of hepatic enzymes. Toxicology 255, 38–44. https://doi.org/10.1016/j.tox.2008.09.026
126. Li, B.-J., Wang, K.-K., Chen, D.-P., Yan, Y., Cai, X.-L., Chen, H.-M., Dong, K., Lin, F., Xu, H.-H., 2021. Distinct roles of two RDL GABA receptors in fipronil action in the diamondback moth (Plutella xylostella). Insect Science 28, 1721–1733. https://doi.org/10.1111/1744-7917.12892

doi:10.6342/NTU202501708

127. Liang, Z., Dong, X., Zhang, Z., Zhang, Q., Zhao, Y., 2022. Age-related thymic involution: Mechanisms and functional impact. Aging Cell 21, e13671. https://doi.org/10.1111/acel.13671
128. Lin, L., Hron, J.D., Peng, S.L., 2004. Regulation of NF-κB, Th Activation, and Autoinflammation by the Forkhead Transcription Factor Foxo3a. Immunity 21, 203–213. https://doi.org/10.1016/j.immuni.2004.06.016
129. Lu, M., Du, J., Zhou, P., Chen, H., Lu, C., Zhang, Q., 2015. Endocrine disrupting potential of fipronil and its metabolite in reporter gene assays. Chemosphere 120, 246–251. https://doi.org/10.1016/j.chemosphere.2014.07.015
130. Luo, C.T., Osmanbeyoglu, H.U., Do, M.H., Bivona, M.R., Toure, A., Kang, D., Xie, Y., Leslie, C.S., Li, M.O., 2017. Ets transcription factor GABP controls T cell homeostasis and immunity. Nat Commun 8, 1062. https://doi.org/10.1038/s41467-017-01020-6
131. Ly, J.D., Grubb, D.R., Lawen, A., 2003. The mitochondrial membrane potential (Δψm) in apoptosis; an update. Apoptosis 8, 115–128. https://doi.org/10.1023/A:1022945107762
132. Lynch, J.W., 2004. Molecular Structure and Function of the Glycine Receptor Chloride Channel. Physiological Reviews 84, 1051–1095. https://doi.org/10.1152/physrev.00042.2003
133. Mackall, C.L., Gress, R.E., 1997. Thymic aging and T-cell regeneration. Immunological Reviews 160, 91–102. https://doi.org/10.1111/j.1600-065X.1997.tb01030.x
134. Maddison, J.E., Page, S.W., Church, D.B. (Eds.), 2008. Chapter 10 - Antiparasitic drugs, in: Small Animal Clinical Pharmacology (Second Edition). W.B. Saunders, Edinburgh, pp. 198–260. https://doi.org/10.1016/B978-070202858-8.50012-9
135. Mahmoud, Y.K., Ali, A.A., Abdelrazek, H.M.A., Aldayel, T.S., Abdel-Daim, M.M., El-Menyawy, M.A.I., 2021. Neurotoxic Effect of Fipronil in Male Wistar Rats: Ameliorative Effect of L-Arginine and L-Carnitine. Biology (Basel) 10, 682. https://doi.org/10.3390/biology10070682
136. Maki, K., Sunaga, S., Ikuta, K., 1996. The V–J Recombination of T Cell Receptor-γ Genes Is Blocked in Interleukin-7 Receptor–deficient Mice. J Exp Med 184, 2423–2428.
137. Maraskovsky, E., O'Reilly, L.A., Teepe, M., Corcoran, L.M., Peschon, J.J., Strasser, A., 1997. Bcl-2 Can Rescue T Lymphocyte Development in Interleukin-7 Receptor–Deficient Mice but Not in Mutant *rag-1*−/− Mice. Cell 89, 1011–1019. https://doi.org/10.1016/S0092-8674(00)80289-5
138. Marshall, F.H., Jones, K.A., Kaupmann, K., Bettler, B., 1999. GABAB receptors – the first 7TM heterodimers. Trends in Pharmacological Sciences 20, 396–399. https://doi.org/10.1016/S0165-6147(99)01383-8
139. Massa, S., Balciunaite, G., Ceredig, R., Rolink, A.G., 2006. Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. European Journal of Immunology 36, 526–532. https://doi.org/10.1002/eji.200535760
140. Matsuzaki, Y., Nakayama, Kei-ichi, Nakayama, Keiko, Tomita, T., Isoda, M., Loh, D.Y., Nakauchi, H., 1997. Role of bcl-2 in the Development of Lymphoid Cells From the Hematopoietic Stem Cell. Blood 89, 853–862. https://doi.org/10.1182/blood.V89.3.853
141. McMahen, R.L., Strynar, M.J., Dagnino, S., Herr, D.W., Moser, V.C., Garantziotis, S., Andersen, E.M., Freeborn, D.L., McMillan, L., Lindstrom, A.B., 2015. Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study. Environ Int 78, 16–23. https://doi.org/10.1016/j.envint.2015.01.016
142. Mendu, S.K., Åkesson, L., Jin, Z., Edlund, A., Cilio, C., Lernmark, Å., Birnir, B., 2011. Increased GABAA channel subunits expression in CD8+ but not in CD4+ T cells in BB rats developing diabetes compared to their congenic littermates. Molecular Immunology 48, 399–407. https://doi.org/10.1016/j.molimm.2010.08.005
143. Mérino, D., Giam, M., Hughes, P.D., Siggs, O.M., Heger, K., O'Reilly, L.A., Adams, J.M., Strasser, A., Lee, E.F., Fairlie, W.D., Bouillet, P., 2009. The role of BH3-only protein Bim extends beyond inhibiting Bcl-2–like prosurvival proteins. The Journal of Cell Biology 186,

doi:10.6342/NTU202501708

355. https://doi.org/10.1083/jcb.200905153
144. Michalek, R.D., Rathmell, J.C., 2010. The metabolic life and times of a T-cell. Immunological Reviews 236, 190–202. https://doi.org/10.1111/j.1600-065X.2010.00911.x
145. Mohamed, F., Senarathna, L., Percy, A., Abeyewardene, M., Eaglesham, G., Cheng, R., Azher, S., Hittarage, A., Dissanayake, W., Sheriff, M.R., Davies, W., Buckley, N., Eddleston, M., 2004. Acute Human Self-Poisoning with the N-Phenylpyrazole Insecticide Fipronil –A GABAA-Gated Chloride Channel Blocker. J Toxicol Clin Toxicol 42, 955–963.
146. Morokata, T., Ishikawa, J., Yamada, T., 2000. Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses. Immunology Letters 72, 119–126. https://doi.org/10.1016/S0165-2478(00)00188-7
147. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4
148. Mossa, A.-T.H., Swelam, E.S., Mohafrash, S.M.M., 2015. Sub-chronic exposure to fipronil induced oxidative stress, biochemical and histopathological changes in the liver and kidney of male albino rats. Toxicology Reports 2, 775–784. https://doi.org/10.1016/j.toxrep.2015.02.009
149. Muegge, K., Vila, M.P., Durum, S.K., 1993. Interleukin-7: a Cofactor For V(D)J Rearrangement of the T Cell Receptor β Gene. Science 261, 93–95. https://doi.org/10.1126/science.7686307
150. Müller, S.M., Ege, M., Pottharst, A., Schulz, A.S., Schwarz, K., Friedrich, W., 2001. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. Blood 98, 1847–1851. https://doi.org/10.1182/blood.V98.6.1847
151. Multiple Sclerosis Information Page: National Institute of Neurological Disorders and Stroke (NINDS) [WWW Document], n.d. URL https://web.archive.org/web/20160213025406/http://www.ninds.nih.gov/disorders/multiple_sclerosis/multiple_sclerosis.htm (accessed 4.6.20).
152. Munoz-Pineiro, M.A., Robouch, P., 2018. Fipronil in eggs: Factsheet – December 2017 [WWW Document]. JRC Publications Repository. URL https://publicationstest.jrc.cec.eu.int/repository/handle/JRC110632 (accessed 2.21.23).
153. Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., Loh, D.Y., 1994. Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc Natl Acad Sci U S A 91, 3700–3704.
154. Narahashi, T., Zhao, X., Ikeda, T., Nagata, K., Yeh, J., 2007. Differential actions of insecticides on target sites: basis for selective toxicity. Hum Exp Toxicol 26, 361–366. https://doi.org/10.1177/0960327106078408
155. Narahashi, T., Zhao, X., Ikeda, T., Salgado, V.L., Yeh, J.Z., 2010. Glutamate-activated chloride channels: Unique fipronil targets present in insects but not in mammals. Pestic Biochem Physiol 97, 149–152. https://doi.org/10.1016/j.pestbp.2009.07.008
156. Nehls, M., Kyewski, B., Messerle, M., Waldschütz, R., Schüddekopf, K., Smith, A.J.H., Boehm, T., 1996. Two Genetically Separable Steps in the Differentiation of Thymic Epithelium. Science 272, 886–889. https://doi.org/10.1126/science.272.5263.886
157. Niizuma, K., Endo, H., Chan, P.H., 2009. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. J Neurochem 109, 133–138. https://doi.org/10.1111/j.1471-4159.2009.05897.x
158. Nohara, K., Ao, K., Miyamoto, Y., Suzuki, T., Imaizumi, S., Tateishi, Y., Omura, S., Tohyama, C., Kobayashi, T., 2008. Arsenite-Induced Thymus Atrophy is Mediated by Cell Cycle Arrest: A Characteristic Downregulation of E2F-Related Genes Revealed by a Microarray Approach. Toxicological Sciences 101, 226–238. https://doi.org/10.1093/toxsci/kfm268
159. Nohara, K., Fujimaki, H., Tsukumo, S., Inouye, K., Sone, H., Tohyama, C., 2002. Effects of

doi:10.6342/NTU202501708

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. Toxicology 172, 49–58. https://doi.org/10.1016/S0300-483X(01)00582-0
160. Ohi, M., Dalsenter, P.R., Andrade, A.J.M., Nascimento, A.J., 2004. Reproductive adverse effects of fipronil in Wistar rats. Toxicology Letters 146, 121–127. https://doi.org/10.1016/j.toxlet.2003.08.008
161. Okazaki, H., Kohro-Ikeda, E., Takeda, S., Ishii, H., Furuta, E., Matsuo, S., Matsumoto, M., Takiguchi, M., Aramaki, H., 2016. Fipronil, an insecticide, acts as an anti-estrogen via the concomitant down-regulation of ERα and PES1. Fundam. Toxicol. Sci. 3, 33–37. https://doi.org/10.2131/fts.3.33
162. Olsen, R.W., Sieghart, W., 2008. International Union of Pharmacology. LXX. Subtypes of γ-Aminobutyric AcidA Receptors: Classification on the Basis of Subunit Composition, Pharmacology, and Function. Update. Pharmacol Rev 60, 243–260. https://doi.org/10.1124/pr.108.00505
163. Omilusik, K., Priatel, J.J., Chen, X., Wang, Y.T., Xu, H., Choi, K.B., Gopaul, R., McIntyre-Smith, A., Teh, H.-S., Tan, R., Bech-Hansen, N.T., Waterfield, D., Fedida, D., Hunt, S.V., Jefferies, W.A., 2011. The CaV1.4 Calcium Channel Is a Critical Regulator of T Cell Receptor Signaling and Naive T Cell Homeostasis. Immunity 35, 349–360. https://doi.org/10.1016/j.immuni.2011.07.011
164. Opferman, J.T., Letai, A., Beard, C., Sorcinelli, M.D., Ong, C.C., Korsmeyer, S.J., 2003. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature 426, 671–676. https://doi.org/10.1038/nature02067
165. Ouyang, W., Liao, W., Luo, C.T., Yin, N., Huse, M., Kim, M.V., Peng, M., Chan, P., Ma, Q., Mo, Y., Meijer, D., Zhao, K., Rudensky, A.Y., Atwal, G., Zhang, M.Q., Li, M.O., 2012. Novel Foxo1-dependent transcriptional programs control Treg cell function. Nature 491, 554–559. https://doi.org/10.1038/nature11581
166. Paal, J.V. der, Neyts, E.C., Verlackt, C.C.W., Bogaerts, A., 2015. Effect of lipid peroxidation on membrane permeability of cancer and normal cells subjected to oxidative stress. Chem. Sci. 7, 489–498. https://doi.org/10.1039/C5SC02311D
167. Packer, L., Witt, E.H., Tritschler, H.J., 1995. Alpha-lipoic acid as a biological antioxidant. Free Radical Biology and Medicine 19, 227–250. https://doi.org/10.1016/0891-5849(95)00017-R
168. Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.-H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., Levine, M., 2003. Vitamin C as an Antioxidant: Evaluation of Its Role in Disease Prevention. Journal of the American College of Nutrition 22, 18–35. https://doi.org/10.1080/07315724.2003.10719272
169. Park, J.H., Lee, J.E., Lee, S.-J., Park, S.J., Park, K.H., Jeong, M., Koh, H.C., 2013. Potential autophagy enhancers protect against fipronil-induced apoptosis in SH-SY5Y cells. Toxicology Letters 223, 25–34. https://doi.org/10.1016/j.toxlet.2013.08.015
170. Park, J.H., Park, Y.S., Lee, J.-B., Park, K.-H., Paik, M., Jeong, M., Koh, H.C., 2016. Meloxicam inhibits fipronil-induced apoptosis via modulation of the oxidative stress and inflammatory response in SH-SY5Y cells. Journal of Applied Toxicology 36, 10–23. https://doi.org/10.1002/jat.3136
171. Pathak, T., Trebak, M., 2018. Mitochondrial Ca2+ signaling. Pharmacology & Therapeutics 192, 112–123. https://doi.org/10.1016/j.pharmthera.2018.07.001
172. Pearce, E.L., Pearce, E.J., 2013. Metabolic Pathways in Immune Cell Activation and Quiescence. Immunity 38, 633–643. https://doi.org/10.1016/j.immuni.2013.04.005
173. Pedre, B., Barayeu, U., Ezeriņa, D., Dick, T.P., 2021. The mechanism of action of N-acetylcysteine (NAC): The emerging role of H2S and sulfane sulfur species. Pharmacology & Therapeutics 228, 107916. https://doi.org/10.1016/j.pharmthera.2021.107916

doi:10.6342/NTU202501708

174. Peng, H.-Y., Lucavs, J., Ballard, D., Das, J.K., Kumar, A., Wang, L., Ren, Y., Xiong, X., Song, J., n.d. Frontiers | Metabolic Reprogramming and Reactive Oxygen Species in T Cell Immunity. https://doi.org/10.3389/fimmu.2021.652687
175. Perales, M.-A., Goldberg, J.D., Yuan, J., Koehne, G., Lechner, L., Papadopoulos, E.B., Young, J.W., Jakubowski, A.A., Zaidi, B., Gallardo, H., Liu, C., Rasalan, T., Wolchok, J.D., Croughs, T., Morre, M., Devlin, S.M., van den Brink, M.R.M., 2012. Recombinant human interleukin-7 (CYT107) promotes T-cell recovery after allogeneic stem cell transplantation. Blood 120, 4882–4891. https://doi.org/10.1182/blood-2012-06-437236
176. Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Willams, D.E., Ware, C.B., Meyer, J.D., Davison, B.L., 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 180, 1955–1960.
177. Pesticides, A., Authority, V.M., 2011. Safety of Fipronil in Dogs and Cats: a review of literature.
178. Pesticides, A., Authority, V.M., n.d. A review of Animal Safety Studies for Fipronil in the dog and cat.
179. Petty, F., Sherman, A.D., 1984. Plasma GABA levels in psychiatric illness. Journal of Affective Disorders 6, 131–138. https://doi.org/10.1016/0165-0327(84)90018-1
180. Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Downs, C.A., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M., Morrissey, C.A., Noome, D.A., Settele, J., Simon-Delso, N., Stark, J.D., Van der Sluijs, J.P., Van Dyck, H., Wiemers, M., 2015. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ Sci Pollut Res 22, 68–102. https://doi.org/10.1007/s11356-014-3471-x
181. Plumb, A.W., Sheikh, A., Carlow, D.A., Patton, D.T., Ziltener, H.J., Abraham, N., 2017. Interleukin-7 in the transition of bone marrow progenitors to the thymus. Immunology & Cell Biology 95, 916–924. https://doi.org/10.1038/icb.2017.68
182. Politikos, I., Kim, H.T., Nikiforow, S., Li, L., Brown, J., Antin, J.H., Cutler, C., Ballen, K., Ritz, J., Boussiotis, V.A., 2015. IL-7 and SCF Levels Inversely Correlate with T Cell Reconstitution and Clinical Outcomes after Cord Blood Transplantation in Adults. PLoS One 10, e0132564. https://doi.org/10.1371/journal.pone.0132564
183. Prevatto, J.P., Torres, R.C., Diaz, B.L., Silva, P.M.R. e, Martins, M.A., Carvalho, V.F., 2017. Antioxidant Treatment Induces Hyperactivation of the HPA Axis by Upregulating ACTH Receptor in the Adrenal and Downregulating Glucocorticoid Receptors in the Pituitary. Oxidative Medicine and Cellular Longevity 2017, 4156361. https://doi.org/10.1155/2017/4156361
184. PubChem, n.d. Fipronil [WWW Document]. URL https://pubchem.ncbi.nlm.nih.gov/compound/3352 (accessed 5.24.25).
185. Puel, A., Ziegler, S.F., Buckley, R.H., Leonard., W.J., 1998. Defective IL7R expression in T-B+NK+ severe combined immunodeficiency. Nat Genet 20, 394–397. https://doi.org/10.1038/3877
186. Puerto, M., Guayerbas, N., Víctor, V.M., De la Fuente, M., 2002. Effects of *N*-acetylcysteine on macrophage and lymphocyte functions in a mouse model of premature ageing. Pharmacology Biochemistry and Behavior 73, 797–804. https://doi.org/10.1016/S0091-3057(02)00902-4
187. Rathmell, J.C., Farkash, E.A., Gao, W., Thompson, C.B., 2001. IL-7 Enhances the Survival and Maintains the Size of Naive T Cells1. The Journal of Immunology 167, 6869–6876. https://doi.org/10.4049/jimmunol.167.12.6869
188. Ratra, G.S., Casida, J.E., 2001. GABA receptor subunit composition relative to insecticide potency and selectivity. Toxicology Letters 122, 215–222. https://doi.org/10.1016/S0378-4274(01)00366-6

doi:10.6342/NTU202501708

189. Ren, W., Liu, G., Yin, J., Tan, B., Wu, G., Bazer, F.W., Peng, Y., Yin, Y., 2017. Amino-acid transporters in T-cell activation and differentiation. Cell Death Dis 8, e2655–e2655. https://doi.org/10.1038/cddis.2016.222
190. Reyes-García, M.G., Hernández-Hernández, F., Hernández-Téllez, B., García-Tamayo, F., 2007. GABA (A) receptor subunits RNA expression in mice peritoneal macrophages modulate their IL-6/IL-12 production. Journal of Neuroimmunology 188, 64–68. https://doi.org/10.1016/j.jneuroim.2007.05.013
191. Ribeiro, D., Melão, A., van Boxtel, R., Santos, C.I., Silva, A., Silva, M.C., Cardoso, B.A., Coffer, P.J., Barata, J.T., 2018. STAT5 is essential for IL-7–mediated viability, growth, and proliferation of T-cell acute lymphoblastic leukemia cells. Blood Adv 2, 2199–2213. https://doi.org/10.1182/bloodadvances.2018021063
192. Richardson, J.A., Little, S.E., 2012. Chapter 31 - Toxicology, in: Little, S.E. (Ed.), The Cat. W.B. Saunders, Saint Louis, pp. 914–933. https://doi.org/10.1016/B978-1-4377-0660-4.00031-4
193. Rikka, S., Quinsay, M.N., Thomas, R.L., Kubli, D.A., Zhang, X., Murphy, A.N., Gustafsson, Å.B., 2011. Bnip3 impairs mitochondrial bioenergetics and stimulates mitochondrial turnover. Cell Death Differ 18, 721–731. https://doi.org/10.1038/cdd.2010.146
194. Roberts, E., Frankel, S., 1950. γ-AMINOBUTYRIC ACID IN BRAIN: ITS FORMATION FROM GLUTAMIC ACID. Journal of Biological Chemistry 187, 55–63. https://doi.org/10.1016/S0021-9258(19)50929-2
195. Roberts, M.S., Cross, S.E., Pellett, M.A., 2002. Skin transport, in: Dermatological and Transdermal Formulations. CRC Press, pp. 107–214.
196. Rochman, Y., Spolski, R., Leonard, W.J., 2009. New insights into the regulation of T cells by γc family cytokines. Nat Rev Immunol 9, 480–490. https://doi.org/10.1038/nri2580
197. Rodewald, H.-R., Waskow, C., Haller, C., 2001. Essential Requirement for C-KIT and Common γ Chain in Thymocyte Development Cannot Be Overruled by Enforced Expression of Bcl-2. J Exp Med 193, 1431–1438. https://doi.org/10.1084/jem.193.12.1431
198. Roggero, E., Pérez, A.R., Tamae-Kakazu, M., Piazzon, I., Nepomnaschy, I., Besedovsky, H.O., Bottasso, O.A., Rey, A. del, 2006. Endogenous glucocorticoids cause thymus atrophy but are protective during acute Trypanosoma cruzi infection. https://doi.org/10.1677/joe.1.06642
199. Romano, R., Palamaro, L., Fusco, A., Giardino, G., Gallo, V., Del Vecchio, L., Pignata, C., 2013. FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program. Frontiers in Immunology 4.
200. Romero, A., Ramos, E., Ares, I., Castellano, V., Martínez, M., Martínez-Larrañaga, M.R., Anadón, A., Martínez, M.A., 2016. Fipronil sulfone induced higher cytotoxicity than fipronil in SH-SY5Y cells: Protection by antioxidants. Toxicology Letters 252, 42–49. https://doi.org/10.1016/j.toxlet.2016.04.005
201. Ruangjaroon, T., Chokchaichamnankit, D., Srisomsap, C., Svasti, J., Paricharttanakul, N.M., 2017. Involvement of vimentin in neurite outgrowth damage induced by fipronil in SH-SY5Y cells. Biochemical and Biophysical Research Communications 486, 652–658. https://doi.org/10.1016/j.bbrc.2017.03.081
202. Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A.C., Horak, I., 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75, 253–261. https://doi.org/10.1016/0092-8674(93)80067-O
203. Sakr, S., Hamed, A., Atef, M., 2022. Betanin ameliorates fipronil-induced nephrotoxicity via activation of Nrf2-HO-1/NQO-1 pathway in albino rat model. Toxicol Res (Camb) 11, 975–986. https://doi.org/10.1093/toxres/tfac076
204. Saleh, H., Nassar, A.M.K., Noreldin, A.E., Samak, D., Elshony, N., Wasef, L., Elewa, Y.H.A., Hassan, S.M.A., Saati, A.A., Hetta, H.F., Batiha, G.E.-S., Umezawa, M., Shaheen, H.M., El-

doi:10.6342/NTU202501708

Sayed, Y.S., 2020. Chemo-Protective Potential of Cerium Oxide Nanoparticles against Fipronil-Induced Oxidative Stress, Apoptosis, Inflammation and Reproductive Dysfunction in Male White Albino Rats. Molecules 25, 3479. https://doi.org/10.3390/molecules25153479
205. Salehzadeh, M., Soma, K.K., 2021. Glucocorticoid production in the thymus and brain: Immunosteroids and neurosteroids. Brain, Behavior, & Immunity - Health 18, 100352. https://doi.org/10.1016/j.bbih.2021.100352
206. Saulsbury, M.D., Heyliger, S.O., Wang, K., Johnson, D.J., 2009. Chlorpyrifos induces oxidative stress in oligodendrocyte progenitor cells. Toxicology 259, 1–9. https://doi.org/10.1016/j.tox.2008.12.026
207. Semyanov, A., Walker, M.C., Kullmann, D.M., 2003. GABA uptake regulates cortical excitability via cell type–specific tonic inhibition. Nat Neurosci 6, 484–490. https://doi.org/10.1038/nn1043
208. Sena, L.A., Li, S., Jairaman, A., Prakriya, M., Ezponda, T., Hildeman, D.A., Wang, C.-R., Schumacker, P.T., Licht, J.D., Perlman, H., Bryce, P.J., Chandel, N.S., 2013. Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. Immunity 38, 225–236. https://doi.org/10.1016/j.immuni.2012.10.020
209. Shaki, F., Hosseini, M.-J., Ghazi-Khansari, M., Pourahmad, J., 2012. Toxicity of depleted uranium on isolated rat kidney mitochondria. Biochimica et Biophysica Acta (BBA) - General Subjects 1820, 1940–1950. https://doi.org/10.1016/j.bbagen.2012.08.015
210. Shan, Y., Zhao, J., Zheng, Y., Guo, S., Schrodi, S.J., He, D., 2023. Understanding the function of the GABAergic system and its potential role in rheumatoid arthritis. Front. Immunol. 14. https://doi.org/10.3389/fimmu.2023.1114350
211. Shanker, A., 2004. Is thymus redundant after adulthood? Immunology Letters 91, 79–86. https://doi.org/10.1016/j.imlet.2003.12.012
212. Shanley, D.P., Aw, D., Manley, N.R., Palmer, D.B., 2009. An evolutionary perspective on the mechanisms of immunosenescence. Trends in Immunology 30, 374–381. https://doi.org/10.1016/j.it.2009.05.001
213. Sheikh, V., Porter, B.O., DerSimonian, R., Kovacs, S.B., Thompson, W.L., Perez-Diez, A., Freeman, A.F., Roby, G., Mican, J., Pau, A., Rupert, A., Adelsberger, J., Higgins, J., Bourgeois, J.S., Jr, Jensen, S.M.R., Morcock, D.R., Burbelo, P.D., Osnos, L., Maric, I., Natarajan, V., Croughs, T., Yao, M.D., Estes, J.D., Sereti, I., 2016. Administration of interleukin-7 increases CD4 T cells in idiopathic CD4 lymphocytopenia. Blood 127, 977–988. https://doi.org/10.1182/blood-2015-05-645077
214. Shi, L., Wan, Y., Liu, J., He, Z., Xu, S., Xia, W., 2021. Insecticide fipronil and its transformation products in human blood and urine: Assessment of human exposure in general population of China. Science of The Total Environment 786, 147342. https://doi.org/10.1016/j.scitotenv.2021.147342
215. Shichkin, V.P., Antica, M., 2022. Key Factors for Thymic Function and Development. Front. Immunol. 13. https://doi.org/10.3389/fimmu.2022.926516
216. Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A.A., Levin, S.D., Geng, Y., von Boehmer, H., Sicinski, P., 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell 4, 451–461. https://doi.org/10.1016/S1535-6108(03)00301-5
217. Sidiropoulou, E., Sachana, M., Hargrreaves, A.J., Woldehiwet, Z., 2010. Immunotoxic properties of pesticides: effects of diazinon-oxon and fipronil on lymphocytic Jurkat cells. Front. Pharmacol. 1. https://doi.org/10.3389/conf.fphar.2010.60.00196
218. Sieghart, W., Sperk, G., 2002. Subunit Composition, Distribution and Function of GABA-A Receptor Subtypes. Current topics in medicinal chemistry 2, 795–816. https://doi.org/10.2174/1568026023393507
219. Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs,

doi:10.6342/NTU202501708

C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environ Sci Pollut Res 22, 5–34. https://doi.org/10.1007/s11356-014-3470-y

220. Singh, J., Phogat, A., Malik, V., 2025. N-acetylcysteine: a potential therapeutic agent against toxicity of pesticides. Mol Biol Rep 52, 539. https://doi.org/10.1007/s11033-025-10635-8
221. Slotkin, T.A., Seidler, F.J., 2010. Oxidative stress from diverse developmental neurotoxicants: Antioxidants protect against lipid peroxidation without preventing cell loss. Neurotoxicology and Teratology 32, 124–131. https://doi.org/10.1016/j.ntt.2009.12.001
222. Smiley, S.T., Grusby, M.J., 1998. Interleukin 4, in: Delves, P.J. (Ed.), Encyclopedia of Immunology (Second Edition). Elsevier, Oxford, pp. 1451–1453. https://doi.org/10.1006/rwei.1999.0368
223. Soltani, N., Qiu, H., Aleksic, M., Glinka, Y., Zhao, F., Liu, R., Li, Y., Zhang, N., Chakrabarti, R., Ng, T., Jin, T., Zhang, H., Lu, W.-Y., Feng, Z.-P., Prud'homme, G.J., Wang, Q., 2011. GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes. Proceedings of the National Academy of Sciences 108, 11692–11697. https://doi.org/10.1073/pnas.1102715108
224. Song, X., Wang, X., Liao, G., Pan, Y., Qian, Y., Qiu, J., 2021. Toxic effects of fipronil and its metabolites on PC12 cell metabolism. Ecotoxicology and Environmental Safety 224, 112677. https://doi.org/10.1016/j.ecoenv.2021.112677
225. Sparrow, E.L., James, S., Hussain, K., Beers, S.A., Cragg, M.S., Bogdanov, Y.D., 2021. Activation of GABA(A) receptors inhibits T cell proliferation. PLOS ONE 16, e0251632. https://doi.org/10.1371/journal.pone.0251632
226. Spidale, N.A., Sylvia, K., Narayan, K., Miu, B., Frascoli, M., Melichar, H.J., Zhihao, W., Kisielow, J., Palin, A., Serwold, T., Love, P., Kobayashi, M., Yoshimoto, M., Jain, N., Kang, J., 2018. Interleukin-17 producing γδ T cells originate from SOX13+ progenitors that are independent of γδTCR signaling. Immunity 49, 857-872.e5. https://doi.org/10.1016/j.immuni.2018.09.010
227. Sprent, J., Surh, C.D., 2011. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nat Immunol 12, 478–484. https://doi.org/10.1038/ni.2018
228. Stafford, E.G., Tell, L.A., Lin, Z., Davis, J.L., Vickroy, T.W., Riviere, J.E., Baynes, R.E., 2018. Consequences of fipronil exposure in egg-laying hens. https://doi.org/10.2460/javma.253.1.57
229. Stehr, C.M., Linbo, T.L., Incardona, J.P., Scholz, N.L., 2006. The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae. Toxicological Sciences 92, 270–278. https://doi.org/10.1093/toxsci/kfj185
230. Steward, F.C., 1949. γ-Aminobutyric acid: a constituent of potato tubers? Science 110, 439–440.
231. Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., Yoshida, H., Nishikawa, S., 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc Natl Acad Sci U S A 90, 9125–9129.
232. Sutherland, J.S., Goldberg, G.L., Hammett, M.V., Uldrich, A.P., Berzins, S.P., Heng, T.S., Blazar, B.R., Millar, J.L., Malin, M.A., Chidgey, A.P., Boyd, R.L., 2005. Activation of Thymic Regeneration in Mice and Humans following Androgen Blockade. The Journal of Immunology 175, 2741–2753. https://doi.org/10.4049/jimmunol.175.4.2741
233. Suzuki, T., Hirai, A., Khidkhan, K., Nimako, C., Ichise, T., Takeda, K., Mizukawa, H., Nakayama, S.M.M., Nomiyama, K., Hoshi, N., Maeda, M., Hirano, T., Sasaoka, K., Sasaki,

doi:10.6342/NTU202501708

N., Takiguchi, M., Ishizuka, M., Ikenaka, Y., 2021. The effects of fipronil on emotional and cognitive behaviors in mammals. Pesticide Biochemistry and Physiology 175, 104847. https://doi.org/10.1016/j.pestbp.2021.104847
234. Szegedi, V., Bárdos, G., Détári, L., Tóth, A., Banczerowski-Pelyhe, I., Világi, I., 2005. Transient alterations in neuronal and behavioral activity following bensultap and fipronil treatment in rats. Toxicology 214, 67–76. https://doi.org/10.1016/j.tox.2005.05.023
235. Tentori, L., Longo, D.L., ZUNIGA-PFLUCKER, J.C., Wing, C., Kruisbeek, A.M., 1988. Essential role of the interleukin 2-interleukin 2 receptor pathway in thymocyte maturation in vivo. J Exp Med 168, 1741–1747.
236. Terçariol, P.R.G., Godinho, A.F., 2011. Behavioral effects of acute exposure to the insecticide fipronil. Pesticide Biochemistry and Physiology 99, 221–225. https://doi.org/10.1016/j.pestbp.2010.12.007
237. THAPA, P., FARBER, D.L., 2019. THE ROLE OF THE THYMUS IN THE IMMUNE RESPONSE. Thorac Surg Clin 29, 123–131. https://doi.org/10.1016/j.thorsurg.2018.12.001
238. The Comparative Toxicogenomics Database | CTD [WWW Document], n.d. URL https://ctdbase.org/ (accessed 6.1.25).
239. Tian, J., Chau, C., Hales, T.G., Kaufman, D.L., 1999. GABAA receptors mediate inhibition of T cell responses. Journal of Neuroimmunology 96, 21–28. https://doi.org/10.1016/S0165-5728(98)00264-1
240. Tian, J., Lu, Y., Zhang, H., Chau, C.H., Dang, H.N., Kaufman, D.L., 2004. γ-Aminobutyric Acid Inhibits T Cell Autoimmunity and the Development of Inflammatory Responses in a Mouse Type 1 Diabetes Model1. The Journal of Immunology 173, 5298–5304. https://doi.org/10.4049/jimmunol.173.8.5298
241. Tingle, C.C.D., Rother, J.A., Dewhurst, C.F., Lauer, S., King, W.J., 2003. Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns, in: Ware, G.W. (Ed.), Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews, Reviews of Environmental Contamination and Toxicology. Springer, New York, NY, pp. 1–66. https://doi.org/10.1007/978-1-4899-7283-5_1
242. Trédan, O., Ménétrier-Caux, C., Ray-Coquard, I., Garin, G., Cropet, C., Verronèse, E., Bachelot, T., Rebattu, P., Heudel, P.E., Cassier, P., Chabaud, S., Croughs, T., Dupont, P., Cadore, A.C., Clapisson, G., Delgado, A., Bardin-dit-Courageot, C., Rigal, C., N'Kodia, A., Gilles-Afchain, L., Morre, M., Pérol, D., Blay, J.Y., Caux, C., 2015. ELYPSE-7: a randomized placebo-controlled phase IIa trial with CYT107 exploring the restoration of CD4+ lymphocyte count in lymphopenic metastatic breast cancer patients. Annals of Oncology 26, 1353–1362. https://doi.org/10.1093/annonc/mdv173
243. Tsai, T.-L., Zhou, T.-A., Hsieh, Y.-T., Wang, J.-C., Cheng, H.-K., Huang, C.-H., Tsai, P.-Y., Fan, H.-H., Feng, H.-K., Huang, Y.-C., Lin, C.-C., Lin, C.-H., Lin, C.-Y., Dzhagalov, I.L., Hsu, C.-L., 2022. Multiomics reveal the central role of pentose phosphate pathway in resident thymic macrophages to cope with efferocytosis-associated stress. Cell Reports 40. https://doi.org/10.1016/j.celrep.2022.111065
244. Vaittinen, S.-L., Komulainen, H., Kosma, V.-M., Julkunen, A., Mäki-Paakkanen, J., Jansson, K., Vartiainen, T., Tuomisto, J., 1995. Subchronic toxicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) in Wistar rats. Food and Chemical Toxicology 33, 1027–1037. https://doi.org/10.1016/0278-6915(95)00079-8
245. Valvassori, S.S., Resende, W.R., Lopes-Borges, J., Mariot, E., Dal-Pont, G.C., Vitto, M.F., Luz, G., de Souza, C.T., Quevedo, J., 2015. Effects of mood stabilizers on oxidative stress-induced cell death signaling pathways in the brains of rats subjected to the ouabain-induced animal model of mania: Mood stabilizers exert protective effects against ouabain-induced activation of the cell death pathway. Journal of Psychiatric Research 65, 63–70. https://doi.org/10.1016/j.jpsychires.2015.04.009

doi:10.6342/NTU202501708

246. Veis, D.J., Sorenson, C.M., Shutter, J.R., Korsmeyer, S.J., 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75, 229–240. https://doi.org/10.1016/0092-8674(93)80065-M
247. Velardi, E., Tsai, J.J., van den Brink, M.R.M., 2021. T cell regeneration after immunological injury. Nat Rev Immunol 21, 277–291. https://doi.org/10.1038/s41577-020-00457-z
248. Veterinary Toxicology, 2018.
249. Vicente, R., Swainson, L., Marty-Grez, S., de Barros, S., Kinet, S., Zimmermann, V.S., Taylor, N., 2010. Molecular and Cellular Basis of T cell Lineage Commitment. Semin Immunol 22, 270–275. https://doi.org/10.1016/j.smim.2010.04.016
250. Vidau, C., González-Polo, R.A., Niso-Santano, M., Gómez-Sánchez, R., Bravo-San Pedro, J.M., Pizarro-Estrella, E., Blasco, R., Brunet, J.-L., Belzunces, L.P., Fuentes, J.M., 2011. Fipronil is a powerful uncoupler of oxidative phosphorylation that triggers apoptosis in human neuronal cell line SHSY5Y. NeuroToxicology 32, 935–943. https://doi.org/10.1016/j.neuro.2011.04.006
251. Wan, J., Martinvalet, D., Ji, X., Lois, C., Kaech, S.M., Von Andrian, U.H., Lieberman, J., Ahmed, R., Manjunath, N., 2003. The Bcl-2 family pro-apoptotic molecule, BNIP3 regulates activation-induced cell death of effector cytotoxic T lymphocytes. Immunology 110, 10–17. https://doi.org/10.1046/j.1365-2567.2003.01710.x
252. Wan, Y.Y., Flavell, R.A., 2009. How Diverse—CD4 Effector T Cells and their Functions. J Mol Cell Biol 1, 20–36. https://doi.org/10.1093/jmcb/mjp001
253. Wang, C., Youle, R.J., 2009. The Role of Mitochondria in Apoptosis. Annu Rev Genet 43, 95–118. https://doi.org/10.1146/annurev-genet-102108-134850
254. Wang, J., He, N., Zhang, N., Quan, D., Zhang, S., Zhang, C., Yu, R.T., Atkins, A.R., Zhu, R., Yang, C., Cui, Y., Liddle, C., Downes, M., Xiao, H., Zheng, Y., Auwerx, J., Evans, R.M., Leng, Q., 2017. NCoR1 restrains thymic negative selection by repressing Bim expression to spare thymocytes undergoing positive selection. Nat Commun 8, 959. https://doi.org/10.1038/s41467-017-00931-8
255. Wang, R., Green, D.R., 2012. Metabolic checkpoints in activated T cells. Nat Immunol 13, 907–915. https://doi.org/10.1038/ni.2386
256. Wang, X., Martínez, M.A., Wu, Q., Ares, I., Martínez-Larrañaga, M.R., Anadón, A., Yuan, Z., 2016. Fipronil insecticide toxicology: oxidative stress and metabolism. Critical Reviews in Toxicology 46, 876–899. https://doi.org/10.1080/10408444.2016.1223014
257. Wang, Xin-quan, Li, Y., Zhong, S., Zhang, H., Wang, Xiang-yun, Qi, P., Xu, H., 2013. Oxidative injury is involved in fipronil-induced G2/M phase arrest and apoptosis in *Spodoptera frugiperda* (Sf9) cell line. Pesticide Biochemistry and Physiology 105, 122–130. https://doi.org/10.1016/j.pestbp.2012.12.008
258. Wang, X.-J., Cao, Q., Zhang, Y., Su, X.-D., 2015. Activation and regulation of caspase-6 and its role in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 55, 553–572. https://doi.org/10.1146/annurev-pharmtox-010814-124414
259. Wang, Y., Luo, Q., Xu, Y., Feng, D., Fei, J., Cheng, Q., Xu, L., 2009. γ-Aminobutyric Acid Transporter 1 Negatively Regulates T Cell Activation and Survival through Protein Kinase C-Dependent Signaling Pathways1. The Journal of Immunology 183, 3488–3495. https://doi.org/10.4049/jimmunol.0900767
260. Webster, M., 1999. Product warning: FRONTLINE. Aust Vet J 77, 202.
261. Wei, Y., Hu, Z., Gu, W., Liu, G., Shi, B., Liu, E., Liu, T., 2017. CD117+CD44+ Stem T Cells Develop in the Thymus and Potently Suppress T-cell Proliferation by Modulating the CTLA-4 Pathway. Stem Cell Research & Therapy 8, 56. https://doi.org/10.1186/s13287-017-0495-4
262. Weidinger, A., Kozlov, A.V., 2015. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. Biomolecules 5, 472–484. https://doi.org/10.3390/biom5020472

doi:10.6342/NTU202501708

263. Wheeler, D.W., Thompson, A.J., Corletto, F., Reckless, J., Loke, J.C.T., Lapaque, N., Grant, A.J., Mastroeni, P., Grainger, D.J., Padgett, C.L., O'Brien, J.A., Miller, N.G.A., Trowsdale, J., Lummis, S.C.R., Menon, D.K., Beech, J.S., 2011. Anaesthetic Impairment of Immune Function Is Mediated via GABAA Receptors. PLOS ONE 6, e17152. https://doi.org/10.1371/journal.pone.0017152
264. Winer, H., Rodrigues, G.O.L., Hixon, J.A., Aiello, F.B., Hsu, T.C., Wachter, B.T., Li, W., Durum, S.K., 2022. IL-7: Comprehensive review. Cytokine 160, 156049. https://doi.org/10.1016/j.cyto.2022.156049
265. Wofford, J.A., Wieman, H.L., Jacobs, S.R., Zhao, Y., Rathmell, J.C., 2008. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. Blood 111, 2101–2111. https://doi.org/10.1182/blood-2007-06-096297
266. World Health Organization, 2022. Pesticide residues in food: 2021: toxicological evaluations: Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, virtual meeting, 6–17 September, 4 and 7 October 2021.
267. Wu, L., Antica, M., Johnson, G.R., Scollay, R., Shortman, K., 1991. Developmental potential of the earliest precursor cells from the adult mouse thymus. Journal of Experimental Medicine 174, 1617–1627. https://doi.org/10.1084/jem.174.6.1617
268. Xia, Y., He, F., Wu, X., Tan, B., Chen, Siyuan, Liao, Y., Qi, M., Chen, Shuai, Peng, Y., Yin, Y., Ren, W., 2021. GABA transporter sustains IL-1β production in macrophages. Science Advances 7, eabe9274. https://doi.org/10.1126/sciadv.abe9274
269. Xue, H.-H., Bollenbacher, J., Rovella, V., Tripuraneni, R., Du, Y.-B., Liu, C.-Y., Williams, A., McCoy, J.P., Leonard, W.J., 2004. GA binding protein regulates interleukin 7 receptor α-chain gene expression in T cells. Nat Immunol 5, 1036–1044. https://doi.org/10.1038/ni1117
270. Xue, H.-H., Bollenbacher-Reilley, J., Wu, Z., Spolski, R., Jing, X., Zhang, Y.-C., McCoy, J.P., Leonard, W.J., 2007. The Transcription Factor GABP Is a Critical Regulator of B Lymphocyte Development. Immunity 26, 421–431. https://doi.org/10.1016/j.immuni.2007.03.010
271. Yadav, A., Mishra, P.C., 2012. Modeling the activity of glutathione as a hydroxyl radical scavenger considering its neutral non-zwitterionic form. Journal of Molecular Modeling 19, 767–777. https://doi.org/10.1007/s00894-012-1601-2
272. Yan, F., Mo, X., Liu, J., Ye, S., Zeng, X., Chen, D., n.d. Thymic function in the regulation of T cells, and molecular mechanisms underlying the modulation of cytokines and stress signaling (Review).
273. Yang, H., Zhou, M., Li, H., Wei, T., Tang, C., Zhou, Y., Long, X., 2020. Effects of Low-level Lipid Peroxidation on the Permeability of Nitroaromatic Molecules across a Membrane: A Computational Study. ACS Omega 5, 4798–4806. https://doi.org/10.1021/acsomega.9b03462
274. Yang, Q., Bell, J.J., Bhandoola, A., 2010. T-Cell Lineage Determination. Immunol Rev 238, 12–22. https://doi.org/10.1111/j.1600-065X.2010.00956.x
275. Young, H.A., Klinman, D.M., Reynolds, D.A., Grzegorzewski, K.J., Nii, A., Ward, J.M., Winkler-Pickett, R.T., Ortaldo, J.R., Kenny, J.J., Komschlies, K.L., 1997. Bone Marrow and Thymus Expression of Interferon-γ Results in Severe B-Cell Lineage Reduction, T-Cell Lineage Alterations, and Hematopoietic Progenitor Deficiencies. Blood 89, 583–595. https://doi.org/10.1182/blood.V89.2.583
276. Youssef, L.A., Rebbaa, A., Pampou, S., Weisberg, S.P., Stockwell, B.R., Hod, E.A., Spitalnik, S.L., 2018. Increased erythrophagocytosis induces ferroptosis in red pulp macrophages in a mouse model of transfusion. Blood 131, 2581–2593. https://doi.org/10.1182/blood-2017-12-822619
277. Yu, Q., Erman, B., Park, J.-H., Feigenbaum, L., Singer, A., 2004. IL-7 Receptor Signals Inhibit Expression of Transcription Factors TCF-1, LEF-1, and RORγt. J Exp Med 200, 797–803. https://doi.org/10.1084/jem.20032183

doi:10.6342/NTU202501708

278. Yu, S., Zhao, D.-M., Jothi, R., Xue, H.-H., 2010. Critical Requirement of GABPα for Normal T Cell Development. J Biol Chem 285, 10179–10188. https://doi.org/10.1074/jbc.M109.088740
279. Zafar, S., Jabeen, I., 2018. Structure, Function, and Modulation of γ-Aminobutyric Acid Transporter 1 (GAT1) in Neurological Disorders: A Pharmacoinformatic Prospective. Frontiers in Chemistry 6.
280. Zafarullah, M., Li, W.Q., Sylvester, J., Ahmad, M., 2003. Molecular mechanisms of N-acetylcysteine actions. CMLS, Cell. Mol. Life Sci. 60, 6–20. https://doi.org/10.1007/s000180300001
281. Zhang, B., Vogelzang, A., Miyajima, M., Sugiura, Y., Wu, Y., Chamoto, K., Nakano, R., Hatae, R., Menzies, R.J., Sonomura, K., Hojo, N., Ogawa, T., Kobayashi, W., Tsutsui, Y., Yamamoto, S., Maruya, M., Narushima, S., Suzuki, K., Sugiya, H., Murakami, K., Hashimoto, M., Ueno, H., Kobayashi, T., Ito, K., Hirano, T., Shiroguchi, K., Matsuda, F., Suematsu, M., Honjo, T., Fagarasan, S., 2021. B cell-derived GABA elicits IL-10+ macrophages to limit anti-tumour immunity. Nature 599, 471–476. https://doi.org/10.1038/s41586-021-04082-1
282. Zhang, B., Xu, Z., Zhang, Y., Shao, X., Xu, X., Cheng, J., Li, Z., 2015. Fipronil induces apoptosis through caspase-dependent mitochondrial pathways in *Drosophila* S2 cells. Pesticide Biochemistry and Physiology 119, 81–89. https://doi.org/10.1016/j.pestbp.2015.01.019
283. Zhang, N., Hartig, H., Dzhagalov, I., Draper, D., He, Y.W., 2005. The role of apoptosis in the development and function of T lymphocytes. Cell Research 15, 749–769. https://doi.org/10.1038/sj.cr.7290345
284. Zhao, X., Salgado, V.L., Yeh, J.Z., Narahashi, T., 2003. Differential Actions of Fipronil and Dieldrin Insecticides on GABA-Gated Chloride Channels in Cockroach Neurons. The Journal of Pharmacology and Experimental Therapeutics 306, 914–924. https://doi.org/10.1124/jpet.103.051839
285. Zhao, X., Yeh, J.Z., Salgado, V.L., Narahashi, T., 2005. Sulfone Metabolite of Fipronil Blocks γ-Aminobutyric Acid- and Glutamate-Activated Chloride Channels in Mammalian and Insect Neurons. The Journal of Pharmacology and Experimental Therapeutics 314, 363–373. https://doi.org/10.1124/jpet.104.077891
286. Zhao, X., Yeh, J.Z., Salgado, V.L., Narahashi, T., 2004. Fipronil Is a Potent Open Channel Blocker of Glutamate-Activated Chloride Channels in Cockroach Neurons. The Journal of Pharmacology and Experimental Therapeutics 310, 192–201. https://doi.org/10.1124/jpet.104.065516
287. Zheng, Y., Sun, J., Luo, Z., Li, Y., Huang, Y., 2024. Emerging mechanisms of lipid peroxidation in regulated cell death and its physiological implications. Cell Death Dis 15, 1–19. https://doi.org/10.1038/s41419-024-07244-x
288. Zhitkovich, A., 2019. N-Acetylcysteine: Antioxidant, Aldehyde Scavenger, and More. Chem. Res. Toxicol. 32, 1318–1319. https://doi.org/10.1021/acs.chemrestox.9b00152
289. Zhu, Y., Zhang, R., Zhang, B., Zhao, T., Wang, P., Liang, G., Cheng, G., 2017. Blood meal acquisition enhances arbovirus replication in mosquitoes through activation of the GABAergic system. Nat Commun 8, 1262. https://doi.org/10.1038/s41467-017-01244-6
290. Zhuang, J., Jiang, Z., Chen, D., Li, J., Crabbe, M.J.C., Qiu, M., Zheng, Y., Qu, W., 2023. Thyroid-Disrupting Effects of Exposure to Fipronil and Its Metabolites from Drinking Water Based on Human Thyroid Follicular Epithelial Nthy-ori 3-1 Cell Lines. Environ. Sci. Technol. 57, 6072–6084. https://doi.org/10.1021/acs.est.2c08627
291. Zohren, F., Souroullas, G.P., Luo, M., Gerdemann, U., Imperato, M.R., Wilson, N.K., Gottgens, B., Lukov, G.L., Goodell, M.A., 2012a. Lyl1 regulates lymphoid specification and maintenance of early T lineage progenitors. Nat Immunol 13, 761–769. https://doi.org/10.1038/ni.2365
292. Zohren, F., Souroullas, G.P., Luo, M., Gerdemann, U., Imperato, M.R., Wilson, N.K.,

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Göttgens, B., Lukov, G.L., Goodell, M.A., 2012b. The transcription factor Lyl-1 regulates lymphoid specification and the maintenance of early T lineage progenitors. Nat Immunol 13, 761–769. https://doi.org/10.1038/ni.2365
293. Zoller, A.L., Kersh, G.J., 2006. Estrogen Induces Thymic Atrophy by Eliminating Early Thymic Progenitors and Inhibiting Proliferation of β-Selected Thymocytes1. The Journal of Immunology 176, 7371–7378. https://doi.org/10.4049/jimmunol.176.12.7371
294. Zubkova, I., Mostowski, H., Zaitseva, M., 2005. Up-Regulation of IL-7, Stromal-Derived Factor-1α, Thymus-Expressed Chemokine, and Secondary Lymphoid Tissue Chemokine Gene Expression in the Stromal Cells in Response to Thymocyte Depletion: Implication for Thymus Reconstitution1. The Journal of Immunology 175, 2321–2330. https://doi.org/10.4049/jimmunol.175.4.2321
295. Žuklys, S., Handel, A., Zhanybekova, S., Govani, F., Keller, M., Maio, S., Mayer, C.E., Teh, H.Y., Hafen, K., Gallone, G., Barthlott, T., Ponting, C.P., Holländer, G.A., 2016. Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells. Nat Immunol 17, 1206–1215. https://doi.org/10.1038/ni.3537

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    b. **Jui-Fang Kuo**, Hsin-Ying Wu, Chun-Wei Tung, Wei-Hsiang Huang, Chen-Si Lin, and Chia-Chi Wang* "Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes" Chem. Res. Toxicol. 2024, 37, 1488−1500. https://doi.org/10.1021/acs.chemrestox.4c00060
    c. **Jui-Fang Kuo**, Yai-Ping Hsiao, Yao-De Wang, Hsin-Pei Weng, and Chia-Chi Wang* "Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes" Toxics 2025, 13, 204. https://doi.org/10.3390/ toxics13030204

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董俊維 Chun-Wei Tung

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Fipronil disturbs the antigen-specific immune responses and GABAergic gene expression in the ovalbumin-immunized BALB/c mice

Jui-Fang Kuo[1], Yin-Hua Cheng[2], Chun-Wei Tung[3] and Chia-Chi Wang[1*]

Abstract

Background Fipronil (FPN) is a broad-spectrum pesticide and commonly known as low toxicity to vertebrates. However, increasing evidence suggests that exposure to FPN might induce unexpected adverse effects in the liver, reproductive, and nervous systems. Until now, the influence of FPN on immune responses, especially T-cell responses has not been well examined. Our study is designed to investigate the immunotoxicity of FPN in ovalbumin (OVA)-sensitized mice. The mice were administered with FPN by oral gavage and immunized with OVA. Primary splenocytes were prepared to examine the viability and functionality of antigen-specific T cells ex vivo. The expression of T cell cytokines, upstream transcription factors, and GABAergic signaling genes was detected by qPCR.

Results Intragastric administration of FPN (1–10 mg/kg) for 11 doses did not show any significant clinical symptoms. The viability of antigen-stimulated splenocytes, the production of IL-2, IL-4, and IFN-γ by OVA-specific T cells, and the serum levels of OVA-specific IgG_1 and IgG_{2a} were significantly increased in FPN-treated groups. The expression of the GABAergic signaling genes was notably altered by FPN. The GAD67 gene was significantly decreased, while the GABAR β2 and GABAR δ were increased.

Conclusion FPN disturbed antigen-specific immune responses by affecting GABAergic genes in vivo. We propose that the immunotoxic effects of FPN may enhance antigen-specific immunity by dysregulation of the negative regulation of GABAergic signaling on T cell immunity.

Keywords Fipronil, Ovalbumin, Immunotoxicity, Antigen-specific immune responses, GABAergic signaling

Background

Fipronil (FPN), an extensively used N-phenylpyrazole pesticide in agriculture and veterinary medicine, induces hyper-excitation neuronal toxicity by antagonizing insect γ-aminobutyric acid ($GABA_A$)-gated chloride channels [1–5]. Although FPN is classified as a Class II moderately hazardous pesticide by the World Health Organization (WHO), FPN has been implicated in adverse health and environmental effects with non-target species toxicity [6]. As honeybees and dragonflies exhibit vulnerability to FPN, leading to its ban in the European Union in 2013 [7]. In addition, FPN has been associated with non-target

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organ toxicity including liver and kidney damage, thyroid dysfunction, and reproductive toxicity in non-target species [3, 8–10].

The increasing use of FPN has raised concerns about potential harm to human health from environmental exposure [11–13]. FPN residues have been found in 40% of U.S. households, and cases of acute illnesses have been reported associated with unintentional exposure, particularly through contact with pets treated with FPN-containing products [14, 15]. An increase in adverse reports of pets treated with FPN has led to increased scrutiny by the U.S. Environmental Protection Agency (EPA) on spot-on insecticides containing FPN. Although FPN is authorized for pest control of pets, it is prohibited in the EU for all food-producing animals. In 2017, FPN contamination was reported in eggs from 45 countries due to illegal use, highlighting the risk of exposure to FPN [13, 16–18]. These reports highlight the potential risks of FPN exposure in humans. Due to the potentially toxic effects of FPN on non-target species or non-target organs, further mechanistic toxicity studies are needed.

Essentially, the neuroendocrine and immune systems are considered to have bidirectional communication [19]. Unfortunately, compared to the well-documented neurotoxicity of FPN, fewer studies have focused on investigating the immunotoxic effects of FPN. Rats were orally administered 10% LD50 (9.7 mg/kg) of FPN for 30 days, resulting in histopathological alteration in the spleen and thymus tissue [20]. In addition, the serum levels of IL-4, IL-12, and IgE were slightly increased after exposure to FPN, suggesting that long-term exposure to FPN could increase allergic and inflammatory responses [20]. Exposure of mice from 4 weeks to 13 weeks of age with 0.5% LD50 FPN (~0.5 mg/kg) didn't alter the spleen weight, however, the mitogenic proliferation of ConA or LPS-stimulated splenocytes was slightly decreased [21]. FPN directly decreased the production of IL-2 and IFN-γ in human lymphocytic Jurkat cells, suggesting that the T cells may be affected by FPN directly at non-cytotoxic concentrations [22]. As these data indicated the adverse effects of FPN on the immune system, however, little is known regarding the immunomodulatory effects of FPN on T helper 1 and T helper 2 immune balance in vivo. Furthermore, the underlying mechanism of immunotoxicity of FPN on T cell-dependent immune responses needs to be urgently clarified.

The critical inhibitory neurotransmitter GABA can be synthesized and released by the immune cells. Additionally, GABAergic signaling genes and functional proteins are expressed in mononuclear phagocytes and lymphocytes [23]. Numerous studies have elucidated the diverse roles of GABA in the immune system. GABA acts as an intercellular signaling molecule to modulate monocyte migration and to suppress T cell activation, proliferation, and cytokine production, through its receptor signaling [23–25]. GABA participates in T cell-mediated immunity *via* GABA transporters (GAT) and GABA receptors [26]. GABA treatment dose-dependently inhibited antigen-specific T cell proliferation and the T cell responses to foreign and self-antigens in vitro [27]. Moreover, the antigen-specific T-cell response could be directly inhibited by the GABAergic agents [28]. Considering the systemic insecticidal properties of FPN, known to antagonize $GABA_A$ receptors in insects, the regulation of GABAergic genes in immune cells might be a potential mechanism of FPN-induced immunotoxicity. This study aimed to study the effects of FPN on antigen-specific T-cell immunity and T helper (Th) 1 and Th2 balance using the ovalbumin (OVA)-sensitized mouse model. The roles of GABAergic genes involved in FPN-induced immunotoxicity were further explored.

Results

Effects of FPN exposure on body weight, spleen index, and spleen cellularity in vivo

Mice exposed to corn oil (VH) or FPN (1, 5, 10 mg/kg) for a total of 11 doses did not exhibit any apparent clinical symptoms. Besides, no mortality was observed in FPN-treated groups. Administration of OVA-immunized mice with 5 and 10 mg/kg of FPN slightly slowed down weight gain and increased the spleen index compared to the VH group (Table 1). The cellularity of $CD4^+$, $CD8^+$, $CD11b^+$, $Gr1^+$, and $B220^+$ in splenocytes was not altered during the administration of FPN (Table 1).

Modulation of antigen-specific antibody production by FPN administration

The BALB/c mice were immunized with OVA to study T-cell-dependent immune responses (Fig. 1). As expected, OVA immunization induced an appreciable increase in the serum levels of all three measured OVA-specific immunoglobulin (Ig), as compared to the non-immunized control (Fig. 2; NA vs. treatment groups). The serum value of OVA-specific IgG_1 and IgG_{2a} was markedly increased in a dose-dependent manner (Fig. 2B-C) but the level of OVA-specific IgM was not altered by FPN (Fig. 2A).

FPN enhanced the cell viability and disturbed IL-2, IL-4, and IFN-γ production ex vivo

Next, we examined the effects of FPN on the proliferation of OVA-stimulated splenocytes using an MTT assay. FPN (5 and 10 mg/kg) robustly enhanced the cell viability of splenocytes in the absence or presence of OVA (100 μg/mL) (Fig. 3A). Naïve T cells proliferate and differentiate into effector Th cells, which are key effectors of the adaptive immune response, based on their secretion of cytokines. Th1 cells secrete IL-2 and IFN-γ, whereas Th2

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Table 1 Effects of FPN exposure on body weight, spleen index, and cellularity of splenocytes

	NA	VH	Fipronil (mg/kg)		
			1	5	10
Body Weight					
Day 1	21.39 ± 0.25	21.56 ± 017	21.24 ± 0.21	21.5 ± 0.21	21.68 ± 0.18
Day 16	23.68 ± 0.31	23.22 ± 0.24	22.46 ± 0.18	22.31 ± 0.23*	21.74 ± 0.19*
Spleen index	3.888 ± 0.07	4.124 ± 0.05	4.205 ± 0.1	4.211 ± 0.96	4.577 ± 0.26
Spleen Cellularity (%)					
$CD4^+$	24.03 ± 0.43	21.9 ± 0.66	17.52 ± 1.63	18.22 ± 1.7	21.21 ± 0.51
$CD8^+$	13.28 ± 0.61	14.23 ± 0.57	13.61 ± 0.7	13.67 ± 0.74	13.22 ± 0.78
$B220^+$	50.94 ± 1.07	51.78 ± 1.4	52.75 ± 1.41	51.87 ± 1.13	49.76 ± 1.33
$CD11b^+$	1.92 ± 0.34	1.49 ± 0.25	1.68 ± 0.3	1.5 ± 0.23	1.72 ± 0.32
$Gr1^+$	1.18 ± 0.25	0.99 ± 0.19	1.03 ± 0.22	0.98 ± 0.2	1.03 ± 0.19
$CD11b^+/Gr1^+$	1.77 ± 0.14	2.29 ± 0.22	2.19 ± 0.21	2.34 ± 0.24	2.4 ± 0.24

[a] Spleen index was calculated as the spleen weight (mg) per body weight (g). Data was expressed as mean ± SEM of 20 mice pooled from four independent experiments

[b] Splenocytes were prepared as described in the Materials and Methods section. The percentage of $CD4^+$, $CD8^+$, $B220^+$, $CD11b^+$, and $Gr1^+$ cells was determined by flow cytometry. Data was expressed as mean ± SEM pooled from four independent experiments ($n = 20$). *$p < 0.05$ as compared with the VH group

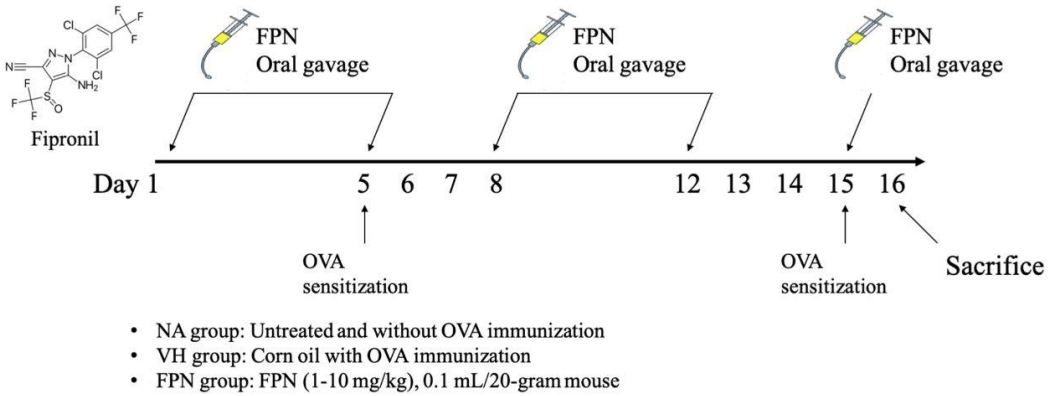

Fig. 1 Protocol for fipronil (FPN) administration and ovalbumin (OVA) immunization. Mice were randomly divided into the following groups: naïve (NA), vehicle-treated and OVA-immunized (VH), and FPN-treated and OVA-immunized (FPN). The dosing regimen for FPN administration and antigen immunization was described in Materials and Methods

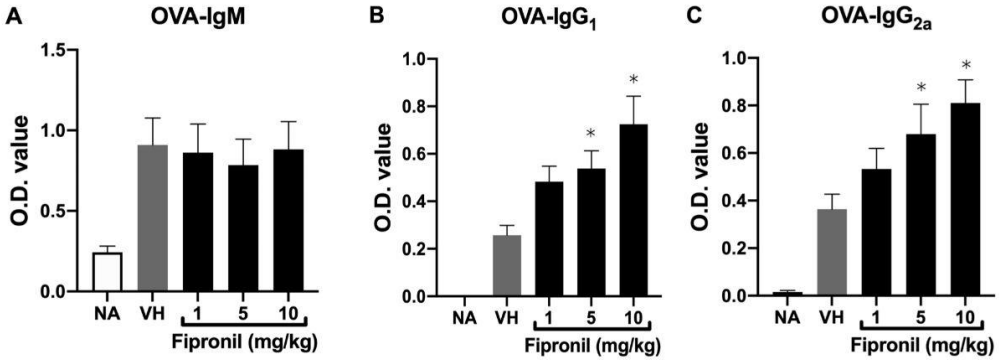

Fig. 2 Induction of OVA-specific IgG_1 and IgG_{2a} production in vivo. The serum levels of OVA-specific IgM, IgG_1, and IgG_{2a} were determined by ELISA. Data was expressed as mean ± SEM of 20 individuals pooled from 4 independent experiments. *$p < 0.05$ as compared with the VH group

doi:10.6342/NTU202501708

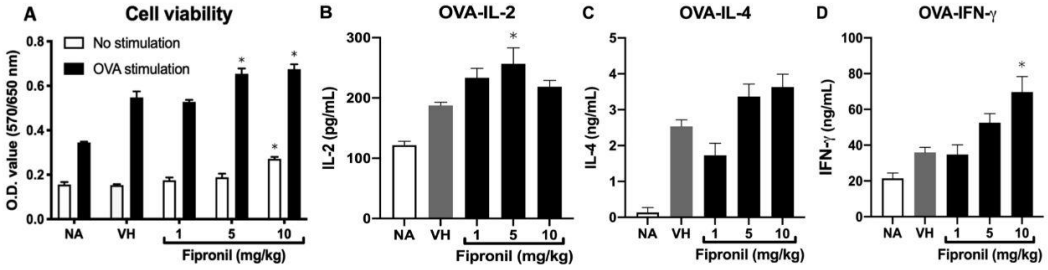

Fig. 3 Enhancement of the cell viability and disturbance of IL-2, IL-4, and IFN-γ production. Splenocytes with the same cell concentration were prepared from each group of mice and cultured in the presence of ovalbumin (100 μg/mL) for 72 h. The supernatants were collected to measure the concentration of IL-2, IFN-γ, and IL-4 by ELISA. Data was expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments ($n = 20$). *$p < 0.05$ was significant compared to the VH group

cells secrete IL-4. The balance between Th1 and Th2 is required in an integrated immune system [29, 30]. Therefore, we investigate the effects of FPN on the production of Th cytokines. As shown in Fig. 3B-D, the production of IL-2, IL-4, and IFN-γ by splenocytes stimulated with OVA (100 μg/mL) was significantly increased at high-dose treatment groups (5, 10 mg/kg of FPN).

FPN slightly down-regulated IL-2, IL-4, and GATA3 expression by OVA-stimulated splenocytes

Th1/Th2 cytokines play pivotal roles in modulating host immune responses [30]. As FPN increased antigen-specific cytokine production including IL-2, IL-4, and IFN-γ, we further examined the effects of FPN on the gene expression of Th1/Th2 cytokines and upstream transcription factors. Surprisingly, the relative mRNA expression of IL-2, IL-4, IFN-γ, GATA3, and T-bet by splenocytes stimulated with OVA (100 μg/mL) for 72 h were not altered (Fig. S1A-E).

FPN altered GABAergic signaling gene expression by primary splenocytes

FPN is a systemic insecticide known to antagonize the $GABA_A$ receptors in insects. To investigate whether the GABAergic signaling genes are involved in the disturbances of immune responses by FPN exposure, the expression of glutamate decarboxylases (GAD65 and GAD67), GABA transporter gene (GAT1), and GABA receptor subunit (GABAR α5, β2, β3, δ) were further examined in splenocytes isolated from FPN-treated mice. The results showed that FPN notably reduced the expression of GAD67 genes in a dose-dependent manner, and then the expression of GABAR β2 and GABAR δ genes were significantly increased at the high dose of FPN compared to VH control. The levels of GAT1 mRNA are also decreased at 10 mg/kg FPN but no remarkable difference (Fig. 4A-G).

Discussion

The present results demonstrated that exposure to FPN disturbed antigen-specific immune responses in vivo. Compared with extensive studies of FPN-induced neurotoxic, reproductive, and cytotoxic effects [9, 31–33], limited studies have explored immunotoxicity associated with lymphocyte functionality [20]. Herein, we focused on mature T cells that primarily reflect T-cell-dependent immune responses. To the best of our knowledge, this study is the first report on the immunotoxic effects of FPN on antigen-specific immunity by oral exposure. Once FPN was administrated to mice by oral gavage, it rapidly transformed into a more toxic metabolite [34, 35], fipronil sulfone, and accumulated for an extended period in the plasma [36]. Furthermore, fipronil sulfone remained at a stable and consistent level in the plasma after 4 times oral administration of FPN [36]. During the same dosing regimen of FPN administration by oral gavage (5 days/week, up to 3 weeks), the plasma level of fipronil sulfone slightly increased. This data indicated the stable bioaccumulation profile of FPN metabolites in mice through oral administration [36]. We speculate that FPN metabolites can stably accumulate in the body to affect the immune responses. However, the intermittent recovery period could have resulted in an underestimation of the effects of the lower dosage of FPN on immune responses. A long-term exposure to FPN needs to be studied to evaluate the prolonged effects of FPN on T-cell immune responses.

In this study, exposure to FPN (1–10 mg/kg) for 11 doses did not induce severe clinical symptoms, and there were no significant changes in spleen index and spleen cellularity. Similar to the findings of Farhad and Banalata (2020), no mortality was recorded [21]. Notably, the body weight gain in the FPN (5 and 10 mg/kg) groups on day 16 exhibited a slight decrease, while the spleen index marginally increased in the 10 mg/kg group. The moderate decline in body weight may be attributed to oxidative stress induced by FPN (the dosage used was ≤9.7 mg/kg

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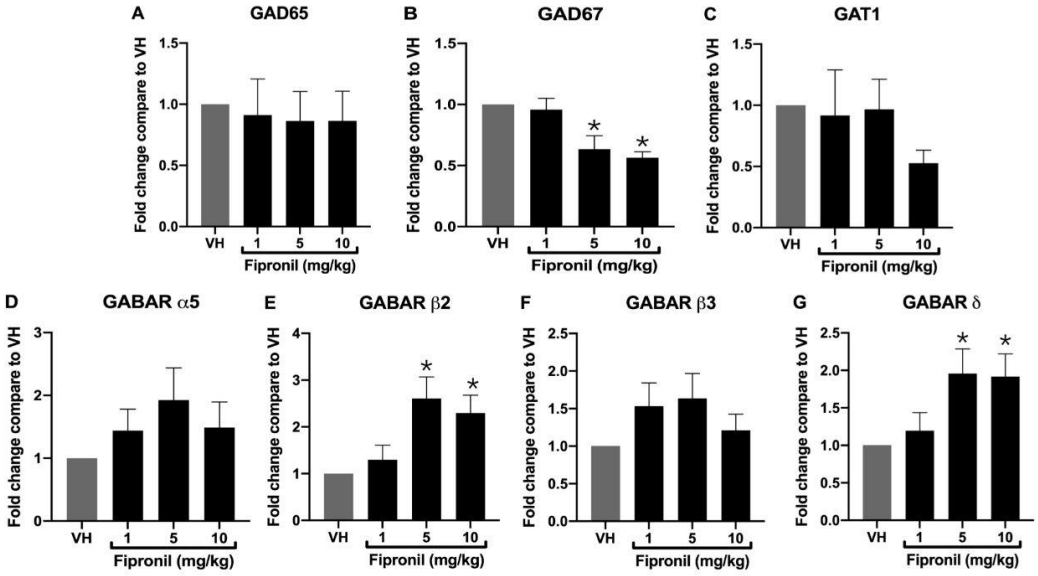

Fig. 4 FPN significantly reduced the mRNA expression of GAD65 and GAT1 and altered the GABA receptor subunit expression. The total RNA of splenocytes (6×10^6 cells) harvested from different treatment groups was extracted to detect the mRNA expression of GAD65, GAD67, GAT1, GABAR α5, GABAR β2, GABAR β3, and GABAR δ by qPCR. The expression level of HPRT was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group ($n = 20$). *$p < 0.05$ was significant compared to the VH group

(1/10 LD50)) [37]. Nevertheless, these observations suggest that administering 10 mg/kg of FPN to mice could potentially induce mild toxicity. Combined with the spleen cellularity data (both populations of lymphocytes and myeloid cells were not significantly altered), a slight increase in the spleen index in the 10 mg/kg group may be due to decreased body weight. Another report showed that exposure of rats to 9.7 mg/kg of FPN for 30 days caused histopathological changes in the spleen. A significant reduction in the proportion of white pulp area was found, accompanied by severe atrophy of lymphoid follicles [20]. Although no changes in splenic composition were observed in our current data, the effects of FPN on lymphopoiesis may become more pronounced with increasing exposure time.

Dysregulated Th1/Th2 cytokine production has the potential to contribute to the development of autoimmune disorders and allergic conditions [29, 30]. In the present study, serum levels of OVA-specific IgG_1 and IgG_{2a} were markedly increased in FPN-treated mice, suggesting that FPN interfered with T cell-dependent antibody production. Furthermore, FPN administration at high dose enhanced the viability and proliferation of antigen-specific T cells in response to OVA stimulation ex vivo, which subsequently led to a significant increase in the production of IL-2, IL-4, and IFN-γ cytokines by OVA-specific T cells. These results revealed the immunostimulatory effects of FPN on adaptive immune responses. The immunostimulatory effects of FPN have been reported in human nasal epithelial cells. Both protein levels and mRNA expression of pro-inflammatory cytokines including IL-1 beta, IL-6, and IL-8 were induced by FPN in vitro through activation of ERK1/2 MAPK, p38 MAPK, and the NF-κB pathway [38]. On the contrary, the mRNA expression of Th1/Th2 cytokines and their respective upstream transcription factors remained unchanged or slightly decreased in the present work. Since the immunostimulatory effects of FPN on Th1/Th2 cytokines are not correlated with the mRNA expression of Th1/Th2 cytokines and differentiation genes, it is suggested that the immunomodulatory effects of FPN on antigen-specific T cell responses may not be through the regulation of Th1/Th2 differentiation and cytokine gene expression.

As FPN is antagonistic to GABA receptors, the regulation of the GABAergic genes by FPN was further investigated to clarify their roles in FPN-mediated immunomodulatory effects in vivo. GABA inhibited antigen-specific T cell proliferation and the T cell responses to foreign and self-antigens in a dose-dependent manner in vitro [27]. The antigen-specific T-cell immune responses were inhibited by the GABAergic agents. The

doi:10.6342/NTU202501708

levels of inflammatory cytokines are reduced after GABA treatment in peripheral macrophages [28]. GABA and/or $GABA_A$ receptor agonists reduced inflammatory responses, antigen-specific cytotoxic immune responses, and antigen-primed delayed-type hypersensitivity reactions, in a non-obese diabetic mouse model of type 1 diabetes [27, 28, 39]. Collectively, these lines of evidence indicate the regulatory roles of GABAergic signaling in the over-reactive adaptive immune responses.

Glutamate decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA. GABA secretion has been observed in stimulated T cells when cultured in a conditioned medium. In addition, dendritic cells and macrophages express GAD65 transcripts to synthesize GABA. Although the main GAD in stimulated T cells has not been well studied, the secretion of the bulk GABA by T cells may be impacted by re-uptaking, storage, and secretion of GABA [28]. In the present study, the expression of GAD67, an enzyme that could be expressed in B cells [40], by antigen-stimulated splenocytes was significantly reduced in FPN-treated mice in a dose-dependent manner. This data indicated that FPN might interfere with the synthesis of GABA through the down-regulation of GAD67 in splenocytes. GABA transporter type 1 (GAT1) is primarily engaged in GABA binding and transport from the cytoplasm to the extracellular space (reverse mode) and back into the cytoplasm (forward mode). Dysfunctional GAT1 may lead to a delay in communication with post-synaptic GABA receptors, resulting in a variety of neurological diseases [41]. The $CD4^+$ T cells isolated from $GAT1^{-/-}$ mice have higher IL-2 and IFN-γ secretion under conditional stimulation, promoting T cell activation and survival through PKC-dependent signaling pathways [42]. These results of previous literature are also consistent with our cytokine data, where a significant increase in the secretion of IL-2 and IFN-γ was observed in the FPN-treated groups. Furthermore, a slight decrease in the expression of GAT1 mRNA has been shown, suggesting the impairment of GABA recycling may be involved in the immunostimulatory effects of FPN.

Emma L. Sparrow et al. identify that the $GABA_A$ receptors are expressed in human and mouse immune cells, and the main subunits of mouse T cells are α5, β2, β3, and δ [43]. Although T cells can form different heteropentameric $GABA_A$ receptor subtypes, the previous study hasn't completely demonstrated the composition and physiological relevance of the $GABA_A$ receptor subunit on T cells, because their affinity for GABA and pharmacological properties are varied in different subunits of the $GABA_A$ receptor. Nevertheless, through functional $GABA_A$ receptors but not $GABA_B$ receptors, higher levels of GABA could reduce antigen-primed T cell proliferation to inhibit immune responses [27]. Our present study demonstrated that the expression of GABA receptor genes was altered after exposure to FPN. The mRNA expression of subunits of β2 and δ were notably increased, and the α5 and β3 transcripts were slightly induced at 1 and 5 mg/kg groups. As FPN competitively binds to GABA receptors, resulting in a reduction of GABA's regulatory effects on immune responses, we speculated that the up-regulation of GABA receptor genes may be a compensatory mechanism to overcome the adverse effects of FPN-induced dysregulation of GABAergic genes. In our data without the obvious changes in spleen composition, the alterations of GABAergic genes were found in concordance with the FPN-mediated abnormal immunostimulatory effects, suggesting the dysregulation of GABAergic genes may play a role in the immunotoxicity of FPN. Figure 5 summarizes the effects of FPN on GABAergic genes in this study.

In conclusion, our study showed that oral exposure to FPN for 11 administration schedule disrupted antigen-specific T-cell responses in vivo, demonstrating immunotoxic effects with intermittent exposure. As GABAergic components regulate T cell-mediated immunity, we hypothesized that FPN may augment the antigen-specific immunity *via* dysregulation of GABAergic genes. This study may open an avenue to investigate the immunotoxic effects of FPN on the dysregulation of GABAergic signaling in primary immune cells.

Materials and methods

Reagents

All reagents were purchased from Sigma (MO, USA) unless otherwise stated. Fipronil (FPN, purity≧97%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and dissolved in corn oil. RPMI 1640 medium (Cat. No. SH30027.02) was purchased from Hyclone (UT, USA). Fetal bovine serum (FBS, Cat. No. 10437-028) and cell culture reagents were purchased from GIBCO BRL (MD, USA) and GE Healthcare (Chicago, IL). Reagents used for ELISA examination were purchased from BD Biosciences (San Jose, CA).

Experimental animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026). Male BALB/c mice aged 5 weeks (weight 18–20 g) were purchased from BioLASCO Experimental Animal Center (Taiwan Co., Ltd, BioLASCO, Taipei, Taiwan). Randomization was carried out as follows: upon arrival, each mouse was assigned to a group and weighed. The total number of mice was then weighed and divided into five groups based on their weight to minimize initial weight differences within each group. Subsequently, the mice were randomly transferred into plastic cages

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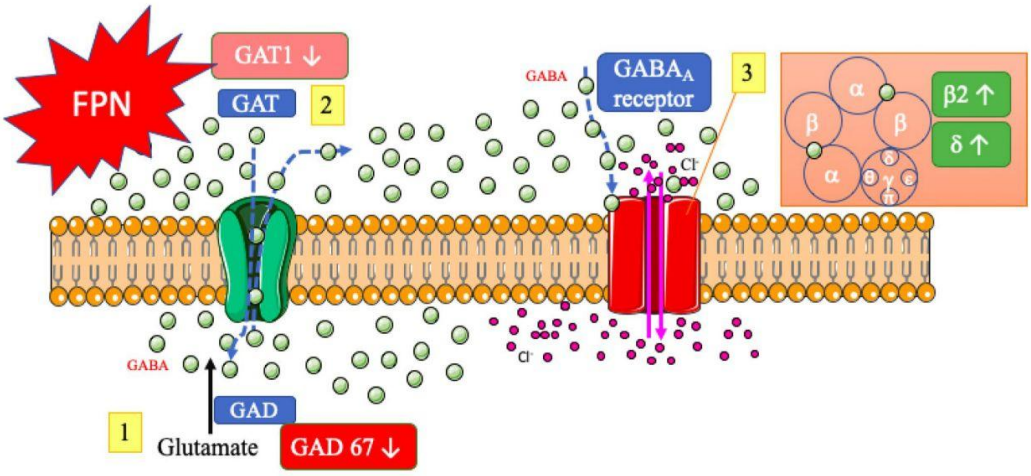

Fig. 5 A schematic diagram of the synthesis and transport of GABA signaling affected by FPN in this study. (1) GABA is produced enzymatically through the activity of glutamate decarboxylase (GAD 65/67). The FPN exposure significantly decreased the mRNA levels of GAD 67, which might reduce GABA synthesis. (2) GABA transporters (GAT) facilitate the transportation of GABA through the cellular space. The deficiency of GAT1 would affect the function of GABA transportation and might be associated with IL-2 and IFN-γ increase by FPN exposure. (3) Activation of $GABA_A$ receptors occurs upon GABA binding, leading to either the efflux or influx of Cl^-. After FPN treatment, the mRNA expression of subunits of β2 and δ were notably increased which might be a compensatory mechanism to overcome the adverse effects of FPN-induced dysregulation of GABAergic genes. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/)

containing sawdust bedding and quarantined for one week (3−6 mice per cage). Mice were raised in temperature (24±2 °C) and humidity (50±20%) controlled room on a 12 h light/dark cycle and given standard laboratory food and water *ad libitum*.

Protocol of animal experiments

The mice (5 animals/group) were either left untreated (naïve; NA) or administered by oral gavage with FPN (1, 5, 10 mg/kg) suspended in corn oil and/ or vehicle (VH; corn oil) 5 days per week for 2 weeks and one more dose before second OVA sensitization on day 15 (Fig. 1). Based on general immunotoxicity assessment, the U.S. Food & Drug Administration recommends a common and ideal functional assay for detecting potential immunotoxicity of drugs, T-cell dependent antigen response (TDAR) assay, triggering by exogenous protein as antigen like ovalbumin (OVA) to induce T-cell–dependent immune responses [44]. This assay assesses immunological function, which is determined by the efficiency of several immune processes, such as antigen uptake and presentation, T cell help, B cell activation, and antibody generation [45, 46]. Except for the NA group, mice were sensitized with OVA twice by intraperitoneal injection, with 0.1 mL sensitization solution containing 100 μg ovalbumin and 1 mg alum (as adjuvant) in saline on day 5 and day 15. Mice were sacrificed on day 16, and their serum samples and spleens were harvested for further experimentation. Because the mice had to be observed for clinical changes after administration of FPN, the experimenter could not be blinded to whether the animal was exposed to FPN or corn oil.

Splenocyte isolation and culture

Mice were sacrificed by cervical dislocation. The spleen was aseptically removed, washed, and extracted into single-cell suspensions, and the erythrocytes in splenocyte cultures were lysed by ACK buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). The cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and cultured at 37 °C in 5% CO_2 for further experiments.

Spleen index

The spleen of each mouse (n=20 in each group) was dissected and weighed immediately after sacrifice. The spleen index was calculated as the spleen weight (mg) per body weight (g).

Flow cytometric analysis for cellularity of splenocytes

The expression of $CD4^+$, $CD8^+$, $CD11b^+$, $Gr\text{-}1^+$, and $B220^+$ by splenocytes was measured by flow cytometry. Briefly, splenocytes were stained with rat anti-mouse

doi:10.6342/NTU202501708

CD4 (BD Biosciences, San Jose, CA) and Gr-1 (eBioscience, Waltham, MA) conjugated with FITC and/or rat anti-mouse CD8 (BD Biosciences, San Jose, CA) and B220 (BD Biosciences, San Jose, CA) conjugated with PE-Cy5 and/or rat anti-mouse CD11b (eBioscience, Waltham, MA) conjugated with APC antibodies in staining buffer (PBS containing 2% FBS and 0.09% sodium azide) avoiding light on ice for 30 min. Appropriate rat anti-mouse antibodies were applied as the isotype control for evaluating non-specific binding. After washing, the single-cell fluorescence of 10,000 cells for each sample was measured by a flow cytometer (BD FACSCalibur, San Jose, CA) Data was analyzed by Flowjo 10.4 software (FlowJo LLC, Ashland, OR).

Metabolic activity assay of splenocyte

The metabolic activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [47]. Splenocytes (6×10^6 cells/mL) were cultured in 96-well plates with the presence of OVA (100 μg/mL) for 72 h. After OVA stimulation for 68 h, an MTT stock solution (5 mg/mL) was added and incubated for 4 h. Then, the formed formazan was dissolved by adding 100 μL Dimethyl sulfoxide (DMSO). The plate was read using an ELISA microplate reader (SpectraMax ® M5 Microplate Reader, Molecular Devices LLC, San Jose, California, USA) at $OD_{570\ nm}$ using $OD_{630\ nm}$ as a background reference.

Measurement of cytokines and OVA-specific ig expression by enzyme-linked immunosorbent assay (ELISA)

Splenocytes (6×10^6 cells/mL) were cultured in quadruplication in 48-well culture plates (0.3 mL/well). The levels of IL-2, IL-4, and IFN-γ in the culture supernatant with/without OVA stimulation for 72 h, OVA-IgM, IgG_1, and IgG_{2a} in serum samples were determined by ELISA as previously described. The optical density was measured at $OD_{450\ nm}$ using an ELISA microplate reader (SpectraMax ® M5 Microplate Reader, Molecular Devices LLC, San Jose, California, USA).

RNA isolation and quantitative polymerase chain reaction (qPCR)

The splenocytes incubated with OVA for 72 h were collected and homogenized in TRIzol reagent. The total RNA was isolated by the GENEzol Pure Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the manufacturer's instructions. The RNA concentration was quantified and qualified using the determination of $OD_{260\ nm}$, $OD_{280\ nm}$, and $OD_{230\ nm}$ by Nanophotometer™ (Implen GmbH, Munich, Germany). One mg of total RNA was reverse-transcribed by SensiFAST cDNA Synthesis Kit (BIOLINE, Memphis, TN) into cDNA products. Real-time PCR was performed by AriaMx Real-Time PCR System (Aglient Technologies, Santa Clara, CA). During the real-time PCR process, SensiFAST SYBR Lo-ROX Kit was provided to quantify mRNA expression. The expression of the HPRT gene was employed as an endogenous control to normalize the expression of target genes. The primers of the target gene used in this study are listed in Table 2.

Table 2 List of quantitative PCR primers

Gene name	Primers (5′ to 3′)
IL-2	F: AGCAGCTGTTGATGGACCTA R: CGCAGAGGTCCAAGTTCAT
IL-4	F: GCTAGTTGTCATCCTGCTCTTC R: GGCGTCCCTTCTCCTGTG
IFN-γ	F: GCCAAGTTTGAGGTCAACAAC R: CCGAATCAGCAGCGACTC
T-bet	F: GCCAGGGAACCGCTTATATG R: GACGATCATCTGGGTCACATTCT
GATA3	F: TACCCTCCGGCTTCATCCT R: TGCACCTGATACTTGAGGCAC
GAD65	F: TCAACTAAGTCCCACCCTAAG R: CCCTGTAGAGTCAATACCTGC
GAD67	F: CGCTTGGCTTTGGAACCGACAA R: GAATGCTCCGTAAACAGTCGTGC
GAT1	F: CAAGCCCAAAACCCTGGTAGT R: CCACGCAGGACATGAGGAA
GABAR α5	F: GATTGTGTTCCCCATCTTGTTTGGC R: TTACTTTGGAGAGGTGGCCCCTTTT
GABAR β2	F: GCTGGTGAGGAAATCTCGGTCCC R: CATGCGCACGGCGTACCAAA
GABAR β3	F: GAGCGTAAACGACCCCGGGAA R: GGGACCCCCGAAGTCGGGTCT
GABAR δ	F: TCAAATCGGCTGGCCAGTTCCC R: GCACGGCTGCCTGGCTAATCC
HPRT	F: TCAGTCAACGGGGGACATAAA R: GGGGCTGTACTGCTTAACCAG

Statistical analysis

All the data were analyzed by SigmaPlot 14.0 (San Jose, CA). The mean ± standard error (SEM) was determined for each treatment group in the individual experiments. All analysis was executed in a blinded manner. Statistical differences between groups were evaluated by one-way ANOVA and Dunnett's two-tailed t-test was employed to compare FPN-treated groups to the control group. P-value < 0.05 was defined as a statistical significance.

Abbreviations

FPN	Fipronil
OVA	Ovalbumin
$GABA_A$	γ-aminobutyric acid
GAD	glutamate decarboxylases
GAT	GABA transporter
GABAR	GABA receptor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-03878-3.

doi:10.6342/NTU202501708

Supplementary Material 1

Acknowledgements
We thank Dr. Han-You Lin, School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan, for providing the service of Agilent Technologies ® Mx3005P qPCR system.

Author contributions
J.F.K. and C.C.W. conceived and designed the experiments; analyzed the data and wrote the manuscript. J.F.K. performed the experiments and drafted the manuscript. Y.H.C and C.W.T. consulted the experiments and tested the concept of this study. All authors read and approved the final manuscript.

Funding
This research was supported by the Ministry of Science and Technology (Taipei, Taiwan) under Grant MOST 106-2320-B-037-002, MOST 107-2320-B-002-065, and National Science and Technology Council (Taipei, Taiwan) under Grant NSTC 112-2313-B-002-005-. The funders had no role in the design of the collection, analysis, and interpretation of data.

Data availability
The raw data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026), and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with the ARRIVE 2.0 guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 31 July 2023 / Accepted: 23 December 2023
Published online: 22 January 2024

References

1. Tingle CCD, Rother JA, Dewhurst CF, Lauer S, King WJ, Fipronil. Environmental Fate, Ecotoxicology, and Human Health Concerns. In: Ware GW, editor. Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews [Internet]. New York, NY: Springer; 2003 [cited 2023 Jan 10]. p. 1–66. (Reviews of Environmental Contamination and Toxicology). https://doi.org/10.1007/978-1-4899-7283-5_1.
2. Wu J, Lu J, Lu H, Lin Y, Chris Wilson P. Occurrence and ecological risks from fipronil in aquatic environments located within residential landscapes. Science of The Total Environment [Internet]. 2015 Jun 15 [cited 2023 Jan 10];518–519:139–47. Available from: https://www.sciencedirect.com/science/article/pii/S0048969715001412.
3. Gunasekara AS, Truong T, Goh KS, Spurlock F, Tjeerdema RS. Environmental fate and toxicology of fipronil. J Pesticide Sci. 2007;32(3):189–99.
4. Ratra GS, Casida JE. GABA receptor subunit composition relative to insecticide potency and selectivity. Toxicology Letters [Internet]. 2001 Jul 6 [cited 2023 Jan 10];122(3):215–22. Available from: https://www.sciencedirect.com/science/article/pii/S0378427401003666.
5. Cole LM, Nicholson RA, Casida JE. Action of Phenylpyrazole Insecticides at the GABA-Gated Chloride Channel. Pesticide Biochemistry and Physiology [Internet]. 1993 May 1 [cited 2023 Jan 10];46(1):47–54. Available from: https://www.sciencedirect.com/science/article/pii/S0048357583710357.
6. Authority (EFSA) EFS. Conclusion regarding the peer review of the pesticide risk assessment of the active substance fipronil. EFSA Journal [Internet]. 2006 [cited 2023 Apr 24];4(5):65r. Available from: https://onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2006.65r.
7. Pisa LW, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Downs CA, Goulson D, et al. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ Sci Pollut Res. 2015;22(1):68–102.
8. Stehr CM, Linbo TL, Incardona JP, Scholz NL. The Developmental neurotoxicity of Fipronil: Notochord degeneration and locomotor defects in zebrafish embryos and larvae. Toxicol Sci. 2006;92(1):270–8.
9. Khan S, Jan MH, Kumar D, Telang AG. Firpronil induced spermotoxicity is associated with oxidative stress, DNA damage and apoptosis in male rats. Pesticide Biochemistry and Physiology [Internet]. 2015 Oct 1 [cited 2023 Jan 10];124:8–14. Available from: https://www.sciencedirect.com/science/article/pii/S0048357515000644.
10. Mossa ATH, Swelam ES, Mohafrash SMM. Sub-chronic exposure to fipronil induced oxidative stress, biochemical and histopathological changes in the liver and kidney of male albino rats. Toxicol Rep. 2015;2:775–84.
11. Bonneau S, Reymond N, Gupta S, Navarro C. Efficacy of a fixed combination of permethrin 54.5% and fipronil 6.1% (Effitix®) in dogs experimentally infested with Ixodes ricinus. Parasites & Vectors. 2015;8(1):204.
12. Dumont P, Liebenberg J, Beugnet F, Fankhauser B. Repellency and acaricidal efficacy of a new combination of fipronil and permethrin against Ixodes ricinus and Rhipicephalus sanguineus ticks on dogs. Parasites & Vectors. 2015;8(1):531.
13. Chen D, Li J, Zhao Y, Wu Y. Human Exposure of Fipronil Insecticide and the Associated Health risk. J Agric Food Chem. 2022;70(1):63–71.
14. Lee SJ, Mulay P, Diebolt-Brown B, Lackovic MJ, Mehler LN, Beckman J, et al. Acute illnesses associated with exposure to fipronil—surveillance data from 11 states in the United States, 2001–2007. Clin Toxicol. 2010;48(7):737–44.
15. Jennings KA, Canerdy TD, Keller RJ, Atieh BH, Doss RB, Gupta RC. Human exposure to fipronil from dogs treated with frontline. Vet Hum Toxicol. 2002;44(5):301–3.
16. Cordeiro F, Bratinova S, Karasek L, Buttinger G, Stroka J, Emteborg H, et al. Can official control laboratories quantify reliably fipronil in eggs? Evidence from a proficiency testing round. Food Addit Contaminants: Part A. 2019;36(6):904–10.
17. Guo Q, Zhao S, Zhang J, Qi K, Du Z, Shao B. Determination of fipronil and its metabolites in chicken egg, muscle and cake by a modified QuEChERS method coupled with LC-MS/MS. Food Addit Contaminants: Part A. 2018;35(8):1543–52.
18. Munoz-Pineiro MA, Robouch P, JRC Publications R. 2018 [cited 2023 Dec 12]. Fipronil in eggs: Factsheet – December 2017. Available from: https://publications.jrc.ec.europa.eu/repository/handle/JRC110632.
19. Ansar Ahmed S. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology [Internet]. 2000 Sep 7 [cited 2023 Jan 10];150(1):191–206. Available from: https://www.sciencedirect.com/science/article/pii/S0300483X00002596.
20. Aldayel TS, Abdel-Rahman HG, Gad EL, Abdelrazek HMA, Mohamed RM, El-Sayed RM. Assessment of modulatory activity of Uncaria tomentosa extract against fipronil immunotoxicity in male rats. Ecotoxicology and Environmental Safety [Internet]. 2021 Nov 1 [cited 2023 Jan 10];224:112674. Available from: https://www.sciencedirect.com/science/article/pii/S0147651321007867.
21. Bano F, Mohanty B. Thyroxine modulation of immune toxicity induced by mixture pesticides mancozeb and fipronil in mice. Life Sciences [Internet]. 2020 Jan 1 [cited 2023 Jan 10];240:117078. Available from: https://www.sciencedirect.com/science/article/pii/S0024320519310057.
22. Z W. Immunotoxic properties of pesticides: effects of diazinon-oxon and fipronil on lymphocytic Jurkat cells. Front Pharmacol [Internet]. 2010 [cited 2023 Jan 10];1. Available from: http://www.frontiersin.org/10.3389/conf.fphar.2010.60.00196/event_abstract.
23. Bhandage AK, Barragan A. GABAergic signaling by cells of the immune system: more the rule than the exception. Cell Mol Life Sci [Internet]. 2021 Aug 1 [cited 2023 Jan 10];78(15):5667–79. https://doi.org/10.1007/s00018-021-03881-z.
24. Barragan A, Weidner JM, Jin Z, Korpi ER, Birnir B. GABAergic signalling in the immune system. Acta Physiologica [Internet]. 2015 [cited 2023 Jan 10];213(4):819–27. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/apha.12467.
25. Kim JK, Kim YS, Lee HM, Jin HS, Neupane C, Kim S et al. GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. Nat Commun [Internet]. 2018 Oct 10 [cited 2023 Jan 10];9(1):4184. Available from: https://www.nature.com/articles/s41467-018-06487-5.

doi:10.6342/NTU202501708

26. Ding X, Chang Y, Wang S, Yan D, Yao J, Zhu G. Transcriptomic Analysis of the Effect of GAT-2 Deficiency on Differentiation of Mice Naïve T Cells Into Th1 Cells In Vitro. Front Immunol [Internet]. 2021 Jun 2 [cited 2023 Jan 10];12:667136. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8208808/.
27. Tian J, Chau C, Hales TG, Kaufman DL. GABAA receptors mediate inhibition of T cell responses. Journal of Neuroimmunology [Internet]. 1999 Apr 1 [cited 2023 Jan 10];96(1):21–8. Available from: https://www.sciencedirect.com/science/article/pii/S0165572898002641.
28. Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, National Academy of Sciences [Internet]. Inhibitory role for GABA in autoimmune inflammation. Proceedings of the. 2010 Feb 9 [cited 2023 Jan 10];107(6):2580–5. Available from: https://www.pnas.org/doi/10.1073/pnas.0915139107.
29. Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. J Autoimmun [Internet]. 2008 Nov [cited 2023 Jan 10];31(3):252–6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3178062/.
30. Wan YY, Flavell RA. How Diverse—CD4 Effector T Cells and their Functions. J Mol Cell Biol [Internet]. 2009 Oct [cited 2023 Jan 10];1(1):20–36. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2841031/.
31. Gupta SK, Pal AK, Sahu NP, Jha AK, Akhtar MS, Mandal SC et al. Supplementation of microbial levan in the diet of Cyprinus carpio fry (Linnaeus, 1758) exposed to sublethal toxicity of fipronil: effect on growth and metabolic responses. Fish Physiol Biochem [Internet]. 2013 Dec 1 [cited 2023 Jan 10];39(6):1513–24. https://doi.org/10.1007/s10695-013-9805-7.
32. Wang X, Martínez MA, Wu Q, Ares I, Martínez-Larrañaga MR, Anadón A et al. Fipronil insecticide toxicology: oxidative stress and metabolism. Critical Reviews in Toxicology [Internet]. 2016 Nov 25 [cited 2023 Jan 10];46(10):876–99. https://doi.org/10.1080/10408444.2016.1223014.
33. Badgujar PC, Pawar NN, Chandratre GA, Telang AG, Sharma AK. Fipronil induced oxidative stress in kidney and brain of mice: Protective effect of vitamin E and vitamin C. Pesticide Biochemistry and Physiology [Internet]. 2015 Feb 1 [cited 2023 Jan 10];118:10–8. Available from: https://www.sciencedirect.com/science/article/pii/S0048357514001953.
34. Romero A, Ramos E, Ares I, Castellano V, Martínez M, Martínez-Larrañaga MR, et al. Fipronil sulfone induced higher cytotoxicity than fipronil in SH-SY5Y cells: Protection by antioxidants. Toxicol Lett. 2016;252:42–9.
35. Song X, Wang X, Liao G, Pan Y, Qian Y, Qiu J. Toxic effects of fipronil and its metabolites on PC12 cell metabolism. Ecotoxicol Environ Saf. 2021;224:112677.
36. Cam M, Durieu E, Bodin M, Manousopoulou A, Koslowski S, Vasylieva N et al. Induction of Amyloid-β42 Production by Fipronil and Other Pyrazole Insecticides. J Alzheimers Dis [Internet]. 2018 [cited 2023 Dec 12];62(4):1663–81. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7065497/.
37. Mossa ATH, Swelam ES, Mohafrash SMM. Sub-chronic exposure to fipronil induced oxidative stress, biochemical and histopathological changes in the liver and kidney of male albino rats. Toxicology Reports [Internet]. 2015 Jan 1 [cited 2023 Jan 10];2:775–84. Available from: https://www.sciencedirect.com/science/article/pii/S2214750015000281.
38. Kwak S, Cho YS, Na HG, Bae CH, Song SY, Kim YD. Fipronil upregulates inflammatory cytokines and MUC5AC expression in human nasal epithelial cells. Rhinology [Internet]. 2022 Apr 25 [cited 2023 Jan 10];58(1):66–73. Available from: https://www.rhinologyjournal.com/Abstract.php?id=2192.
39. Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL. γ-Aminobutyric Acid Inhibits T Cell Autoimmunity and the Development of Inflammatory Responses in a Mouse Type 1 Diabetes Model1. The Journal of Immunology [Internet]. 2004 Oct 15 [cited 2023 Jan 10];173(8):5298–304. https://doi.org/10.4049/jimmunol.173.8.5298.
40. Zhang B, Vogelzang A, Miyajima M, Sugiura Y, Wu Y, Chamoto K et al. B cell-derived GABA elicits IL-10+ macrophages to limit anti-tumour immunity. Nature [Internet]. 2021 Nov [cited 2023 Jan 10];599(7885):471–6. Available from: https://www.nature.com/articles/s41586-021-04082-1.
41. Zafar S, Jabeen I, Structure. Function, and Modulation of γ-Aminobutyric Acid Transporter 1 (GAT1) in Neurological Disorders: A Pharmacoinformatic Prospective. Frontiers in Chemistry [Internet]. 2018 [cited 2023 Jan 10];6. Available from: https://www.frontiersin.org/articles/https://doi.org/10.3389/fchem.2018.00397.
42. Ren W, Liu G, Yin J, Tan B, Wu G, Bazer FW et al. Amino-acid transporters in T-cell activation and differentiation. Cell Death Dis [Internet]. 2017 Mar [cited 2023 Jan 10];8(3):e2655–e2655. Available from: https://www.nature.com/articles/cddis2016222.
43. Sparrow EL, James S, Hussain K, Beers SA, Cragg MS, Bogdanov YD. Activation of GABA(A) receptors inhibits T cell proliferation. PLOS ONE [Internet]. 2021 May 20 [cited 2023 Jan 10];16(5):e0251632. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0251632.
44. Bugelski PJ, Kim C. T-Dependent Antigen Response (TDAR) Tests: Meta-Analysis of Results Generated Across Multiple Laboratories. Journal of Immunotoxicology [Internet]. 2007 Jan 1 [cited 2023 Jan 10];4(2):159–64. https://doi.org/10.1080/15476910701337126.
45. Morokata T, Ishikawa J, Yamada T. Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses. Immunology Letters [Internet]. 2000 May 1 [cited 2023 Jan 10];72(2):119–26. Available from: https://www.sciencedirect.com/science/article/pii/S0165247800001887.
46. Nohara K, Fujimaki H, Tsukumo S, ichi, Inouye K, Sone H, Tohyama C. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. Toxicology [Internet]. 2002 Mar 5 [cited 2023 Jan 10];172(1):49–58. Available from: https://www.sciencedirect.com/science/article/pii/S0300483X01005820.
47. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods [Internet]. 1983 Dec 16 [cited 2023 Jan 10];65(1):55–63. Available from: https://www.sciencedirect.com/science/article/pii/0022175983903034.

Publisher's Note

doi:10.6342/NTU202501708

Chemical Research in Toxicology

pubs.acs.org/crt

Article

Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes

Published as part of Chemical Research in Toxicology special issue "Women in Toxicology".

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Cite This: *Chem. Res. Toxicol.* 2024, 37, 1488−1500

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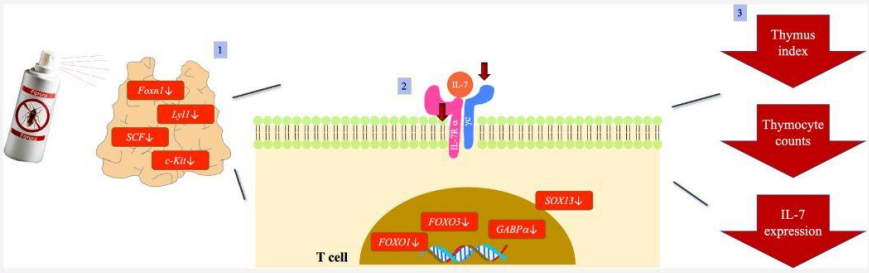

ABSTRACT: The susceptibility of the immune system to immunotoxic chemicals is evident, particularly in the thymus, a vital primary immune organ prone to atrophy due to exposure to toxicants. Fipronil (FPN), a widely used insecticide, is of concern due to its potential neurotoxicity, hepatotoxicity, and immunotoxicity. Our previous study showed that FPN disturbed the antigen-specific T-cell functionality *in vivo*. As T-cell lineage commitment and thymopoiesis are closely interconnected with the normal function of the T-cell-mediated immune responses, this study aims to further examine the toxic effects of FPN on thymocyte development. In this study, 4-week-old BALB/c mice received seven doses of FPN (1, 5, 10 mg/kg) by gavage. Thymus size, medulla/cortex ratio, total thymocyte counts, double-positive thymocyte population, and IL-7-positive cells decreased dose-dependently. IL-7 aids the differentiation of early T-cell precursors into mature T cells, and several essential genes contribute to the maturation of T cells in the thymus. *Foxn1* ensures that the thymic microenvironment is suitable for the maturation of T-cell precursors. *Lyl1* is involved in specifying lymphoid cells and maintaining T-cell development in the thymus. The *c-Kit/SCF* collaboration fosters a supportive thymic milieu to promote the formation of functional T cells. The expression of *IL-7*, *IL-7R*, *c-Kit*, *SCF*, *Foxn1*, and *Lyl1* genes in the thymus was significantly diminished in FPN-treated groups with the concordance with the reduction of IL-7 signaling proteins (IL-7, IL-7R, c-KIT, SCF, LYL1, FOXO3A, and GABPA), suggesting that the dysregulation of T-cell lineage-related genes may contribute to the thymic atrophy induced by FPN. In addition, FPN disturbed the functionality of thymocytes with an increase of IL-4 and IFN-γ production and a decrease of IL-2 secretion after T-cell mitogen stimulation *ex vivo*. Collectively, FPN significantly deregulated genes related to T-cell progenitor differentiation, survival, and expansion, potentially leading to impaired thymopoiesis.

■ INTRODUCTION

Fipronil (FPN) is a Class II moderately hazardous pesticide.[1−5] Its widespread use raised concerns regarding its impact on nontarget organisms and various organ systems. Cumulative pollution and toxicity have been found in the natural ecosystem as well as in beneficial insects like bees and dragonflies.[6] Accordingly, the European Union imposed a ban on its use in 2013.[7,8] Furthermore, FPN pesticides are also strictly prohibited for use in food-producing animals in the EU and other countries. Although FPN has been considered a low-toxicity pesticide, several studies have shown that FPN has neurotoxic, hepatotoxic, and reproductive effects on nontarget organisms, including mammals, birds, and aquatic species.[9−12]

Humans are exposed to FPN in a variety of ways, including occupational exposure (155/159 workers),[13] unintentional

Received: February 14, 2024
Revised: July 27, 2024
Accepted: August 5, 2024
Published: August 14, 2024

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

exposure (FPN and its metabolite can be detected within 25% of sampling serum, $n = 96$),[14−16] self-poisoning (6 cases),[17] or consumption of contaminated food or drinking water.[15,18−21] Approximately 40% of FPN residues in American households were detected through contact with pets treated with FPN-containing products.[14,22] With a surge in adverse outcome reports of pets treated with FPN, the U.S. Environmental Protection Agency (EPA) has intensified its examination of spot-on insecticides containing FPN. It has been reported that FPN could be rapidly absorbed through the gastrointestinal tract,[23,24] and its more toxic metabolite, FPN sulfone, exhibits a persistent accumulation in the body for up to 7 days in acute self-poisoning humans.[25] Fipronil sulfone can be detected in the serum of newborns, implying that if women are exposed to FPN during pregnancy, its metabolites can transfer through the placenta to newborn infants and lead to adverse effects on thyroid function and Apgar score.[26] Therefore, the toxic effects of exposure to FPN pesticides remain a significant public health concern and require mechanistic studies of their potential hazards to vertebrates.

The immune system displays heightened sensitivity to toxic responses induced by various chemicals, particularly affecting the thymus, which is prone to atrophy upon exposure to compounds like immunosuppressive drugs and environmental chemicals.[27−31] Serving as a vital immune organ, the thymus plays a pivotal role in coordinating the maturation, selection, and differentiation of the majority of naive T cells.[32] Despite its functionality declining with age, the thymus remains crucial for T-cell-repertoire reconstitution, ensuring immune responses in diverse situations until late adulthood.[28] Potential threats to immune function arise from the impact of compounds that induce atrophy on the thymus, making the thymus a sensitive indicator of the immunotoxicity of toxicants.

The process of T-cell lineage commitment necessitates collaboration between thymocytes and thymic epithelial cells (TECs) within the thymic microenvironment. This intricate interaction is tightly regulated by several transcription factors and the IL-7 signaling pathway.[33] In T-cell development, thymic IL-7, produced by TECs, binds to IL-7R on immature T lymphoid progenitor cells to further promote their proliferation, differentiation, and survival.[34,35] In addition, TECs may release stem cell factor (SCF) to drive thymocyte expansion at several stages through activation of Kit receptors. Disruption of Foxn1 may impede the maturation of TECs and indirectly result in the loss of intrathymic T-cell development and the manifestation of immunodeficiency.[36] *Lyl1* is involved in the regulation of lymphoid specification.[37] These genes play crucial roles in shaping the microenvironment required for T-cell development and facilitating progression through various stages of thymopoiesis.

Recent studies revealed the immunotoxic effects of FPN on mammals. The oral administration of 10% LD_{50} (9.7 mg/kg) FPN to rats resulted in histopathological alterations in the spleen and thymus tissues. Additionally, an increase in proinflammatory cytokines and antibodies in the serum suggested that FPN triggered allergic and inflammatory responses in male rats, concurrently impairing lymphocyte function.[38] In human lymphocytic Jurkat cells, FPN demonstrated a direct reduction in the synthesis of IL-2 and IFN-γ, indicating a potential direct impact on T cells even at noncytotoxic concentrations.[39] In our previous study, we demonstrated that FPN treatment disturbed antigen-specific immune responses through dysregulation of GABAergic genes *in vivo*.[40] Despite the disclosed adverse effects of FPN on the immune system, limited knowledge exists regarding how FPN modulates T-cell lineage commitment and maturation in the thymus. Regarding the adverse effects of FPN on mature T-cell function, this study aims to further examine the effects of FPN on thymocyte development *in vivo*.

MATERIALS AND METHODS

Reagents. Fipronil (FPN, 97%) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). RPMI 1640 medium (catalog no. SH30027.02) was purchased from Hyclone (UT). Fetal bovine serum (FBS, cat. no. 10437-028) and cell culture reagents were purchased from GIBCO BRL (MD) and GE Healthcare (Chicago, IL). Reagents for enzyme-linked immunosorbent assay (ELISA) analysis were provided by BD Biosciences (San Jose, CA). All other reagents were acquired from Sigma (MO) unless otherwise specified.

Experimental Animals. The BALB/c mice (3 weeks old; weighing 12−14 g) were supplied by the BioLASCO Experimental Animal Center (BioLASCO, Taipei, Taiwan). After their arrival, the mice were randomly assigned to groups and weighed for randomization. To minimize initial weight differences within each category, the mice were then categorized based on their combined weight into five groups. Individual housing was provided, maintaining controlled conditions, including a 12-h light/dark cycle, temperature (22 ± 2 °C), humidity (40 ± 15%), and unrestricted access to standard laboratory food and water *ad libitum*. Animal experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026).

Protocol of Animal Experiment. Following a 1-week acclimatization period, 4-week-old mice (5 animals/group) were randomly assigned to five groups, including no treatment groups (naïve; NA), vehicle control group (VH; corn oil), and oral gavage with fipronil (FPN) at doses of 1, 5, or 10 mg/kg suspended in corn oil for a total of seven doses (Figure 1). Based on previous studies, 10 mg/kg of

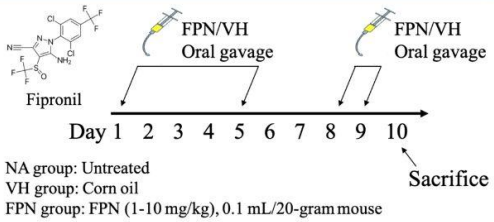

Figure 1. Protocol for fipronil (FPN) administration. Mice were randomly divided into the following groups: naïve (NA), vehicle-treated (VH), and FPN-treated group. The dosing regimen for FPN administration is described in Materials and Methods.

FPN (equivalent to 1/10 of the oral LD_{50} in mice) was chosen to minimize the risk of acute toxicity and mortality while still inducing subchronic toxic effects over a seven-dose treatment period.[1,40] The other doses of 1 mg/kg (1/100 LD_{50}) and 5 mg/kg (1/20 LD_{50}) were selected to demonstrate dose-related effects of FPN. On day 10, the mice were euthanized, and their thymus was harvested for studying the systemic immune responses. Since the mice needed to be monitored for clinical changes following exposure to FPN, the experimenter could not be blinded to whether the animals were exposed to FPN or corn oil.

Thymocyte Isolation and Culture. The thymus was aseptically removed from mice, washed, and then processed into a single-cell suspension. The culture medium was RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. In all cases, thymocytes were cultured

https://doi.org/10.1021/acs.chemrestox.4c00060
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doi:10.6342/NTU202501708

at 37 °C in 5% CO_2. Thymocyte counts were determined using a Moxi Z Mini Automated Cell Counter (ORFLO, ID).

Thymus Index. The thymus from each mouse (n = 20 in each group) was aseptically dissected and weighed immediately upon euthanasia. The thymus index was calculated as the thymus weight (mg) divided by the body weight (g) of the mouse.

Flow Cytometric Analysis for Cellularity of Thymocytes. The primary thymocytes were stained with rat antimouse CD4 conjugated with FITC (BD Biosciences, San Jose, CA), and/or rat antimouse CD8 conjugated with PE-Cy5 (BD Biosciences, San Jose, CA), and/or rat antimouse TCR$\alpha\beta$ conjugated with APC (BioLegend, San Diego, CA), and/or rat antimouse TCR$\gamma\delta$ conjugated with PE (BioLegend, San Diego, CA) antibodies in phosphate-buffered saline (PBS) containing 2% FBS. Appropriate rat antimouse antibodies were applied as the isotype control. For each sample, 10,000 events were collected and measured by a flow cytometer (BD FACSCalibur, San Jose, CA). Data was analyzed by Flowjo 10.4 software (FlowJo LLC, Ashland, OR).

Cytotoxicity Assay. The cytotoxicity of FPN to thymocytes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[41] Thymocytes (6×10^6 cells/mL) were cultured in triplicate (100 μL/well) in 96-well culture plates followed by stimulation with concanavalin A (ConA) for 44 h. Following stimulation, a stock solution of MTT (5 mg/mL) was added and incubated for 4 h. Subsequently, the resultant formazan was dissolved with the dimethyl sulfoxide (DMSO). The absorbance was read by an ELISA microplate reader (SpectraMax M5Microplate Reader, Molecular Devices LLC, San Jose, California) at an OD_{570nm}, with OD_{630nm} utilized as a background reference for accurate measurements.

Measurement of Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA). Thymocytes (6×10^6 cells/mL) were cultured in quadruplicate in a 48-well culture plate (0.3 mL/well). The culture supernatant with concanavalin A (ConA) stimulation (48 h) was collected to examine the level of IL-2, IL-4, and IFN-γ by ELISA kit (BD Biosciences, San Jose, CA) as previously described.[40] The optical density was measured at OD_{450nm} by using an ELISA microplate reader (SpectraMax M5Microplate Reader, Molecular Devices LLC, San Jose, CA).

RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR). Total RNA from thymus tissue and isolated thymocytes (stimulated by ConA for 24 h) were homogenized using TRIzol reagent and isolated using the GENEzol Pure Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), following the manufacturer's instructions. Subsequently, cDNA was prepared, and quantitative PCR (qPCR) was conducted as previously described.[40] Expression levels of target genes were determined by using the $\Delta\Delta C_t$ method and normalized to the HRPT mRNA content. The primers for the target genes used in this study are listed in Table 1.

Histological Examination. Formalin-fixed tissue sections of the thymus were subjected to hematoxylin and eosin (H&E) staining for histological evaluation. The slides were visualized using an optical microscope (ZIESS, Oberkochen, Germany). Morphometric analysis was performed to objectively measure alterations in the cortical and medullary sizes of the thymus. The ratio of cortex to medulla was calculated using the ImageJ image processing and analysis software (Bethesda, MD).

Immunohistochemical (IHC) Analysis. Tissue sections of the thymus were dewaxed, rehydrated, and then antigen-retrieved in Trilogy (Cell Marque, AR) at 121 °C for 15 min. The sections were separately incubated with ice methanol containing 3% H_2O_2 and blocked in PBS with 2.5% goat serum to reduce the endogenous peroxidase activity and nonspecific reactions. The slides were incubated with antimouse IL-7 antibody (ThermoFisher, MA; OriGene, MD) at 4 °C overnight and then treated with ImmPRESS HRP Goat Anti-Rabbit Polymer (Vector Laboratories, Burlingame, CA) for 30 min. For visualization, slides were incubated with the horseradish peroxidase (HRP) substrate 3,3′diaminobenzidine for 3–7 min followed by hematoxylin counterstaining for 1 min in the dark. The dark-brown positive signals were counted manually. All photos were captured using an optical microscope (ZIESS, Oberkochen, Germany).

Table 1. List of Quantitative PCR Primers

gene name	primers (5′ to 3′)
IL-7	F: TCTGCTGCCTGTCACATCATC
	R: GGACATTGAATTCTTCACTGATATTCA
IL-7 receptor	F: CACAGCCAGTTGGAAGTGGATG
	R: GGCATTTCACTCGTAAAAGAGCC
SCF	F: CCCTGAAGACTCGGGCCTA
	R: CAATTACAAGCGAAATGAGAGCC
c-Kit	F: GAGTTCCATAGACTCCAGCGTC
	R: AATGAGCAGCGGCGTGAACAGA
GABPα	F: CCGCTACACCGACTACGATT
	R: ACCTTCATCACCAACCCAAG
FOXO1	F: CTACGAGTGGATGGTGAAGAGC
	R: CCAGTTCCTTCATTCTGCACTCG
FOXO3	F: CCTACTTCAAGGATAAGGGCGAC
	R: GCCTTCATTCTGAACGCGCATG
*Foxn*1	F: TGACGGAGCACTTCCCTTAC
	R: GACAGGTTATGGCGAACAGAA
*Lyl*1	F: CAGCTAACTGCCTTGGGAAG
	R: CCAGCTCACTATGGCTTGGT
*SOX*13	F: GATGCCACCAACGCTAAAGC
	R: TTGCGGTTGAAGTCCAGGC
HPRT	F: TCAGTCAACGGGGGACATAAA
	R: GGGGCTGTACTGCTTAACCAG

Preparation of Thymus Protein Extracts and Western Blotting. Following the manufacturer's instructions, the thymus was harvested from each experimental group and homogenized in a mammalian cell lysis buffer supplemented with protease inhibitors (GoldBio, MO). Total protein was extracted and quantified using the bicinchoninic acid (BCA) protein assay (ThermoFisher, MA). Equal amounts of protein (40 μg) were loaded on 10–12% SDS-PAGE gels (polyacrylamide gel electrophoresis) and subsequently transferred to a poly(vinylidene fluoride) (PVDF) membrane using the protocol of the Trans-Blot Turbo Transfer System (Bio-Rad, München, Germany) with Trans-Blot Turbo RTA Transfer Packs. After being blocked with EveryBlot Blocking Buffer at room temperature, the PVDF membranes were incubated overnight at 4 °C with primary antibodies against IL-7 (ThermoFisher), IL-7R (Origene), SCF (ThermoFisher), c-KIT, FOXO3A, GABPA (Genetex, Hsinchu, Taiwan), LYL1 (ThermoFisher), or β-actin (Genetex). After washing with TBST buffer, the membranes were incubated for 1 h at room temperature with HRP-conjugated goat antirabbit secondary antibodies (Bio-Rad, CA). Following three washes with TBST, the protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad) on a Bio-Rad ChemiDoc XRS+ System. Densitometric analysis of the bands was performed using Bio-Rad Image Lab software with β-actin serving as loading control.

Statistical Analysis. Statistical analyses were conducted using GraphPad Prism version 9 software (GraphPad Software, Inc., La Jolla, CA). Data were expressed as the mean ± standard error mean (SEM) and were determined for each treatment group in individual experiments. To assess the impacts of FPN compared to the VH group, statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed t test. A p-value <0.05 was considered statistically significant. All analyses were carried out in a blinded fashion.

RESULTS

FPN Affected Body Weight, Thymus Index, and Population of Thymocytes *In Vivo*. After being administered corn oil (VH) or three different dosages of FPN (1, 5, 10 mg/kg) for a total of seven doses, mice showed no clinical

https://doi.org/10.1021/acs.chemrestox.4c00060
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doi:10.6342/NTU202501708

Table 2. Effects of FPN on Body Weight, Thymus Index, and Cellularity of Thymocytes[a,b,c,d]

			fipronil (mg/kg)		
	NA	VH	1	5	10
body weight					
day 1	17.5 ± 0.72	17.19 ± 0.54	17.52 ± 0.6	17.58 ± 0.54	17.01 ± 0.47
day 10	21.07 ± 0.64	20.24 ± 0.46	20.69 ± 0.44	20.56 ± 0.35	18.77 ± 0.25*
thymus index					
index[d]	3.82 ± 0.16	3.68 ± 0.13	3.26 ± 0.14*	3.06 ± 0.1*	2.81 ± 0.2*
total number ($\times 10^8$)[d]	1.392 ± 0.58	1.464 ± 0.74	1.217 ± 0.78	1.155 ± 0.64*	0.941 ± 0.6*
thymus cellularity (%)					
$CD4^+$	13.54 ± 0.32	12.61 ± 0.48	15.02 ± 0.17*	16.01 ± 0.24*	15.58 ± 0.31*
$CD8^+$	5.80 ± 0.35	6.06 ± 0.47	7.58 ± 0.11*	7.25 ± 0.20*	6.79 ± 0.28
$CD4^+/CD8^+$	76.61 ± 0.71	78.24 ± 0.93	73.84 ± 0.76*	70.98 ± 1.04*	71.18 ± 1.21*
$CD4^-/CD8^-$	3.70 ± 0.19	3.53 ± 0.15	3.73 ± 0.12	4.74 ± 0.28*	5.45 ± 0.32*
TCR α/β^+	34.58 ± 6.6	35 ± 7.08	34.85 ± 6.52	33.01 ± 7.26	34 ± 6.46
TCR γ/δ^+	0.59 ± 0.23	0.39 ± 0.08	0.3 ± 0.05	0.33 ± 0.07	0.4 ± 0.09
number of different subsets of thymocytes ($\times 10^6$)[d]					
$CD4^+$	17.94 ± 1.02	17.29 ± 1.34	18.28 ± 1.18	19.32 ± 1.22	15.85 ± 1.15
$CD8^+$	7.685 ± 0.43	8.309 ± 0.64	9.228 ± 0.59	8.749 ± 0.55	6.908 ± 0.5
$CD4^+/CD8^+$	101.5 ± 5.79	107.2 ± 8.32	89.9 ± 5.82	85.66 ± 5.44	72.42 ± 5.27*
$CD4^-/CD8^-$	4.902 ± 0.27	4.84 ± 0.37	4.541 ± 0.29	5.72 ± 0.36	5.545 ± 0.4
TCR α/β^+	45.81 ± 2.61	47.99 ± 3.72	42.42 ± 2.75	39.83 ± 2.53	34.59 ± 2.52*
TCR γ/δ^+	0.781 ± 0.044	0.5347 ± 0.041	0.365 ± 0.023*	0.398 ± 0.025*	0.407 ± 0.029*

[a]Data were expressed as mean ± SEM of triplicate samples pooled from four independent experiments ($N = 20$/group). *$p < 0.05$ as compared with the VH group. [b]Thymus index was calculated as the thymus weight (mg) per body weight (g). Data are expressed as mean ± SEM of 20 samples pooled from 4 independent experiments. [c]Thymocytes were prepared as described in the Materials and Methods section. The percentage of $CD4^+/CD8^+$, TCR α/β^+, and TCR γ/δ^+ cells was determined by flow cytometry. [d]Percent values and total number of thymus were used to calculate the total number of each cell population in the thymus.

signs or mortality. However, the group that received 10 mg/kg of FPN exhibited a remarkable weight reduction, and all FPN-treated groups displayed a significant decrease in the thymus index compared to that of the VH group. During FPN administration, the population of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ double-negative (DN) thymocytes significantly increased, while the proportion of $CD4^+/CD8^+$ double-positive (DP) cells decreased. Notably, the T-cell receptor (TCR) subunit percentages remained unaltered (Table 2). To avoid potential misinterpretations caused by evaluating FPN toxicity based on percentage changes alone, we also calculated absolute thymocyte counts and the number of different subsets of thymocytes. Our analysis revealed that in the 10 mg/kg FPN group, the absolute number of $CD4^+/CD8^+$ DP thymocytes was significantly reduced, aligning with the slight decrease in the absolute number of $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes. There was a slight increase in the number of $CD4^-/CD8^-$ DN thymocytes. Interestingly, although the proportion of TCR α/β^+ and TCR γ/δ^+ cells remained unchanged, the absolute cell numbers of TCR α/β^+ and TCR γ/δ^+ cells were decreased in FPN-treated groups.

FPN Leads to a Dose-Dependent Reduction in Thymocyte Numbers in Mice. We subsequently investigated the impact of FPN on the total number of thymocytes. In the FPN treatment groups, we observed a dose-dependent reduction in the total number of thymocytes, with a significant decrease noted in the 5 and 10 mg/kg groups. In the highest dose group (10 mg/kg), the total number of thymocytes reduced to only 64.3% (about 9.41×10^7 cells/thymus) compared with the VH control group (about 1.46×10^8 cells/thymus), representing a 35.7% decrease (Figure 2A).

Effects of the Cortex/Medulla Ratio and Thymus Atrophy. Histological analysis of thymic H&E sections included an assessment of the cortex/medulla ratio and overall thymic area ratio. The results revealed a significant decrease in the medullary proportion and a corresponding increase in the cortical proportion in the 5 mg/kg group (Figure 2B,C). Mice treated with 10 mg/kg FPN exhibited a marked decrease in thymocyte numbers and the overall thymic area proportions (the overall area reduced to approximately 80.06% compared to the VH group) without alteration of the cortex/medulla ratio.

FPN Significantly Attenuated mRNA Expression of IL-7 in the Thymus. The level of IL-7 mRNA was significantly decreased in the FPN treatment groups. Therefore, the IL-7 protein levels were further confirmed by immunohistochemistry. The analysis reveals that the number of IL-7 positive cells was significantly attenuated in both the cortex and medulla area at high-dose FPN treatment groups (Figure 3).

FPN Significantly Decreased mRNA Expression of Transcription Factors of T-Cell Lineage and IL-7 Signaling in the Thymus. The total mRNA of the thymus tissues was directly extracted for qPCR detection. The analysis reveals a significant decrease in the mRNA expression levels related to the IL-7 signaling pathway, including IL-7, IL-7R, GABPα, FOXO1, and FOXO3. Furthermore, in the key transcription factors essential for T-cell maturation and development, a downward trend in the expression levels of Foxn1, Lyl1, SCF, and c-Kit was observed (Figure 4).

Reduction of T-Cell Lineage Transcription Factors and IL-7 Signaling-Associated Proteins in the Thymus by FPN. Given the significant reduction in the expression of genes related to IL-7 signaling, we further investigated whether protein levels in the thymus were similarly affected by FPN. Consistent with the mRNA results, the protein levels of IL-7, IL-7R, GABPA, FOXO3A, SCF, c-KIT, and LYL1 were

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

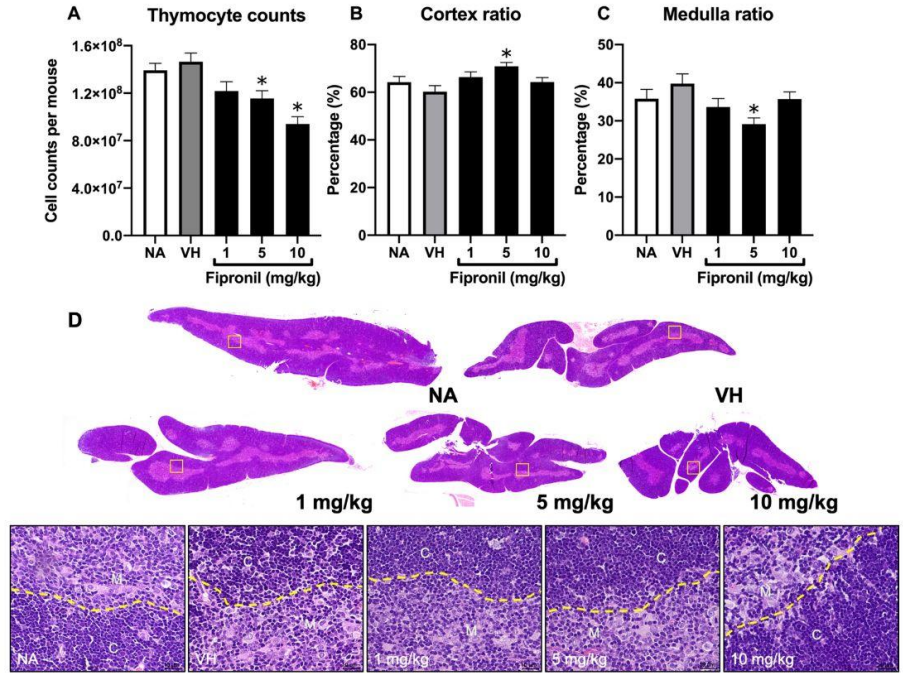

Figure 2. Reduction of thymocyte counts, thymus size, and medulla/cortex ratio by FPN. (A) Total thymocyte counts were isolated from each mouse and expressed as means ± SEM. (B, C) Ratio of cortex/medulla was quantified using ImageJ software as described in Materials and Methods. The cortex/medulla ratio was expressed as the mean ± SEM of thymus sections pooled from four independent experiments. *$p < 0.05$ as compared with the VH group. (D) Representative H&E-stained histological images of thymus sections in each treatment group were shown (spliced from original magnification 100×). The lower panels are enlarged images of the area of yellow boxes (original magnification, 400×). Areas marked by the dashed line distinguish the cortex or medulla regions.

reduced to varying degrees when normalized to the intensity of the VH group (Figure 5).

FPN Significantly Decreased mRNA Expression of IL-7R, *SCF*, *GABPα*, *Lyl*1, and *SOX*13 in ConA-Stimulated Thymocytes. The primary thymocytes were isolated and stimulated by ConA for 24 h to further assess mRNA expression of IL-7 signaling genes and transcription factors of the T-cell lineage, which are essential for the development and differentiation of T cells into mature T cells with specific functions in the immune system. The results showed that the mRNA expression of, *IL*-7 receptor, *SCF*, *GABPα*, Lyl1, and SOX13 was notably decreased in the high dose of FPN compared to the VH control (Figure 6).

Differential Effects of FPN on the Production of IL-2, IL-4, and IFN-γ *Ex Vivo*. The cytotoxic effect of FPN on thymocytes was assessed by using the MTT assay. We assessed the cytokine levels under ConA stimulation. ConA induces T-cell mitosis by binding to mannose and glucose residues on glycoproteins. These bindings lead to the cross-linking of cell surface receptors, activation of signaling pathways, calcium influx, and subsequent activation of transcription factors. These factors drive cell cycle progression, ultimately resulting in mitosis. There was a significant decrease in the production of IL-2, accompanied by an increase in both IL-4 and IFN-γ by FPN (Figure 7).

■ DISCUSSION

Extensive research has delved into the neurotoxic, reproductive, and cytotoxic effects induced by fipronil,[10,42−44] but studies of immunotoxicity specifically related to lymphocyte function are lacking. Administration of fipronil induces inflammatory responses, demonstrating immunotoxic effects in Wistar rats. After exposure to FPN for 30 days, the general architecture of the thymus was changed with the pale medulla and thick cortex, indicating the developing T lymphocytes were trapped in the outer cortex. Additionally, the corticomedullary junction appeared indistinct, with obvious aggregation of proteinaceous eosinophilic cells and numerous phagocytosed apoptotic bodies present in the medulla.[38] However, none of these studies have comprehensively investigated the effects of FPN on the T-cell functionality and its underlying mechanisms. In our previous study, we determined the immunotoxic effects of FPN on T-cell-mediated immune responses and demonstrated the interference of FPN with mature T-cell function.[40] In this study, we aim to study the deleterious impacts of FPN on thymic development and T-cell lineage commitment, thereby the young age BALB/c mice (4-week-old) were applied in this study.[45]

The thymus is one of the primary lymphoid organs that generates self-tolerant and immunocompetent T lymphocytes. Thymopoiesis is an intricate process that involves the

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

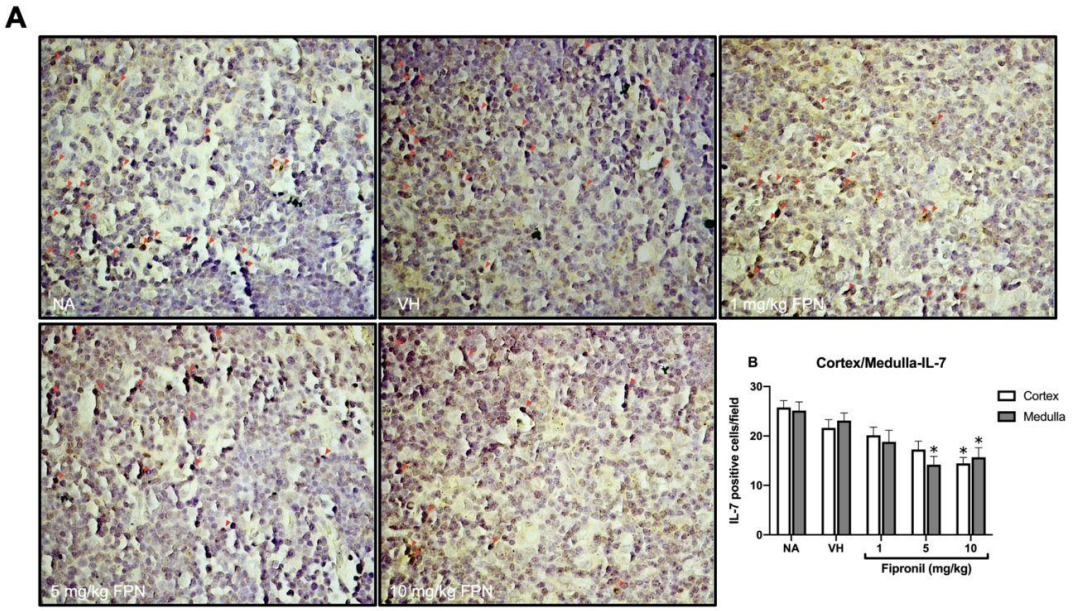

Figure 3. Fipronil significantly decreased IL-7-positive cells in the thymus. (A) Representative immunohistological images of thymus sections in each treatment group were shown (original magnification, 400×). Arrows indicate IL-7$^+$ cells with red signals. (B) Quantified data for the number of IL-7 positive cells from the cortex or medulla area were expressed as the mean ± SEM of 20 samples per group (N = 20/group). *p < 0.05 was significant compared to the VH group.

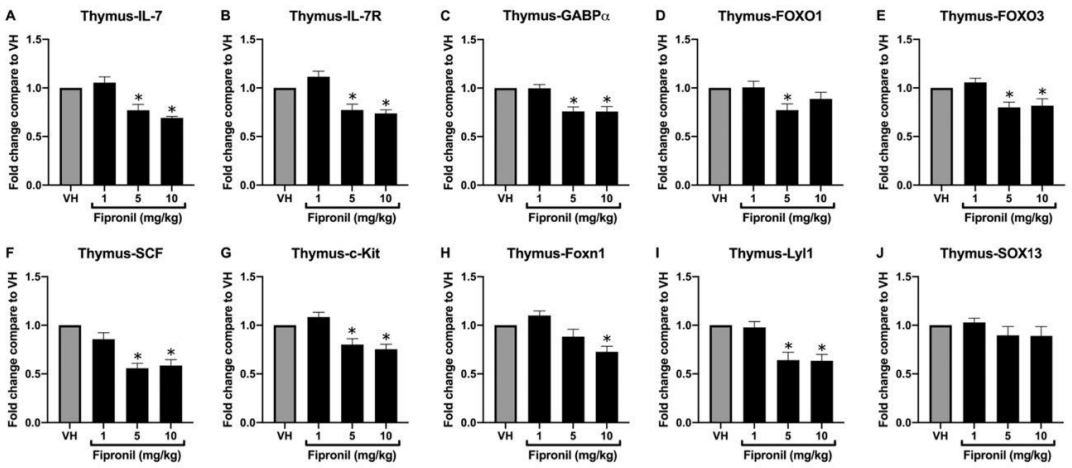

Figure 4. Fipronil significantly decreased the mRNA expression of transcription factors of T-cell lineage and IL-7 signaling in the thymus. The total RNA of the thymus harvested from different treatment groups was extracted to detect the mRNA expression of transcription factors of T-cell lineage and IL-7 signaling by qPCR. The expression level of *HPRT* was used as the control for semiquantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N = 20/group). *p < 0.05 was significant compared to the VH group.

maturation and differentiation of thymocytes (immature T cells) into functional and diverse T-cell subsets, which are tightly regulated by various signaling pathways, transcription factors, and interactions with stromal thymic stromal cells. The functionality of the thymus will remain developed for the whole lifetime, especially following hematopoietic cell stress, despite that thymus size will decline with age, named thymic involution (age-related atrophy).[28,46] Human thymic involution is thought to begin at 1 year of age, whereas murine thymic involution peaks at 4 weeks of age and gradually

https://doi.org/10.1021/acs.chemrestox.4c00060
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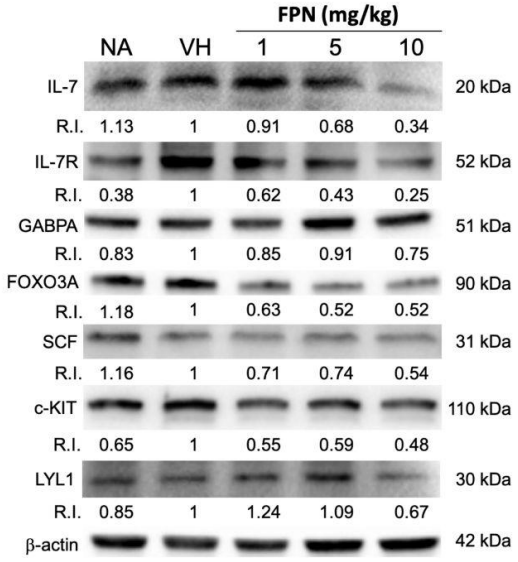

Figure 5. Fipronil markedly decreased T-cell lineage transcription factors and IL-7 signaling-associated proteins in the thymus. The total protein of the thymus harvested from different treatment groups was extracted to detect the protein expression of transcription factors of T-cell lineage and IL-7 signaling by Western blotting. The expression level of β-actin was used as the loading control for semiquantification. The different protein/β-actin ratio in the treatment group was divided by the protein/β-actin ratio in VH as the relative intensity (RI). The result was representative of three independent experiments (N = 9/group).

decreases thereafter.[47] The impacts of thymic atrophy are most profound in clinical conditions that result in severe loss of peripheral T cells that could contribute to a reduction of pathogen defense, a high incidence of autoimmune responses, and the attenuation of immunosurveillance.[48,49] The current data demonstrated that FPN induced thymus atrophy and downregulated important signaling pathways crucial for thymocyte selection and differentiation. To the best of our knowledge, this study represents the first investigation of the immunotoxic effects of FPN on thymic development through the dysregulation of essential development transcription factors and the IL-7-associated genes following short-term oral exposure.

Our results demonstrated that despite exposure to FPN (1−10 mg/kg), seven doses did not elicit severe clinical symptoms and there were significant changes in both thymic index and thymocyte cellularity. Intriguingly, the thymus index was notably decreased in each FPN treatment group, while the body weight gain was significantly decreased only in the 10 mg/kg FPN groups on day 10. These results suggest that the dosage of 10 mg/kg of FPN may have more pronounced toxicity in mice. The slight decrease in body weight could be linked to oxidative stress provoked by FPN (the administered dosage was ≤9.7 mg/kg (1/10 LD_{50})).[12] In parallel with the decline of the thymus index, the total number of thymocytes isolated from each mouse of FPN-treated groups significantly decreased in a dose-dependent manner. Histological analysis showed a reduction of the medulla−cortex ratio, suggesting atrophy in the medulla area of the thymus. In the 10 mg/kg FPN group, although the cortex/medulla ratio did not exhibit the most substantial difference, the total size of the thymus retained only about 80% consistent with a significant reduction of 35% of the total number of thymocytes, indicating an overall atrophy of the thymus.

Furthermore, based on the alteration of thymus cellularity in FPN-treated mice, there is a clear imbalance in the ratio of CD4/CD8 thymocyte population. Distribution confusion of the CD4/CD8 subsets was similarly verified in the absolute number from each cell population. The immature thymocytes were blocked in the $CD4^-/CD8^-$ double-negative (DN) stage, leading to the deficiency of the $CD4^+/CD8^+$ double-positive (DP) T-cell population. This data suggested that FPN-induced cell arrest in the DN stage without successfully transitioning to the DP stage may be due to the toxic effect of FPN on the early stage of thymocyte development. Interestingly, the proportion of CD4 and CD8 SP thymocytes remained unchanged or slightly increased during the FPN exposure. We speculate that thymocyte development led to an increase in the number of mature CD4 and CD8 SP thymocytes, which may be a compensatory response to early developmental disruptions. Collectively, these findings suggest the potential immune toxic effects of FPN on the thymus, accompanied by induction of thymus atrophy and disruption of DP thymocyte expansion.[50] The pre-TCR is expressed during the DN3 stage following TCRβ rearrangement, and subsequent TCRα rearrangement leads to the development of DP T cells. After the positive and negative selection processes, these cells are converted into CD4 or CD8 SP thymocytes. TCR integrity and signaling are crucial for thymocyte proliferation, development, and maturation.[51,52] Our results showed that the number of TCR α/β^+ and TCR γ/δ^+ cells was decreased in FPN-treated groups, indicating that FPN may downregulate the expansion of TCR α/β^+ and TCR γ/δ^+ cells during development.

The dynamic regulation of IL-7 signaling profoundly influences thymus and T-cell development. IL-7 is a fundamental requirement for each lymphocyte during its initial developmental stage.[53−56] During the DN stage, immature thymocytes experience their initial interaction with IL-7, which is essential for the survival of DN thymocytes to progress into the subsequent developmental stage.[57] IL-7Rα expression commences upon entry into the DN2 stage.[58] Experimental depletion of IL-7 through injections of IL-7 or IL-7R antibodies *in vivo* resulted in a profound reduction in overall thymocyte numbers (>99%).[59−61] Additionally, genetic deletion of IL-7, IL-7R, or proximal signaling molecules of IL-7R led to a severe defect in thymopoiesis and a developmental block at the DN3 stage. These data strongly support the indispensability of IL-7 for the survival of post-β-selection DN thymocytes.[59,61−63] Furthermore, initiating a STAT5-dependent opening of the TCR γ-chain locus for TCR rearrangement in $\gamma\delta$ T-cell development also necessitates IL-7 signaling.[54,55] This process has also been proposed for the TCR β-chain locus during $\alpha\beta$ T-cell development.[56] In our results, a series of genes and proteins related to IL-7 are significantly decreased by FPN exposure, suggesting that FPN-mediated immunodeficiency effects may be closely associated with the dysregulation of the IL-7 signaling pathway. Oral administration of tributyltin acetate resulted in a decrease in CD4 and CD8 SP T-cell populations and blocked the thymocyte differentiation at the DP and DN stages by down-regulating IL-7 mRNA in thymic epithelial cells.[64,65] During

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

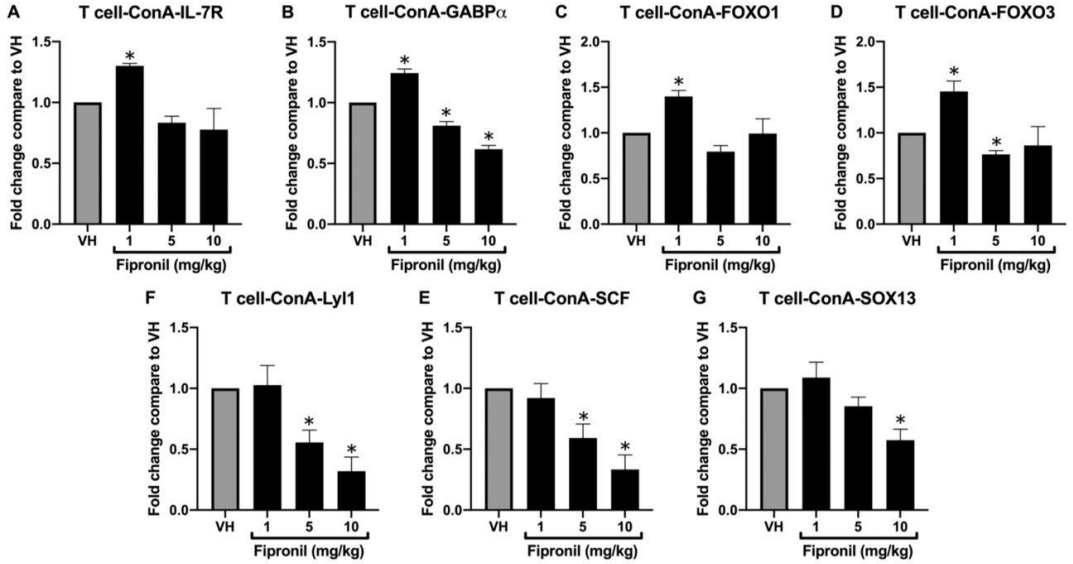

Figure 6. Fipronil significantly decreased mRNA expression of *Lyl*1, SOX13, SCF, IL-7*R*, and *GABPα* in ConA-stimulated thymocytes. The total RNA of thymocytes (6×10^6 cells/mL) harvested from different treatment groups stimulated by ConA was extracted to detect the mRNA expression of *Lyl*1, *SOX*13, *SCF*, and *IL-7* receptors by qPCR. The expression level of *HPRT* was used as the control for semiquantification. The expression level of *HPRT* was used as the control for semiquantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group ($N = 20$/group). *$p < 0.05$ was significant compared to the VH group.

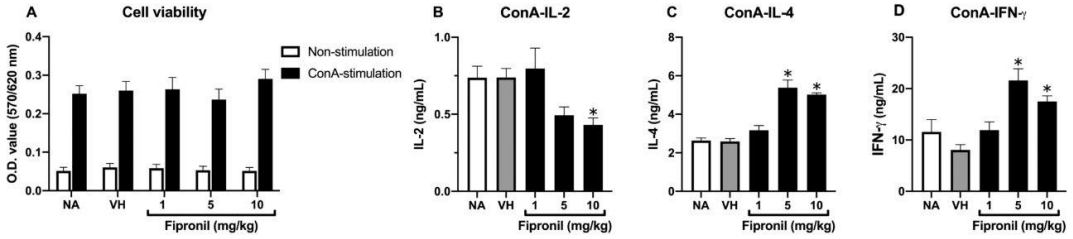

Figure 7. Fipronil exhibited an increase in IL-4 and IFN-γ and a decrease in IL-2 production stimulated by ConA. Thymocytes (6×10^6 cells/mL) were prepared from each group of mice and cultured in the absence or presence of concanavalin A (ConA; 5 μg/mL) for 48 h. The metabolic activity of cells was determined by an MTT assay, and the level of IL-2, IL-4, and IFN-γ in the supernatants was measured by ELISA. Data was expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments ($N = 20$/group). *$p < 0.05$ was significant compared to the VH group.

severe thymic atrophy induced by dexamethasone or irradiation, the regeneration of the thymus occurred through upregulation of IL-7 expression.[66] Collectively, these data suggest that the regulation of IL-7 plays a crucial role in compensating for different chemical stimuli. Disruption of crosstalk between thymic epithelial cells and thymocytes may lead to a reduction in the level of mature T-cell development.

Several transcription factors have already been recognized, whose interaction and cross-regulatory network might be associated with the IL-7Rα expression. Such GA-binding protein (GABP), the Ets family transcription factor, has been identified as essential for the upregulation of IL-7Rα in immature DN thymocytes.[67] Forkhead box O (*FOXO*) 1 transcription factor deficiency resulted in a severe defect in IL-7Rα expression.[68] *FOXO*1-deficient mice can develop a lethal inflammatory disorder and lead to an elevation in CD4 and CD8 single-positive (SP) thymocyte populations.[69,70] *FOXO*3*a*-deficient mice lead to mild lymphoproliferative syndrome and the formation of inflammatory lesions. Furthermore, *FOXO*3*a* deficiency will also develop a systemic and spontaneous autoimmune syndrome attributed to hyperactive NF-κB signaling in T cells.[71] These pieces of evidence suggest that the well-regulation of the FOXO family will direct the normal development of thymocytes in different stages associated with *IL-7R* expression. In the present study, the expression of *IL-7*, IL-7R, *GABPα*, *FOXO*1, and *FOXO*3 was decreased in thymus tissues of 5 and 10 mg/kg FPN-treated groups. Interestingly, only *GABPα* was dose-dependently decreased in ConA-stimulated thymocytes isolated from FPN-treated mice. After ConA stimulation, the expression of *IL-7R*, *FOXO*1, and *FOXO*3 by thymocytes was not altered in high-dose FPN groups, while it exhibited a slight or even

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

significant increase in the 1 mg/kg FPN group. We hypothesize that when mice are exposed to a lower dosage of FPN, a defense mechanism may be activated to counteract the potential immunotoxicity induced by FPN. However, the defense mechanism may become ineffective at higher dosages. Another speculation may be that these genes expressed by other parenchymal cells, such as thymic epithelial cells (TECs), in the thymus are more sensitive to FPN treatment than thymocytes.

A decrease in the IL-7 positive signals has been observed in high-dose groups (5 and/or 10 mg/kg of FPN) by IHC staining. Meanwhile, the Western blot results also showed a downward trend in the levels of key functional proteins, including IL-7, IL-7R, SCF, c-KIT, GABPA, FOXO3A, and LYL1, which is consistent with the observed mRNA trends. Given the reduction in IL-7 positive cells and the significant downregulation of IL-7 signaling-related genes and proteins, the proliferation, development, and T-cell lineage commitment are markedly impaired, ultimately resulting in immunodeficiency. Accordingly, we hypothesized that FPN progressively reduced expression levels of genes and proteins associated with IL-7 signaling, which was coregulated by downstream transcription factors. Therefore, the decline in these genes and proteins mainly results in a reduction in thymocyte numbers and a disruption of the thymic microenvironment. In our results, the decreased levels of *IL-7* and *FOXO*1 might also contribute to the slight activation of CD4 transcription, promoting an increased population of $CD4^+$ T cells.[69,72] Dysregulation of the CD4/CD8 ratio can lead to various immune dysregulations, impacting adaptive immune responses and potentially leading to immunodeficiency or autoimmune disorders.[48,49] Additionally, we propose that the immunotoxic effects of FPN may involve the regulation of apoptotic pathways by FOXO, such as the upregulation of Fas ligand (FasL) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), as well as pro-apoptotic/preapoptotic adjustment by Bcl-2 family member (Fu and Tindall, 2008). This speculation requires further research.

Besides IL-7 signaling genes, several transcription factors are essential to thymic development. The full growth and differentiation of TECs have relied on *Foxn*1 activation. *Foxn*1 also promotes the downstream transcription of genes implicated in thymus organogenesis.[73] In the absence of *Foxn*1 expression, the intrathymic lymphopoiesis of affected patients is completely blocked,[74,75] leading to severe primary T-cell immunodeficiency,[76−78] which is also observed on the $Foxn1^{-/-}$ mice model.[79] *Lyl*1 has been recognized as a critical component responsible for orchestrating lymphoid specification in multipotent bone marrow progenitors. Additionally, *Lyl*1 plays a vital role in sustaining the survival and expansion of thymic cell progenitors, particularly during the crucial stages of pro-T-cell expansion. Lack of *Lyl*1 in early T lineage progenitors and DN thymocyte progenitors exhibits in increased apoptosis, blocked differentiation, and impaired population expansion.[37,80] Stem cell factor (*SCF*) is produced by stromal cells and interacts with its ligand *c-Kit* expressed by DN thymocytes. Proper coordination between *SCF*, *c-Kit*, and IL-7 signaling pathways is essential for the progression of thymopoiesis and the production of functional T cells in the thymus.[34,35] In the present results, the mRNA expression of these transcription factors associated with T-cell progenitors' differentiation, survival, and expansion were significantly reduced by FPN. These findings highlight the immunotoxic effects of FPN, presumably resulting in the disorder of thymopoiesis. As FPN exhibits persistent bioaccumulation of a prolonged presence of FPN and its metabolites in the body,[38,81−83] exposure to FPN may contribute to a disturbance in thymic functionality and homeostasis.

Under the ConA stimulation *ex vivo*, the secretions of IL-2 were decreased, and the IL-4 and IFN-γ were increased, suggesting the disturbance of thymocyte function by FPN. The dysregulation of cytokine production can play a pivotal role in the development of immunodeficiency syndromes and various T-cell lymphoproliferative disorders. IL-2 regulates T-cell growth, proliferation, differentiation, and the maturation of different subsets of T cells in the thymus.[84] The absence of IL-2 can lead to conditions such as anemia, abnormal lymphoproliferation, and an inflammatory bowel disease akin to ulcerative colitis.[85] IL-4 may also influence T-cell maturation in the thymus. *In vivo*, overexpression of IL-4 has been associated with a reduction in the total number of immature thymocytes, accompanied by an increase in the number of mature $CD8^+$ thymocytes, mirroring the observed trends in the cellularity of $CD8^+$.[86,8786,87] Similar outcomes have been reported in IFN-γ transgenic mice, where CD4 or CD8 single-positive T-cell populations were elevated.[88] Additionally, the increased secretion of IFN-γ might be mediated by the decreased levels of the *FOXO*3*a* gene.[89] Our data showed a diminishment of IL-2 production with a disturbance of Th1 or Th2 cytokine production. We speculate that the thymus might be compensating for FPN-induced toxicity by accelerating the maturation process to maintain the SP thymocyte population. However, this accelerated maturation might lead to functional dysregulation in these rapidly matured thymocytes.

Survival, maturation, and trafficking of T cells in the thymus are regulated by the thymic hypersensitivity to glucocorticoids (GC).[90] High GC levels can induce T-cell apoptosis and have an immunosuppressive effect on T cells, potentially affecting T-cell selection and causing thymus atrophy.[27,91−93] Treatment of Wistar rats with 1/20 LD_{50} of FPN for 6 weeks significantly induced higher serum corticosterone levels (approximately 141.31 pg/mL),[94] which is the major stress hormone controlled by corticotropin-releasing hormone and adrenocorticotropic hormone in the Hypothalamus−Pituitary−Adrenal (HPA) axis. This potential immunotoxic mechanism of GC is related to oxidative damage.[94−96] GC acts by binding to the glucocorticoid receptor (GR), which is expressed by all thymocytes during their development, albeit at different levels in each CD4/CD8 subset 94,95. Despite $CD4^+/CD8+$ with the lowest GR level, they exhibit the highest sensitivity to GC-induced apoptosis 88,89. As FPN may induce corticosterone in serum, the elevated cortisol effects on thymus atrophy may be one of the potential mechanisms involved in FPN-induced thymus atrophy. Further studies are needed to evaluate how FPN regulates corticosterone levels within the thymus and to elucidate their roles in thymocyte development.

Although the acceptable daily intake (ADI) of FPN is 0.0002 mg/kg, a very conservative safety threshold for risk management of chronic exposure, in the real world, accidental or occupational exposure may occur at high doses. Cam et al. summarized different FPN exposure conditions and the serum level of the major FPN metabolite, fipronil sulfone, in human cases. In a self-poisoning case, the maximum fipronil and fipronil sulfone levels could reach 3.74 μg/mL.[25] In comparison to a pharmacokinetics study, the plasma levels of

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

FPN or fipronil sulfone concentration reached around 0.6 and 1.2 μg/mL, respectively, after a single oral dose of FPN (10 mg/kg).[83] As previous toxicology studies applied similar or higher doses to elucidate the effects of FPN on different biological systems, the present study included 1/100 to 1/10 of the oral LD_{50} (1−10 mg/kg) to minimize the risk of acute toxicity and mortality while still inducing subchronic toxic effects to demonstrate dose-dependent effects of FPN on thymopoiesis.[42,97] Collectively, our study may still be valid to provide scientific evidence for further evaluation of the immunotoxicity of FPN due to intentional or unintentional exposure.

■ CONCLUSIONS

This study demonstrated that oral exposure to FPN for seven doses induced thymic atrophy and altered both the thymic cellularity and absolute thymocyte numbers across different subpopulations. These immunotoxic effects are attributed to the dysregulation of genes and proteins involved in IL-7 signaling transduction as well as the impaired functionality of crucial transcription factors essential for thymocyte survival, thymic development, and T-cell lineage commitment. This study may open an avenue to investigate the immunotoxic effects of FPN on T-cell development. Combined with our previous research that FPN disturbed antigen-specific T-cell responses *in vivo*, the cumulative immunotoxicity of FPN needs to be given more attention.

■ ASSOCIATED CONTENT

Data Availability Statement

The original data employed or analyzed in this present study can be obtained from the corresponding author upon making a reasonable request.

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Author Contributions

J.-F.K. and C.-C.W. conceived and designed the experiments, analyzed the data, and wrote the manuscript. J.-F.K. performed the experiments and drafted the manuscript. H.-Y.W. and W.-H.H. consulted the experiments and tested the concept of this study. All authors read and approved the final manuscript. CRediT: **Jui-Fang Kuo** conceptualization, data curation, formal analysis, investigation, visualization, writing-original draft; **Hsin-Ying Wu** conceptualization, methodology; **Chun-Wei Tung** formal analysis, funding acquisition; **Wei-Hsiang Huang** formal analysis, resources, validation; **Chen-Si Lin** formal analysis, methodology; **Chia-Chi Wang** conceptualization, methodology, project administration, supervision, visualization, writing-review & editing.

Funding

This research was supported by the Ministry of Science and Technology (Taipei, Taiwan) under Grant MOST 106-2320-B-037-002, MOST 107-2320-B-002-065, MOST-110-2221-E-400-004-MY3, and NSTC-112-2321-B-400-003. The funders had no role in the design of the collection, analysis, and interpretation of data.

Notes

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026) and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with the ARRIVE 2.0 guidelines. The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Han-You Lin for providing the service of Agilent Technologies Mx3005P qPCR system and Dr. Hui-Wen Chen for providing the service of ChemiDoc XRS+ System, both from the School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan.

■ ABBREVIATIONS

FPN: fipronil
ConA: concanavalin A
TEC: thymic epithelial cells
ETP: early T lineage progenitors
SCF: stem cell factors
c-Kit: tyrosine-protein kinase KIT
GABPα: GA-binding protein α
FOXO: forkhead box-O
*Foxn*1: forkhead box protein N1
*Lyl*1: lymphoblastomic leukemia 1
*SOX*13: SRY-box transcription factor 13

■ REFERENCES

(1) Tingle, C. C. D.; Rother, J. A.; Dewhurst, C. F.; Lauer, S.; King, W. J. Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns. In *Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews*; Ware, G. W., Ed.; Springer: New York, NY, 2003; pp 1−66.

(2) Wu, J.; Lu, J.; Lu, H.; Lin, Y.; Chris Wilson, P. Occurrence and Ecological Risks from Fipronil in Aquatic Environments Located within Residential Landscapes. *Sci. Total Environ.* **2015**, *518*−*519*, 139−147.

(3) Gunasekara, A. S.; Truong, T.; Goh, K. S.; Spurlock, F.; Tjeerdema, R. S. Environmental Fate and Toxicology of Fipronil. *J. Pestic. Sci.* **2007**, *32* (3), 189−199.

(4) Ratra, G. S.; Casida, J. E. GABA Receptor Subunit Composition Relative to Insecticide Potency and Selectivity. *Toxicol. Lett.* **2001**, *122* (3), 215−222.

(5) Cole, L. M.; Nicholson, R. A.; Casida, J. E. Action of Phenylpyrazole Insecticides at the GABA-Gated Chloride Channel. *Pestic. Biochem. Physiol.* **1993**, *46* (1), 47−54.

doi:10.6342/NTU202501708

(6) Authority (EFSA). Conclusion Regarding the Peer Review of the Pesticide Risk Assessment of the Active Substance Fipronil. *EFSA J.* **2006**, *4* (5), 65r.
(7) EFSA assesses risks to bees from fipronil | EFSA. https://www.efsa.europa.eu/en/press/news/130527 (accessed December 18, 2023).
(8) Pisa, L. W.; Amaral-Rogers, V.; Belzunces, L. P.; Bonmatin, J. M.; Downs, C. A.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C.; Liess, M.; McField, M.; Morrissey, C. A.; Noome, D. A.; Settele, J.; Simon-Delso, N.; Stark, J. D.; Van der Sluijs, J. P.; Van Dyck, H.; Wiemers, M. Effects of Neonicotinoids and Fipronil on Non-Target Invertebrates. *Environ. Sci. Pollut. Res.* **2015**, *22* (1), 68−102.
(9) Stehr, C. M.; Linbo, T. L.; Incardona, J. P.; Scholz, N. L. The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae. *Toxicol. Sci.* **2006**, *92* (1), 270−278.
(10) Khan, S.; Jan, M. H.; Kumar, D.; Telang, A. G. Firpronil Induced Spermotoxicity Is Associated with Oxidative Stress, DNA Damage and Apoptosis in Male Rats. *Pestic. Biochem. Physiol.* **2015**, *124*, 8−14.
(11) Yu, F.; Wang, Z.; Ju, B.; Wang, Y.; Wang, J.; Bai, D. Apoptotic Effect of Organophosphorus Insecticide Chlorpyrifos on Mouse Retina in Vivo via Oxidative Stress and Protection of Combination of Vitamins C and E. *Exp. Toxicol. Pathol.* **2008**, *59* (6), 415−423.
(12) Mossa, A.-T. H.; Swelam, E. S.; Mohafrash, S. M. M. Sub-Chronic Exposure to Fipronil Induced Oxidative Stress, Biochemical and Histopathological Changes in the Liver and Kidney of Male Albino Rats. *Toxicol. Rep.* **2015**, *2*, 775−784.
(13) Herin, F.; Boutet-Robinet, E.; Levant, A.; Dulaurent, S.; Manika, M.; Galatry-Bouju, F.; Caron, P.; Soulat, J.-M. Thyroid Function Tests in Persons with Occupational Exposure to Fipronil. *Thyroid* **2011**, *21* (7), 701−706.
(14) Jennings, K. A.; Canerdy, T.; Keller, R.; Atieh, B.; Doss, R.; Gupta, R. Human Exposure to Fipronil from Dogs Treated with Frontline. *Vet. Hum. Toxicol.* **2002**, *44*, 301−303.
(15) Chen, D.; Li, J.; Zhao, Y.; Wu, Y. Human Exposure of Fipronil Insecticide and the Associated Health Risk. *J. Agric. Food Chem.* **2022**, *70* (1), 63−71.
(16) McMahen, R. L.; Strynar, M. J.; Dagnino, S.; Herr, D. W.; Moser, V. C.; Garantziotis, S.; Andersen, E. M.; Freeborn, D. L.; McMillan, L.; Lindstrom, A. B. Identification of Fipronil Metabolites by Time-of-Flight Mass Spectrometry for Application in a Human Exposure Study. *Environ. Int.* **2015**, *78*, 16−23.
(17) Leghait, J.; Gayrard, V.; Picard-Hagen, N.; Camp, M.; Perdu, E.; Toutain, P.-L.; Viguié, C. Fipronil-Induced Disruption of Thyroid Function in Rats Is Mediated by Increased Total and Free Thyroxine Clearances Concomitantly to Increased Activity of Hepatic Enzymes. *Toxicology* **2009**, *255* (1), 38−44.
(18) van der Sluijs, J. P.; Amaral-Rogers, V.; Belzunces, L. P.; Bijleveld van Lexmond, M. F. I. J.; Bonmatin, J.-M.; Chagnon, M.; Downs, C. A.; Furlan, L.; Gibbons, D. W.; Giorio, C.; Girolami, V.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C.; Liess, M.; Long, E.; McField, M.; Mineau, P.; Mitchell, E. A. D.; Morrissey, C. A.; Noome, D. A.; Pisa, L.; Settele, J.; Simon-Delso, N.; Stark, J. D.; Tapparo, A.; Van Dyck, H.; van Praagh, J.; Whitehorn, P. R.; Wiemers, M. Conclusions of the Worldwide Integrated Assessment on the Risks of Neonicotinoids and Fipronil to Biodiversity and Ecosystem Functioning. *Environ. Sci. Pollut. Res.* **2015**, *22* (1), 148−154.
(19) Bonneau, S.; Reymond, N.; Gupta, S.; Navarro, C. Efficacy of a Fixed Combination of Permethrin 54.5% and Fipronil 6.1% (Effitix) in Dogs Experimentally Infested with Ixodes Ricinus. *Parasites Vectors* **2015**, *8* (1), No. 204.
(20) Dumont, P.; Liebenberg, J.; Beugnet, F.; Fankhauser, B. Repellency and Acaricidal Efficacy of a New Combination of Fipronil and Permethrin against Ixodes Ricinus and Rhipicephalus Sanguineus Ticks on Dogs. *Parasites Vectors* **2015**, *8* (1), No. 51.
(21) He, X.; Chen, J.; Li, X.; Wang, J.; Xin, M.; Sun, X.; Cao, W.; Wang, B. Pollution Status, Influencing Factors and Environmental Risks of Neonicotinoids, Fipronil and Its Metabolites in a Typical Semi-Closed Bay in China. *Environ. Pollut.* **2021**, *291*, No. 118210.
(22) Lee, S.-J.; Mulay, P.; Diebolt-Brown, B.; Lackovic, M. J.; Mehler, L. N.; Beckman, J.; Waltz, J.; Prado, J. B.; Mitchell, Y. A.; Higgins, S. A.; Schwartz, A.; Calvert, G. M. Acute Illnesses Associated with Exposure to Fipronil—Surveillance Data from 11 States in the United States, 2001−2007. *Clin. Toxicol.* **2010**, *48* (7), 737−744.
(23) World Health Organization. Pesticide Residues in Food: 2021: Toxicological Evaluations: Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, Virtual Meeting, 6−17 September, 4 and 7 October 2021. 2022.
(24) California Environmental Protection Agency. FIPRONIL RISK CHARACTERIZATION DOCUMENT. 2023.
(25) Mohamed, F.; Senarathna, L.; Percy, A.; Abeyewardene, M.; Eaglesham, G.; Cheng, R.; Azher, S.; Hittarage, A.; Dissanayake, W.; Sheriff, M. R.; Davies, W.; Buckley, N.; Eddleston, M. Acute Human Self-Poisoning with the N-Phenylpyrazole Insecticide Fipronil − A GABAA-Gated Chloride Channel Blocker. *J. Toxicol. Clin. Toxicol.* **2004**, *42* (7), 955−963.
(26) Kim, Y. A.; Yoon, Y. S.; Kim, H. S.; Jeon, S. J.; Cole, E.; Lee, J.; Kho, Y.; Cho, Y. H. Distribution of Fipronil in Humans, and Adverse Health Outcomes of in Utero Fipronil Sulfone Exposure in Newborns. *Int. J. Hyg. Environ. Health* **2019**, *222* (3), 524−532.
(27) Ashwell, J. D.; Lu, F. W. M.; Vacchio, M. S. Glucocorticoids in T Cell Development and Function. *Annu. Rev. Immunol.* **2000**, *18* (1), 309−345.
(28) Shanker, A. Is Thymus Redundant after Adulthood? *Immunol. Lett.* **2004**, *91* (2), 79−86.
(29) Drela, N. Xenobiotic-Induced Alterations in Thymocyte Development. *APMIS* **2006**, *114* (6), 399−419.
(30) Zoller, A. L.; Kersh, G. J. Estrogen Induces Thymic Atrophy by Eliminating Early Thymic Progenitors and Inhibiting Proliferation of β-Selected Thymocytes1. *J. Immunol.* **2006**, *176* (12), 7371−7378.
(31) Nohara, K.; Ao, K.; Miyamoto, Y.; Suzuki, T.; Imaizumi, S.; Tateishi, Y.; Omura, S.; Tohyama, C.; Kobayashi, T. Arsenite-Induced Thymus Atrophy Is Mediated by Cell Cycle Arrest: A Characteristic Downregulation of E2F-Related Genes Revealed by a Microarray Approach. *Toxicol. Sci.* **2008**, *101* (2), 226−238.
(32) Ladi, E.; Yin, X.; Chtanova, T.; Robey, E. A. Thymic Microenvironments for T Cell Differentiation and Selection. *Nat. Immunol.* **2006**, *7* (4), 338−343.
(33) Thapa, P.; Farber, D. L. The Role of the Thymus in the Immune Response. *Thorac. Surg. Clin.* **2019**, *29* (2), 123−131.
(34) Chung, B.; Min, D.; Joo, L. W.; Krampf, M. R.; Huang, J.; Yang, Y.; Shashidhar, S.; Brown, J.; Dudl, E. P.; Weinberg, K. I. Combined Effects of Interleukin-7 and Stem Cell Factor Administration on Lymphopoiesis after Murine Bone Marrow Transplantation. *Biol. Blood Marrow Transplant.* **2011**, *17* (1), 48−60.
(35) Politikos, I.; Kim, H. T.; Nikiforow, S.; Li, L.; Brown, J.; Antin, J. H.; Cutler, C.; Ballen, K.; Ritz, J.; Boussiotis, V. A. IL-7 and SCF Levels Inversely Correlate with T Cell Reconstitution and Clinical Outcomes after Cord Blood Transplantation in Adults. *PLoS One* **2015**, *10* (7), No. e0132564.
(36) Žuklys, S.; Handel, A.; Zhanybekova, S.; Govani, F.; Keller, M.; Maio, S.; Mayer, C. E.; Teh, H. Y.; Hafen, K.; Gallone, G.; Barthlott, T.; Ponting, C. P.; Holländer, G. A. Foxn1 Regulates Key Target Genes Essential for T Cell Development in Postnatal Thymic Epithelial Cells. *Nat. Immunol.* **2016**, *17* (10), 1206−1215.
(37) Zohren, F.; Souroullas, G. P.; Luo, M.; Gerdemann, U.; Imperato, M. R.; Wilson, N. K.; Gottgens, B.; Lukov, G. L.; Goodell, M. A. Lyl1 Regulates Lymphoid Specification and Maintenance of Early T Lineage Progenitors. *Nat. Immunol.* **2012**, *13* (8), 761−769.
(38) Aldayel, T. S.; Abdel-Rahman, H. G.; Gad EL-Hak, H. N.; Abdelrazek, H. M. A.; Mohamed, R. M.; El-Sayed, R. M. Assessment of Modulatory Activity of Uncaria Tomentosa Extract against Fipronil Immunotoxicity in Male Rats. *Ecotoxicol. Environ. Saf.* **2021**, *224*, No. 112674.

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

(39) Sidiropoulou, E.; Sachana, M.; Hargrreaves, A. J.; Woldehiwet, Z. In *Immunotoxic Properties of Pesticides: Effects of Diazinon-Oxon and Fipronil on Lymphocytic Jurkat Cells*, Front. Pharmacol. Conference Abstract: 8th Southeast European Congress on Xenobiotic Metabolism and Toxicity—XEMET 2010, 2010.
(40) Kuo, J.-F.; Cheng, Y.-H.; Tung, C.-W.; Wang, C.-C. Fipronil Disturbs the Antigen-Specific Immune Responses and GABAergic Gene Expression in the Ovalbumin-Immunized BALB/c Mice. *BMC Vet. Res.* **2024**, *20* (1), No. 30.
(41) Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65* (1), 55−63.
(42) Badgujar, P. C.; Pawar, N. N.; Chandratre, G. A.; Telang, A. G.; Sharma, A. K. Fipronil Induced Oxidative Stress in Kidney and Brain of Mice: Protective Effect of Vitamin E and Vitamin C. *Pestic. Biochem. Physiol.* **2015**, *118*, 10−18.
(43) Gupta, S. K.; Pal, A. K.; Sahu, N. P.; Jha, A. K.; Akhtar, M. S.; Mandal, S. C.; Das, P.; Prusty, A. K. Supplementation of Microbial Levan in the Diet of Cyprinus Carpio Fry (Linnaeus, 1758) Exposed to Sublethal Toxicity of Fipronil: Effect on Growth and Metabolic Responses. *Fish Physiol. Biochem.* **2013**, *39* (6), 1513−1524.
(44) Wang, X.; Martínez, M. A.; Wu, Q.; Ares, I.; Martínez-Larrañaga, M. R.; Anadón, A.; Yuan, Z. Fipronil Insecticide Toxicology: Oxidative Stress and Metabolism. *Crit. Rev. Toxicol.* **2016**, *46* (10), 876−899.
(45) Bano, F.; Mohanty, B. Thyroxine Modulation of Immune Toxicity Induced by Mixture Pesticides Mancozeb and Fipronil in Mice. *Life Sci.* **2020**, *240*, No. 117078.
(46) Shanley, D. P.; Aw, D.; Manley, N. R.; Palmer, D. B. An Evolutionary Perspective on the Mechanisms of Immunosenescence. *Trends Immunol.* **2009**, *30* (7), 374−381.
(47) Sutherland, J. S.; Goldberg, G. L.; Hammett, M. V.; Uldrich, A. P.; Berzins, S. P.; Heng, T. S.; Blazar, B. R.; Millar, J. L.; Malin, M. A.; Chidgey, A. P.; Boyd, R. L. Activation of Thymic Regeneration in Mice and Humans Following Androgen Blockade. *J. Immunol.* **2005**, *175* (4), 2741−2753.
(48) Liang, Z.; Dong, X.; Zhang, Z.; Zhang, Q.; Zhao, Y. Age-related Thymic Involution: Mechanisms and Functional Impact. *Aging Cell* **2022**, *21* (8), No. e13671.
(49) Velardi, E.; Tsai, J. J.; van den Brink, M. R. M. T Cell Regeneration after Immunological Injury. *Nat. Rev. Immunol.* **2021**, *21* (5), 277−291.
(50) Gruver, A. L.; Sempowski, G. D. Cytokines, Leptin, and Stress-Induced Thymic Atrophy. *J. Leukocyte Biol.* **2008**, *84* (4), 915−923.
(51) Carpenter, A. C.; Bosselut, R. Decision Checkpoints in the Thymus. *Nat. Immunol.* **2010**, *11* (8), 666−673.
(52) Cui, Z.; Zhao, F.; Chen, X.; Li, J.; Jin, X.; Han, Y.; Wang, L.; Zhou, Y.; Lu, L. NPAT Supports CD8+ Immature Single-Positive Thymocyte Proliferation and Thymic Development. *J. Immunol.* **2022**, *209* (5), 916−925.
(53) Corcoran, A. E.; Riddell, A.; Krooshoop, D.; Venkitaraman, A. R. Impaired Immunoglobulin Gene Rearrangement in Mice Lacking the IL-7 Receptor. *Nature* **1998**, *391* (6670), 904−907.
(54) Durum, S. K.; Candèias, S.; Nakajima, H.; Leonard, W. J.; Baird, A. M.; Berg, L. J.; Muegge, K. Interleukin 7 Receptor Control of T Cell Receptor γ Gene Rearrangement: Role of Receptor-Associated Chains and Locus Accessibility. *J. Exp. Med.* **1998**, *188* (12), 2233−2241.
(55) Maki, K.; Sunaga, S.; Ikuta, K. The V−J. Recombination of T Cell Receptor-γ Genes Is Blocked in Interleukin-7 Receptor−Deficient Mice. *J. Exp. Med.* **1996**, *184* (6), 2423−2428.
(56) Muegge, K.; Vila, M. P.; Durum, S. K. Interleukin-7: A Cofactor For V(D)J. Rearrangement of the T Cell Receptor β Gene. *Science* **1993**, *261* (5117), 93−95.
(57) Vicente, R.; Swainson, L.; Marty-Gres, S.; de Barros, S.; Kinet, S.; Zimmermann, V. S.; Taylor, N. Molecular and Cellular Basis of T Cell Lineage Commitment. *Semin. Immunol.* **2010**, *22* (5), 270−275.
(58) Yu, Q.; Erman, B.; Park, J.-H.; Feigenbaum, L.; Singer, A. IL-7 Receptor Signals Inhibit Expression of Transcription Factors TCF-1, LEF-1, and RORγt. *J. Exp. Med.* **2004**, *200* (6), 797−803.
(59) Bhatia, S. K.; Tygrett, L. T.; Grabstein, J. H.; Waldschmidt, T. J. The Effect of in Vivo IL-7 Deprivation on T Cell Maturation. *J. Exp. Med.* **1995**, *181* (4), 1399−1409.
(60) Grabstein, K. H.; Waldschmidt, T. J.; Finkelman, F. D.; Hess, B. W.; Alpert, A. R.; Boiani, N. E.; Namen, A. E.; Morrissey, P. J. Inhibition of Murine B and T Lymphopoiesis in Vivo by an Anti-Interleukin 7 Monoclonal Antibody. *J. Exp. Med.* **1993**, *178* (1), 257−264.
(61) Sudo, T.; Nishikawa, S.; Ohno, N.; Akiyama, N.; Tamakoshi, M.; Yoshida, H.; Nishikawa, S. Expression and Function of the Interleukin 7 Receptor in Murine Lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (19), 9125−9129.
(62) Peschon, J. J.; Morrissey, P. J.; Grabstein, K. H.; Ramsdell, F. J.; Maraskovsky, E.; Gliniak, B. C.; Park, L. S.; Ziegler, S. F.; Williams, D. E.; Ware, C. B.; Meyer, J. D.; Davison, B. L. Early Lymphocyte Expansion Is Severely Impaired in Interleukin 7 Receptor-Deficient Mice. *J. Exp. Med.* **1994**, *180* (5), 1955−1960.
(63) von Freeden-Jeffry, U.; Vieira, P.; Lucian, L. A.; McNeil, T.; Burdach, S. E. G.; Murray, R. Lymphopenia in Interleukin (IL)-7 Gene-Deleted Mice Identifies IL-7 as a Nonredundant Cytokine. *J. Exp. Med.* **1995**, *181* (4), 1519−1526.
(64) Im, E.; Kim, H.; Kim, J.; Lee, H.; Yang, H. Tributyltin Acetate-Induced Immunotoxicity Is Related to Inhibition of T Cell Development in the Mouse Thymus. *Mol. Cell. Toxicol.* **2015**, *11* (2), 231−239.
(65) da Silva, R. C.; Teixeira, M. P.; de Paiva, L. S.; Miranda-Alves, L. Environmental Health and Toxicology: Immunomodulation Promoted by Endocrine-Disrupting Chemical Tributyltin. *Toxics* **2023**, *11* (8), 696.
(66) Zubkova, I.; Mostowski, H.; Zaitseva, M. Up-Regulation of IL-7, Stromal-Derived Factor-1α, Thymus-Expressed Chemokine, and Secondary Lymphoid Tissue Chemokine Gene Expression in the Stromal Cells in Response to Thymocyte Depletion: Implication for Thymus Reconstitution1. *J. Immunol.* **2005**, *175* (4), 2321−2330.
(67) Xue, H.-H.; Bollenbacher, J.; Rovella, V.; Tripuraneni, R.; Du, Y.-B.; Liu, C.-Y.; Williams, A.; McCoy, J. P.; Leonard, W. J. GA Binding Protein Regulates Interleukin 7 Receptor α-Chain Gene Expression in T Cells. *Nat. Immunol.* **2004**, *5* (10), 1036−1044.
(68) Kerdiles, Y. M.; Beisner, D. R.; Tinoco, R.; Dejean, A. S.; Castrillon, D. H.; DePinho, R. A.; Hedrick, S. M. Foxo1 Links Homing and Survival of Naive T Cells by Regulating L-Selectin, CCR7 and Interleukin 7 Receptor. *Nat. Immunol.* **2009**, *10* (2), 176−184.
(69) Gubbels Bupp, M. R.; Edwards, B.; Guo, C.; Wei, D.; Chen, G.; Wong, B.; Masteller, E.; Peng, S. L. T Cells Require Foxo1 to Populate the Peripheral Lymphoid Organs. *Eur. J. Immunol.* **2009**, *39* (11), 2991−2999.
(70) Ouyang, W.; Liao, W.; Luo, C. T.; Yin, N.; Huse, M.; Kim, M. V.; Peng, M.; Chan, P.; Ma, Q.; Mo, Y.; Meijer, D.; Zhao, K.; Rudensky, A. Y.; Atwal, G.; Zhang, M. Q.; Li, M. O. Novel Foxo1-Dependent Transcriptional Programs Control Treg Cell Function. *Nature* **2012**, *491* (7425), 554−559.
(71) Lin, L.; Hron, J. D.; Peng, S. L. Regulation of NF-κB, Th Activation, and Autoinflammation by the Forkhead Transcription Factor Foxo3a. *Immunity* **2004**, *21* (2), 203−213.
(72) Winer, H.; Rodrigues, G. O. L.; Hixon, J. A.; Aiello, F. B.; Hsu, T. C.; Wachter, B. T.; Li, W.; Durum, S. K. IL-7: Comprehensive Review. *Cytokine* **2022**, *160*, No. 156049.
(73) Romano, R.; Palamaro, L.; Fusco, A.; Giardino, G.; Gallo, V.; Del Vecchio, L.; Pignata, C. FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program. *Front. Immunol.* **2013**, *4*, No. 187.
(74) Nehls, M.; Kyewski, B.; Messerle, M.; Waldschütz, R.; Schüddekopf, K.; Smith, A. J. H.; Boehm, T. Two Genetically Separable Steps in the Differentiation of Thymic Epithelium. *Science* **1996**, *272* (5263), 886−889.

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

(75) Blackburn, C. C.; Augustine, C. L.; Li, R.; Harvey, R. P.; Malin, M. A.; Boyd, R. L.; Miller, J. F.; Morahan, G. The Nu Gene Acts Cell-Autonomously and Is Required for Differentiation of Thymic Epithelial Progenitors. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93* (12), 5742−5746.
(76) Amorosi, S.; D'Armiento, M.; Calcagno, G.; Russo, I.; Adriani, M.; Christiano, A.; Weiner, L.; Brissette, J.; Pignata, C. FOXN1 Homozygous Mutation Associated with Anencephaly and Severe Neural Tube Defect in Human Athymic Nude/SCID Fetus. *Clin. Genet.* **2008**, *73* (4), 380−384.
(77) Cunningham-Rundles, C.; Ponda, P. P. Molecular Defects in T- and B-Cell Primary Immunodeficiency Diseases. *Nat. Rev. Immunol.* **2005**, *5* (11), 880−892.
(78) Frank, J.; Pignata, C.; Panteleyev, A. A.; Prowse, D. M.; Baden, H.; Weiner, L.; Gaetaniello, L.; Ahmad, W.; Pozzi, N.; Cserhalmi-Friedman, P. B.; Aita, V. M.; Uyttendaele, H.; Gordon, D.; Ott, J.; Brissette, J. L.; Christiano, A. M. Exposing the Human Nude Phenotype. *Nature* **1999**, *398* (6727), 473−474.
(79) Müller, S. M.; Ege, M.; Pottharst, A.; Schulz, A. S.; Schwarz, K.; Friedrich, W. Transplacentally Acquired Maternal T Lymphocytes in Severe Combined Immunodeficiency: A Study of 121 Patients. *Blood* **2001**, *98* (6), 1847−1851.
(80) Zohren, F.; Souroullas, G. P.; Luo, M.; Gerdemann, U.; Imperato, M. R.; Wilson, N. K.; Göttgens, B.; Lukov, G. L.; Goodell, M. A. The Transcription Factor Lyl-1 Regulates Lymphoid Specification and the Maintenance of Early T Lineage Progenitors. *Nat. Immunol.* **2012**, *13* (8), 761−769.
(81) Romero, A.; Ramos, E.; Ares, I.; Castellano, V.; Martínez, M.; Martínez-Larrañaga, M. R.; Anadón, A.; Martínez, M. A. Fipronil Sulfone Induced Higher Cytotoxicity than Fipronil in SH-SY5Y Cells: Protection by Antioxidants. *Toxicol. Lett.* **2016**, *252*, 42−49.
(82) Song, X.; Wang, X.; Liao, G.; Pan, Y.; Qian, Y.; Qiu, J. Toxic Effects of Fipronil and Its Metabolites on PC12 Cell Metabolism. *Ecotoxicol. Environ. Saf.* **2021**, *224*, No. 112677.
(83) Cam, M.; Durieu, E.; Bodin, M.; Manousopoulou, A.; Koslowski, S.; Vasylieva, N.; Barnych, B.; Hammock, B. D.; Bohl, B.; Koch, P.; Omori, C.; Yamamoto, K.; Hata, S.; Suzuki, T.; Karg, F.; Gizzi, P.; Erakovic Haber, V.; Bencetic Mihaljevic, V.; Tavcar, B.; Portelius, E.; Pannee, J.; Blennow, K.; Zetterberg, H.; Garbis, S. D.; Auvray, P.; Gerber, H.; Fraering, J.; Fraering, P. C.; Meijer, L. Induction of Amyloid-B42 Production by Fipronil and Other Pyrazole Insecticides. *J. Alzheimer's Dis.* **2018**, *62* (4), 1663−1681.
(84) Tentori, L.; Longo, D. L.; Zuniga-Pflucker, J. C.; Wing, C.; Kruisbeek, A. M. Essential Role of the Interleukin 2-Interleukin 2 Receptor Pathway in Thymocyte Maturation in Vivo. *J. Exp. Med.* **1988**, *168* (5), 1741−1747.
(85) Sadlack, B.; Merz, H.; Schorle, H.; Schimpl, A.; Feller, A. C.; Horak, I. Ulcerative Colitis-like Disease in Mice with a Disrupted Interleukin-2 Gene. *Cell* **1993**, *75* (2), 253−261.
(86) Carty, S. A.; Koretzky, G. A.; Jordan, M. S. Interleukin-4 Regulates Eomesodermin in CD8+ T Cell Development and Differentiation. *PLoS One* **2014**, *9* (9), No. e106659.
(87) Smiley, S. T.; Grusby, M. J. Interleukin 4. In *Encyclopedia of Immunology*, 2nd ed.; Delves, P. J., Ed.; Elsevier: Oxford, 1998; pp 1451−1453.
(88) Young, H. A.; Klinman, D. M.; Reynolds, D. A.; Grzegorzewski, K. J.; Nii, A.; Ward, J. M.; Winkler-Pickett, R. T.; Ortaldo, J. R.; Kenny, J. J.; Komschlies, K. L. Bone Marrow and Thymus Expression of Interferon-γ Results in Severe B-Cell Lineage Reduction, T-Cell Lineage Alterations, and Hematopoietic Progenitor Deficiencies. *Blood* **1997**, *89* (2), 583−595.
(89) Kerdiles, Y. M.; Stone, E. L.; Beisner, D. L.; McGargill, M. A.; Ch'en, I. L.; Stockmann, C.; Katayama, C. D.; Hedrick, S. M. Foxo Transcription Factors Control Regulatory T Cell Development and Function. *Immunity* **2010**, *33* (6), 890−904.
(90) Salehzadeh, M.; Soma, K. K. Glucocorticoid Production in the Thymus and Brain: Immunosteroids and Neurosteroids. *Brain, Behav., Immun.: Health* **2021**, *18*, No. 100352.
(91) Deobagkar-Lele, M.; Chacko, S. K.; Victor, E. S.; Kadthur, J. C.; Nandi, D. Interferon-γ- and Glucocorticoid-Mediated Pathways Synergize to Enhance Death of CD4+ CD8+ Thymocytes during Salmonella Enterica Serovar Typhimurium Infection. *Immunology* **2013**, *138* (4), 307−321.
(92) Roggero, E.; Perez, A. R; Tamae-Kakazu, M.; Piazzon, I.; Nepomnaschy, I.; Besedovsky, H. O; Bottasso, O. A; del Rey, A. Endogenous Glucocorticoids Cause Thymus Atrophy but Are Protective during Acute Trypanosoma Cruzi Infection. *J. Endocrinol.* **2006**, *190*, 495−503.
(93) Yan, F.; Mo, X.; Liu, J.; Ye, S.; Zeng, X.; Chen, D. Thymic Function in the Regulation of T Cells, and Molecular Mechanisms Underlying the Modulation of Cytokines and Stress Signaling (Review). *Mol. Med. Rep.* **2017**, *16*, 7175.
(94) Mahmoud, Y. K.; Ali, A. A.; Abdelrazek, H. M. A.; Aldayel, T. S.; Abdel-Daim, M. M.; El-Menyawy, M. A. I. Neurotoxic Effect of Fipronil in Male Wistar Rats: Ameliorative Effect of L-Arginine and L-Carnitine. *Biology* **2021**, *10* (7), 682.
(95) Gallo-Payet, N.; Battista, M.-C. Steroidogenesis—Adrenal Cell Signal Transduction. In *Comprehensive Physiology*; John Wiley & Sons, Ltd, 2014; pp 889−964.
(96) Prevatto, J. P.; Torres, R. C.; Diaz, B. L.; e Silva, P. M. R.; Martins, M. A.; Carvalho, V. F. Antioxidant Treatment Induces Hyperactivation of the HPA Axis by Upregulating ACTH Receptor in the Adrenal and Downregulating Glucocorticoid Receptors in the Pituitary. *Oxid. Med. Cell. Longevity* **2017**, *2017* (1), No. 4156361.
(97) Koslowski, S.; Latapy, C.; Auvray, P.; Blondel, M.; Meijer, L. Long-Term Fipronil Treatment Induces Hyperactivity in Female Mice. *Int. J. Environ. Res. Public Health* **2020**, *17* (5), 1579.

https://doi.org/10.1021/acs.chemrestox.4c00060
Chem. Res. Toxicol. 2024, 37, 1488−1500

doi:10.6342/NTU202501708

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Article

Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes

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Abstract: Fipronil (FPN), a widely used pesticide, is associated with significant immunotoxic effects, particularly impacting thymocyte survival and immune homeostasis. This study explores the mechanistic pathways underlying FPN-induced apoptosis and oxidative stress. Short-term FPN exposure (1–10 mg/kg) notably suppressed the expression of both anti-apoptotic (*Bcl-2*, *Bcl-6*, *Mcl-1*) and pro-apoptotic (*Bnip3*, *Bim*) genes in thymic tissues in vivo. Additionally, in isolated primary thymocytes, FPN directly decreased the expression of *Bcl-2*, *Bcl-6*, *Mcl-1*, and *Bnip3* expression, coupled with a significant increase in pro-apoptotic *Bim* expression in a dose-dependent manner. FPN treatment directly led to elevated reactive oxygen species (ROS), lipid peroxidation, mitochondrial membrane depolarization, reduced cellular metabolic activity, and depleted intracellular calcium and glutathione (GSH) levels, indicating mitochondrial dysfunction and oxidative stress. Annexin V/PI staining confirmed that FPN induced late-stage apoptosis and necrosis in primary thymocytes. These findings elucidate the immunotoxic effects of FPN on thymocytes, highlighting its detrimental impact on immune system integrity, thymic development, and T cell maturation through oxidative damage and mitochondrial-mediated apoptosis.

Keywords: fipronil; immunotoxicity; apoptosis; BCL-2 family; reactive oxygen species; mitochondrial membrane potential; glutathione; lipid peroxidation

Academic Editor: Joshua R. Edwards

Received: 26 January 2025
Revised: 5 March 2025
Accepted: 11 March 2025
Published: 12 March 2025

Citation: Kuo, J.-F.; Hsiao, Y.-P.; Wang, Y.-D.; Weng, H.-P.; Wang, C.-C. Fipronil Triggers Immunotoxicity Through Reactive Oxygen Species-Driven Mitochondrial Apoptosis in Thymocytes. *Toxics* **2025**, *13*, 204. https://doi.org/10.3390/toxics13030204

1. Introduction

Fipronil (FPN) is known for its high specificity towards insect γ-aminobutyric acid ($GABA_A$) receptors. The widespread use of FPN has consequently raised significant concerns about its harmful effects on non-target organisms. The contamination of water and soil by FPN and its metabolites poses an important and pressing public health and environmental concern [1–3]. FPN has been shown to cause significant mortality in aquatic organisms such as fish and amphibians, leading to disruptions in aquatic ecosystems [4]. Furthermore, evidence has shown that FPN exerts neurotoxic, hepatotoxic, and reproductive toxic effects on vertebrates [4–12]. Together, these findings underscore urgent public health concerns stemming from its unanticipated toxic effects.

The thymus serves as the primary lymphoid organ, integral to the maturation of T-cells and the establishment of immunological tolerance. It plays a pivotal role in the positive selection, ensuring T cells that recognize self-MHC molecules are retained, and in the negative selection, eliminating autoreactive T cells to prevent autoimmunity. Disruption of thymic homeostasis can significantly impair systemic immune function, increasing susceptibility to immunodeficiencies and autoimmune disorders. Our previous studies demonstrated

doi:10.6342/NTU202501708

that FPN induces immunotoxic effects in mature and immature T cells. FPN disrupts antigen-specific immune responses through the modulation of GABAergic signaling [13]. FPN has been shown to induce thymic atrophy and thymocyte depletion by modulating the IL-7 signaling pathway, a critical regulator of thymic development, and by disrupting the expression of transcription factors essential for T-cell survival and lineage commitment [14]. These findings strongly suggest that FPN exerts potent immunotoxic effects, potentially leading to immunosuppression. This highlights the critical need for further investigation into the precise mechanisms by which FPN impacts thymocyte homeostasis.

Apoptosis, or programmed cell death, is a key mechanism in sustaining cellular homeostasis, tissue integrity, and immune regulation [15]. The thymus removes dysfunctional or autoreactive thymocytes, thereby preserving immune tolerance and systemic integrity [16,17]. This mechanism depends on the regulation of the mitochondrial apoptotic pathway by the BCL-2 protein family. Anti-apoptotic BCL-2 family members, including BCL-2 and MCL-1, maintain mitochondrial integrity by inhibiting the pro-apoptotic proteins BAX and BAK. This inhibition prevents cytochrome *c* release and the subsequent activation of caspases. Conversely, pro-apoptotic proteins like Bim and bNIP3 facilitate mitochondrial membrane permeabilization in response to various cellular stressors [18]. Dysregulation of these pathways during thymic development can severely compromise immune homeostasis, potentially leading to immunodeficiencies or autoimmune conditions.

Prior research has demonstrated that FPN causes oxidative stress and a variety of off-target toxicities in animals, mostly due to an imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to oxidative damage [6,8,19–22]. This stress compromises the antioxidant defense system, generating hydroxyl radicals that cause damage to cellular macromolecules [6,23]. Mice were orally administered FPN at 10 mg/kg (approximately 10% of the LD_{50}) for 28 days. The liver, brain, and kidney tissues showed an apparent decline in the expression of antioxidant genes and a notable reduction in the activity of antioxidant enzymes including glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) as a result of this exposure. FPN-induced lipid peroxidation was mitigated by supplementation with vitamin E or vitamin C [24,25]. These findings suggest that prolonged exposure to FPN induces oxidative stress, with lipid peroxidation likely serving as a key mechanism behind the oxidative damage observed. Meanwhile, ROS is crucial for T cell activation, expansion, and effector function interface [26–28]. Since thymocyte development relies heavily on ROS homeostasis [29], we focused on determining whether FPN exposure induces oxidative stress in thymocytes and exploring the underlying mechanisms of cell death.

Despite growing evidence of FPN's broad-spectrum toxicity, the specific effects of FPN on thymocyte apoptosis and oxidative stress remain unclear. This study first investigates whether FPN alters apoptosis-regulating genes in vivo. Considering the continuous renewal and elimination of thymocytes within the thymus in vivo, it is challenging to directly observe the effects of toxicants on thymocyte death. Subsequently, in vitro experiments are conducted to explore the direct toxic effects of FPN on thymocyte death in the presence of the antioxidant N-acetylcysteine (NAC). This study aims to elucidate the complex interplay between oxidative stress and apoptosis in thymocytes, offering critical insights that could inform strategies to mitigate the immunotoxic effects of FPN exposure.

2. Materials and Methods

2.1. Chemicals and Reagents

Fipronil (FPN, 97%) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). RPMI 1640 medium (Cat. No. SH30027.02) was sourced from HyClone (Logan, UT, USA). Fetal bovine serum (FBS, Cat. No. 10437-028) and other cell culture reagents were

doi:10.6342/NTU202501708

provided by GIBCO BRL (Gaithersburg, MD, USA) and GE Healthcare (Chicago, IL, USA). Reagents for ELISA analysis were supplied by BD Biosciences (San Jose, CA, USA). All other reagents, unless otherwise specified, were obtained from Sigma (St. Louis, MO, USA).

2.2. Experimental Animals

Male BALB/c mice (3 weeks old, approximately 12–14 g body weight) were obtained from the BioLASCO Experimental Animal Center (BioLASCO, Taipei, Taiwan). Upon arrival, the mice were stratified by body weight to reduce initial weight variation and then randomly assigned to five groups. Each mouse was housed individually under controlled environmental conditions (temperature: 20–25 °C, humidity: 50–60%, 12-h light/dark cycle) with unrestricted access to food and water ad libitum. All animal experiments complied with the ethical guidelines of the Institutional Animal Care and Use Committee of National Taiwan University (IACUC Approval No: NTU108-EL-00026).

2.3. Protocol of Sub-Chronic Animal Experiment and Preparation of Thymocytes from Mice Thymus

Four-week-old mice (five animals per group) were randomly assigned to one of five groups after one week of acclimatization (Figure 1). The highest dose of FPN, 10 mg/kg (equivalent to 1/10 of the oral LD_{50} in mice), was chosen based on previous studies to minimize the risk of acute toxicity and mortality while inducing sub-chronic toxic effects during the seven-dose treatment period [9,13]. To investigate dose-dependent effects, lower doses of 1 mg/kg (equivalent to 1/100 of the LD_{50}) and 5 mg/kg (equivalent to 1/20 of the LD_{50}) were conducted.

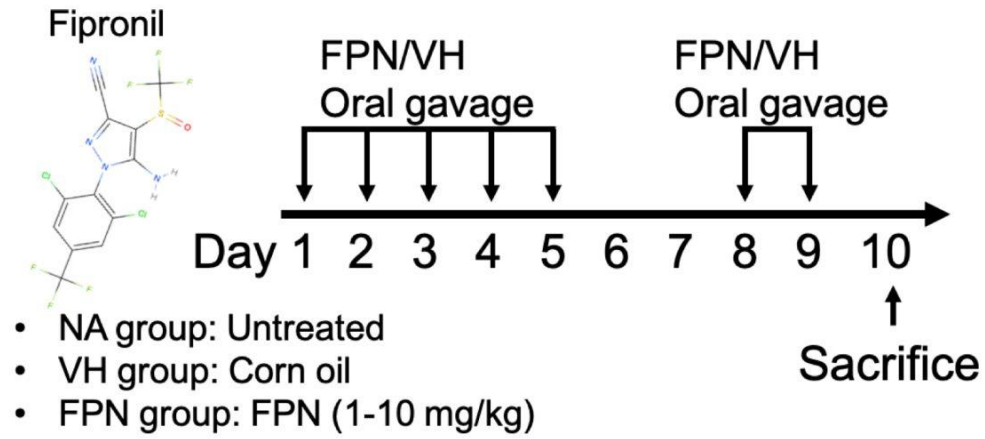

Figure 1. Protocol for fipronil (FPN) administration.

Following euthanasia, the thymus was harvested to assess oxidative stress. Each thymus was rapidly excised, mechanically dissociated, and processed into a single-cell suspension. The cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FBS in a 37 °C incubator with 5% CO_2.

The mice were randomly divided into the naïve (NA), vehicle-treated (VH), and FPN-treated groups. The dosing regimen for FPN administration is described in Materials and Methods.

2.4. RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

Total mRNA was extracted from thymus tissue and isolated thymocytes (stimulated with or without ConA for 24 h). The samples were homogenized using TRIzol reagent and

doi:10.6342/NTU202501708

purified with the GENEzol Pure Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the manufacturer's protocol. The extracted mRNA was then reverse-transcribed into cDNA, which was subsequently used for quantitative PCR (qPCR) as described previously. Gene expression levels were quantified using the $\Delta\Delta C_t$ method, with normalization to *Hprt* mRNA levels. The primers used for target gene analysis are listed in Table 1.

Table 1. List of quantitative PCR primers.

Gene Name	Primers (5′ to 3′)
Bcl-2	F: CCTGTGGATGACTGAGTACCTG R: AGCCAGGAGAAATCAAACAGAGG
Mcl-1	F: AGCTTCATCGAACCATTAGCAGAA R: CCTTCTAGGTCCTGTACGTGGA
Bcl-6	F: CAGAGATGTGCCTCCATACTGC R: CTCCTCAGAGAAACGGCAGTCA
Bnip3	F: GCTCCAAGAGTTCTCACTGTGAC R: GTTTTTCTCGCCAAAGCTGTGGC
Bim	F: GGAGATACGGATTGCACAGGAG R: CTCCATACCAGACGGAAGATAAAG
Hprt	F: TCAGTCAACGGGGGACATAAA R: GGGGCTGTACTGCTTAACCAG

2.5. Evaluation of Mitochondrial Function

The mitochondrial metabolic activity of primary thymocytes following FPN treatment was analyzed using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Thymocytes were seeded into a 96-well plate for 24 h. To assess cellular metabolism, the treated thymocytes were incubated with an MTT stock solution (5 mg/mL), enabling the conversion of MTT into formazan crystals, which served as an indicator of mitochondrial function. The purple formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was recorded to determine cell viability [16]. After incubation, the absorbance was measured at 570 nm using an ELISA microplate reader (SpectraMax® M5 Microplate Reader, Molecular Devices LLC, San Jose, CA, USA), with 630 nm used as a background reference to ensure accurate quantification.

2.6. Evaluation of Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

Thymocytes were isolated and cultured at a concentration of 5×10^6 cells/mL in 48-well plates, with 0.3 mL of cell suspension per well. Cultures were performed in quadruplicate. The cells were stimulated with PMA/Iono for 24 h. Following incubation, cell culture supernatants were collected, and IL-2 levels were quantified using a commercially available ELISA kit (BD Biosciences, San Jose, CA, USA). Optical density was measured at 450 nm using a SpectraMax® M5 Microplate Reader (Molecular Devices LLC, San Jose, CA, USA).

2.7. Assessment of Apoptotic/Necrotic Indicators

Apoptosis in primary thymocytes following FPN treatment was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Dojindo, Kumamoto, Japan) following the manufacturer's protocol. A total of 1 × 106 primary thymocytes were harvested, resuspended in 100 µL of the staining solution, and incubated for 15 min at room temperature in the dark. After staining, flow cytometry (BD FACSCalibur, San Jose, CA, USA) was performed to analyze apoptosis, using a 488 nm laser for excitation. FITC fluorescence was detected through a 515 nm bandpass filter, while PI fluorescence was recorded using a filter > 600 nm.

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2.8. Measurement of Mitochondrial Depolarization

The mitochondrial membrane potential ($\Delta\psi m$) of primary thymocytes was evaluated using the JC-1 detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The JC-1 stock solution (2.5 mg/mL) was prepared in DMSO and diluted to prepare the working solution. Cells were harvested and incubated in the dark with the JC-1 working solution for 30 min at 37 °C. Fluorescence signals (red aggregates and green monomers) were measured using a flow cytometer.

2.9. Measurement of Intracellular Calcium

The intracellular calcium of primary thymocytes was evaluated using the Fluo-4 AM detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The Fluo-4 AM stock solution was prepared in DMSO and diluted to prepare the working solution (final concentration: 1 μM). Cells were harvested and incubated in the dark with the Fluo-4 working solution containing 0.02% of the non-ionic detergent Pluronic ®F-127 for 30 min at 37 °C. Following incubation, excess Fluo-4 dye was removed by washing the cells, and calcium levels were subsequently analyzed using a flow cytometer.

2.10. Detection of Glutathione (GSH) Activity

Primary thymocytes' intracellular glutathione (GSH) levels were measured using the fluorescent probe 5-chloromethyl fluorescein diacetate (CMFDA; CellTracker Green, Invitrogen). A CMFDA stock solution (10 mM) was prepared in DMSO. After harvesting, the cells were centrifuged to remove the supernatant, and the pellet was resuspended in the CMFDA working solution. The cells were incubated in the dark at 37 °C for 30 min. Following incubation, excess CMFDA dye was removed by washing the cells, and GSH levels were subsequently analyzed using a flow cytometer.

2.11. Quantification of Intracellular ROS Levels

The levels of intracellular ROS were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA; Invitrogen). The FPN-treated primary thymocytes were incubated with 5 μM H_2-DCFDA for 30 min at 37 °C. Following incubation, excess dye was removed by washing with warm PBS, and the cells were analyzed using a flow cytometer.

2.12. Evaluation of Lipid Peroxidation (LPO)

Lipid peroxidation in FPN-treated thymocytes was quantified using the C11-Bodipy$^{581/591}$ fluorescent probe (Cayman Chemical, Ann Arbor, MI, USA). This sensor specifically detects LPO by undergoing oxidation in the presence of intracellular lipid peroxides, emitting a distinct bright green fluorescence signal that indicates oxidative stress. The cells were incubated with the working solution (final concentration: 20 μM) at 37 °C for 30 min in the dark. Post-incubation, fluorescence changes reflecting LPO levels were analyzed by flow cytometry.

2.13. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as mean ± standard error of the mean (SEM) for each treatment group in individual experiments. To determine the effects of FPN compared to the vehicle control (VH) group, the data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. A p-value < 0.05 was considered statistically significant. All analyses were conducted in a blinded manner.

doi:10.6342/NTU202501708

3. Results

3.1. Deregulation of BCL-2 Protein Family Gene Expression by FPN In Vivo, Ex Vivo, and In Vitro

3.1.1. FPN Significantly Attenuated Bcl-2 Family mRNA Expression in the Thymus

Our previous study demonstrated that sub-chronic exposure to FPN induced severe thymic atrophy in mice, accompanied by a substantial reduction in total thymocyte count. To further investigate the underlying mechanisms, we analyzed the expression of genes involved in the regulation of apoptosis and T-cell development. Total mRNA was extracted from thymus tissues to determine the expression levels of critical *Bcl-2* family genes involved in apoptosis regulation by qPCR, including the anti-apoptotic genes *Bcl-2*, *Bcl-6*, and *Mcl-1*, as well as the intrinsic pathway-associated pro-apoptotic gene *Bnip3* and *Bim* (Figure 2A–E). Following FPN administration, a significant reduction was observed in the mRNA expression of *Bcl-2*, *Mcl-1*, and *Bcl-6*, implicating a weakened anti-apoptotic defense. Interestingly, the expression of the pro-apoptotic gene *Bim* and *Bnip3* was significantly reduced as well.

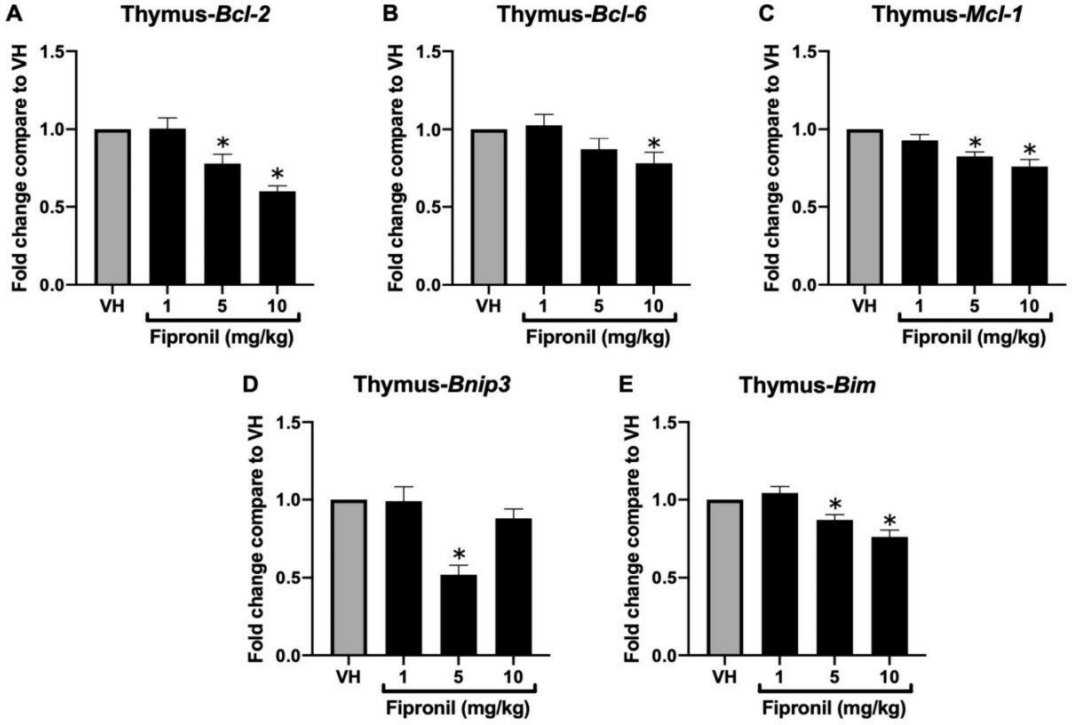

Figure 2. Fipronil significantly decreased mRNA expression of the *Bcl-2* family of thymus in vivo. The total mRNA harvested from different thymus treatment groups was extracted to detect the mRNA expression by qPCR. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N = 20/group). * $p < 0.05$ was significant compared to the VH group.

3.1.2. FPN Significantly Attenuated Bcl-2 Family mRNA Expression in ConA-Stimulated Thymocytes Ex Vivo

RNA analysis of whole thymic tissue extracts revealed significant downregulation of *Bcl-2* family target genes following FPN exposure. However, recognizing that whole thymic tissue comprises a heterogeneous population of cell types, we sought to minimize

doi:10.6342/NTU202501708

potential confounding effects arising from this cellular heterogeneity. To this end, mice were administered either VH or varying doses of FPN, and thymocytes were subsequently isolated and stimulated with concanavalin A (ConA) to assess the direct effects of FPN on these cells. Since in vivo FPN exposure may have already influenced thymocyte functions at the time of sampling, we aimed to investigate whether the thymocytes responding to ConA stimulation, which mimics T cell activation through T cell receptor (TCR) crosslinking, exhibit consistent alterations in apoptosis-related gene expression. By analyzing the expression of key apoptotic regulators in ConA-stimulated thymocytes, we aimed to determine if the transcriptional changes observed in vivo persist under ex vivo activation, potentially indicating dysregulation of survival pathways following FPN exposure.

Primary thymocytes were isolated and stimulated with ConA (final concentration, 5 μg/mL) for 24 h to further evaluate the impact of FPN on *Bcl-2* family gene expression in a more homogenous cell population. ConA, a mitogen that induces T-cell proliferation by cross-linking cell surface glycoprotein receptors, activates intracellular signaling pathways and transcription factors, thereby promoting cell cycle progression. Consistent with the results obtained from whole thymic tissue, high-dose FPN treatment significantly reduced the mRNA expression of *Bcl-2*, *Bcl-6*, *Mcl-1*, *Bnip3*, and *Bim* compared to the vehicle control (Figure 3A–E).

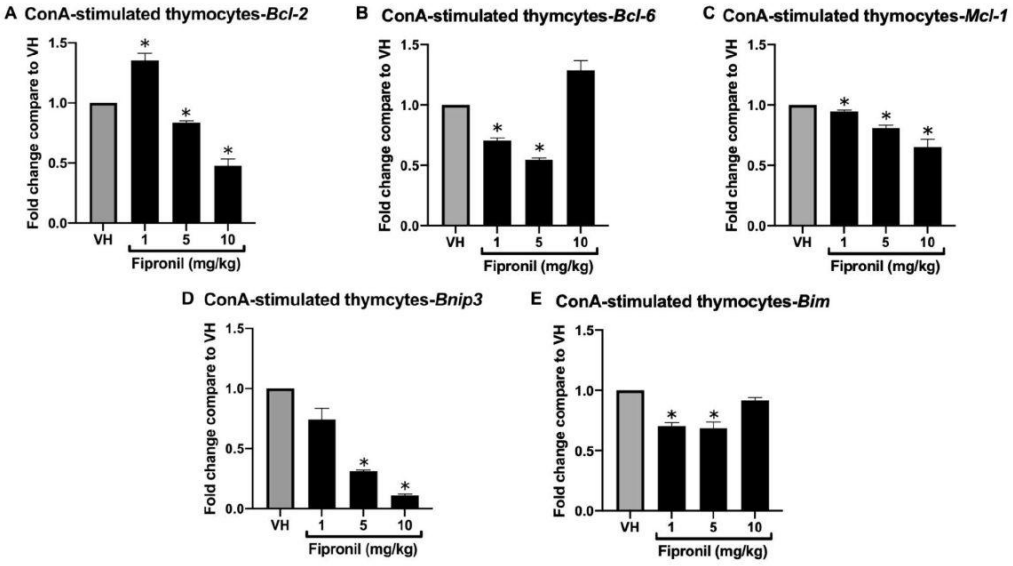

Figure 3. Fipronil significantly decreased mRNA expression of the *Bcl-2* family ex vivo. Total RNA was extracted from primary thymocytes following ConA stimulation to quantify mRNA expression by qPCR. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group (N = 20/group). * $p < 0.05$ was significant compared to the VH group.

3.1.3. Acute Exposure of FPN Significantly Attenuated Anti-Apoptotic mRNA Expression in the Primary Thymocytes In Vitro

Our previous sub-chronic in vivo studies demonstrated that FPN exposure induces thymic atrophy and disrupts the expression of genes and proteins essential for T cell development and maturation. To further explore the underlying cellular and molecular mechanisms, we used an in vitro acute exposure model by treating isolated primary thymocytes with FPN. The anti-apoptotic capacity of primary thymocytes was also evaluated

doi:10.6342/NTU202501708

by assessing the mRNA expression. The analysis revealed a significant reduction in the expression of *Bcl-2*, *Bcl-6*, and *Mcl-1* genes following FPN treatment compared to the vehicle control, the same as the qPCR results of the in vivo model. In contrast, the expression of the pro-apoptotic gene *Bim* was significantly upregulated in a dose-dependent manner (Figure 4A–E). These findings highlight that FPN exposure disrupts multiple apoptotic regulatory mechanisms, not only diminishing the anti-apoptotic capacity of thymocytes but also selectively influencing pathways associated with mitochondrial integrity and stress-induced apoptosis.

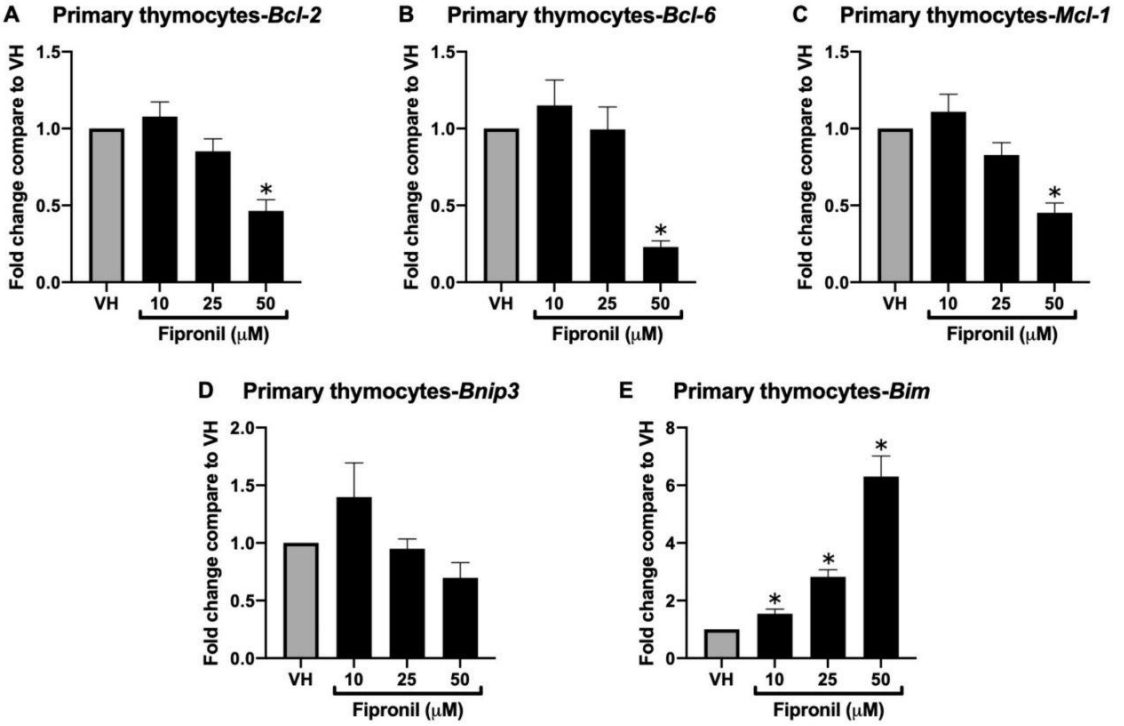

Figure 4. Fipronil significantly decreased mRNA expression of the *Bcl-2* family in vitro. The total mRNA harvested from different treatment groups was extracted to detect the mRNA expression by qPCR. The expression level of *Hprt* was used as the control for semi-quantification. Results were expressed as the mean ± SEM pooled from four independent experiments with technological duplication in each group. * $p < 0.05$ was significant compared to the VH group.

3.2. Cytotoxic and Apoptotic Effects by FPN in an Acute In Vitro Model

3.2.1. Cytotoxic and Immunosuppressive Effects of FPN on Primary Thymocytes

The cytotoxic effect of FPN on primary thymocytes was assessed using the MTT assay under PMA/Iono stimulation. Ionomycin facilitates Ca^{2+} release from the endoplasmic reticulum (ER), activating Ca^{2+}-sensitive enzymes and synergizing with PMA to enhance protein kinase C (PKC) activation. This combined action effectively induces T cell activation, proliferation, and cytokine production, providing a robust model for studying T cell function and signaling pathways. After being treated with FPN at concentrations of 5, 10, 25, and 50 μM, the thymocyte viability was significantly reduced in a dose-dependent manner (Figure 5A). Similarly, under PMA/Iono stimulation, high concentrations of FPN (50 μM) significantly decreased IL-2 secretion (Figure 5B). Supple-

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mentation with N-acetylcysteine (NAC, final concentration: 1 mM) effectively reversed the toxic effects induced by FPN.

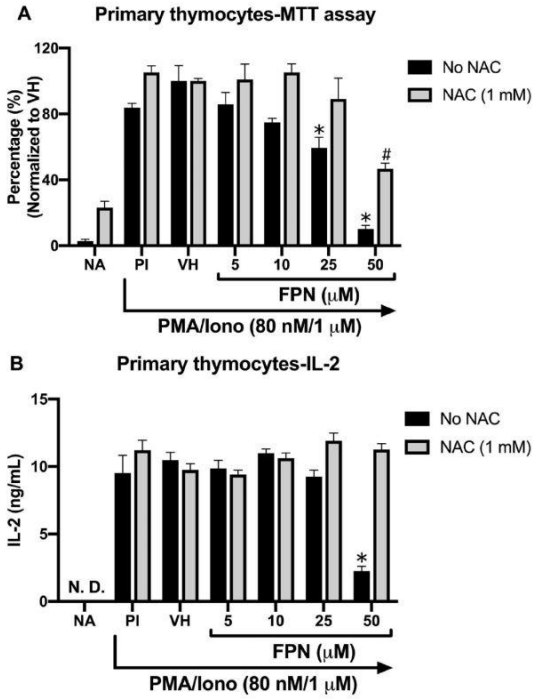

Figure 5. Fipronil exhibited a reduction of primary thymocyte viability and IL-2 production stimulated by PMA/Iono. The primary thymocytes (5×10^6 cells/mL) were treated with 0.05% DMSO (VH) or FPN in different concentrations and stimulated with PMA/Iono (PMA/Iono: 80 nM/1 μM) for 24 h. (**A**) The viability of thymocytes was measured by MTT assay. (**B**) The level of IL-2 in the supernatants was measured by ELISA. Data were expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments. * $p < 0.05$ was significant compared to the VH without the NAC group. # $p < 0.05$ was significant compared to the VH with the NAC group.

3.2.2. Effects of FPN Treatment on Apoptosis in the Primary Thymocytes In Vitro

Apoptosis was evaluated using Annexin V/PI double staining. As shown in Figure 6A–C, cells in the Q4 quadrant (Annexin V^-/PI^-) represent viable thymocytes with minimal Annexin V and PI binding, indicating intact cell membranes. In contrast, cells in the Q2 and Q3 quadrants (Annexin V^+/PI^+ and Annexin V^+/PI^-) correspond to populations undergoing early and late stages of apoptosis, respectively. Our findings revealed a concentration- and time-dependent increase in thymocyte apoptosis following FPN exposure (Figure 6).

doi:10.6342/NTU202501708

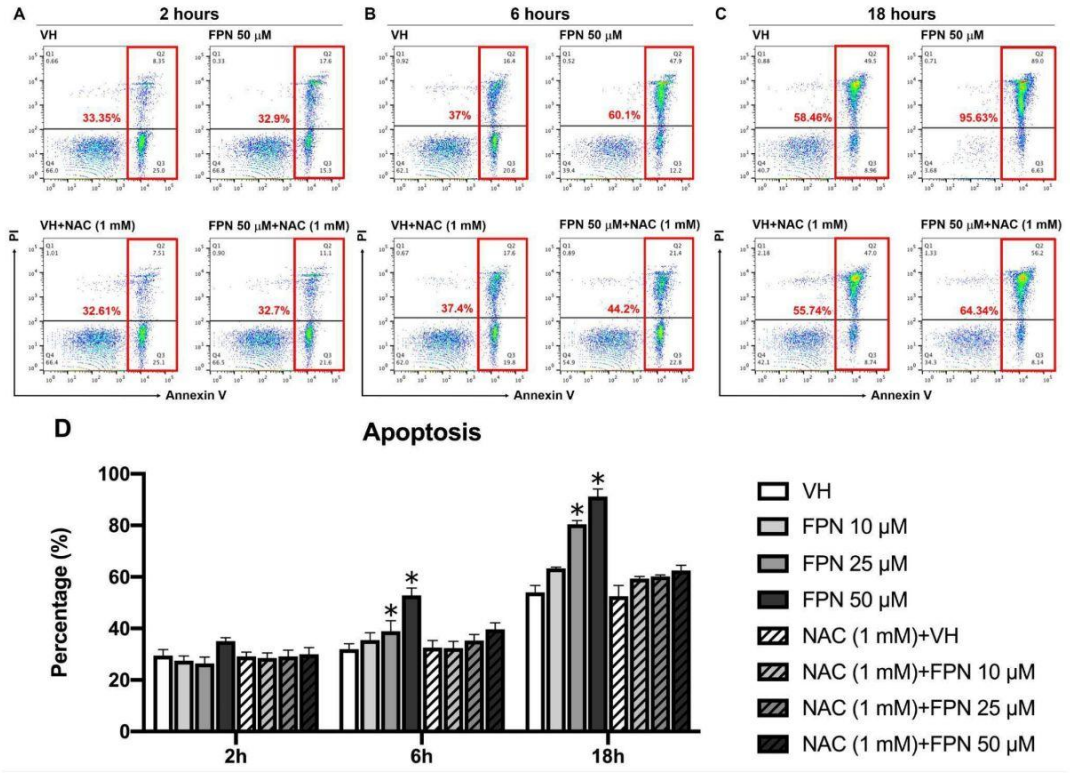

Figure 6. Fipronil inducted of thymocyte apoptosis in vitro. The apoptosis indicator was measured by Annexin V/PI staining. (**A**–**C**) The representative dot plot shows either the VH group and 50 μM FPN with or without NAC treatment at different time points. (**D**) Statistical data represent the sum of Annexin V^+/PI^+ and Annexin V^+/PI^- populations. The primary thymocytes (5×10^6 cells/mL) were treated with 0.05% DMSO (VH) or FPN in different concentrations for 2, 6, and 18 h. Data were expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments. * $p < 0.05$ was significant compared to the VH group of each time point.

3.3. Mitochondrial Dysfunction and Oxidative Stress Dysregulation Induced by FPN Exposure in an Acute In Vitro Model

3.3.1. Induction of Mitochondrial Depolarization on Primary Thymocytes by Fipronil

Mitochondrial depolarization was evaluated using JC-1 staining. In the primary thymocytes treated with 50 μM FPN, a significant increase in the proportion of depolarized cells was observed starting at 6 h. Additionally, starting from 18 h, a marked increase in mitochondrial depolarization was also evident in the cells treated with 10–50 μM FPN (Figure 7A).

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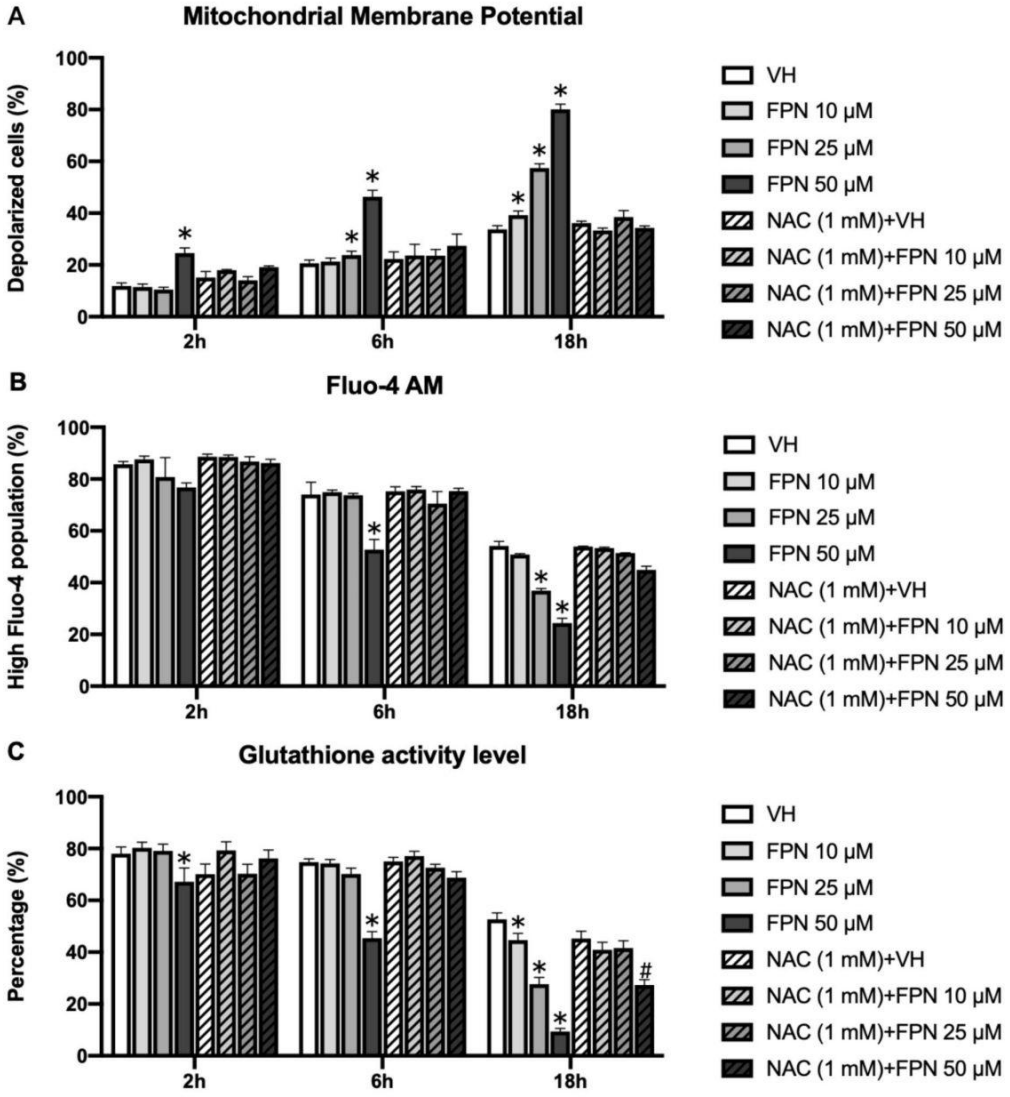

Figure 7. Effects of FPN on mitochondrial membrane potential, intracellular calcium levels, and GSH levels in primary thymocytes. Primary thymocytes (5×10^6 cells/mL) were treated with 0.05% DMSO (VH) or FPN at different concentrations for 2, 6, and 18 h. (**A**) Mitochondrial membrane potential was assessed using JC-1 staining, (**B**) intracellular calcium levels were measured using Fluo-4 AM staining, and (**C**) intracellular GSH levels were determined using CMF-DA staining. Data are expressed as the mean ± SEM of quadruplicate cultures and are representative of four independent experiments. * $p < 0.05$ compared to the VH group of each time point. # $p < 0.05$ compared to the VH with the NAC group.

3.3.2. Depletion of Intracellular Calcium on Primary Thymocytes by Fipronil

To investigate potential disruptions in calcium homeostasis resulting from mitochondrial membrane potential loss, intracellular calcium levels were assessed using Fluo-4 AM staining. In the primary thymocytes treated with 50 μM FPN, a significant reduction in the level of calcium was observed starting at 6 h. Additionally, starting from 18 h, a

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marked decrease in calcium level was also evident in the cells treated with 10–50 μM FPN (Figure 7B).

3.3.3. Reduction of Glutathione by Fipronil on Primary Thymocytes

The antioxidant glutathione (GSH) was assessed using CellTracker CMF-DA staining. The results indicated a significant decrease in GSH levels in the 50 μM FPN treatment group over a time course of 2 to 18 h, and the lower doses of FPN (10 and 25 μM) were decreased starting at 18 h (Figure 7C), which were consistent with the results of Annexin V/PI. This reduction in GSH activity indicated an increase in oxidative stress within the cells, suggesting that FPN exposure disrupted the redox balance. The supplementation of the antioxidant N-acetylcysteine (NAC, final concentration: 1 mM) effectively mitigated FPN-induced mitochondrial membrane potential changes, calcium, and GSH depletion.

3.3.4. Accumulation of Intracellular ROS by Fipronil on Primary Thymocytes

The levels of intracellular ROS were determined by the H_2-DCFDA detection kit. The results revealed a marked H_2O_2 accumulation in the 50 μM FPN treatment group at an early point of 0.5 to 2 h (Figure 8A). After six hours, the effects of increased oxidative damage by FPN are no longer apparent. The treatment of NAC could prevent the oxidative stress induced by FPN.

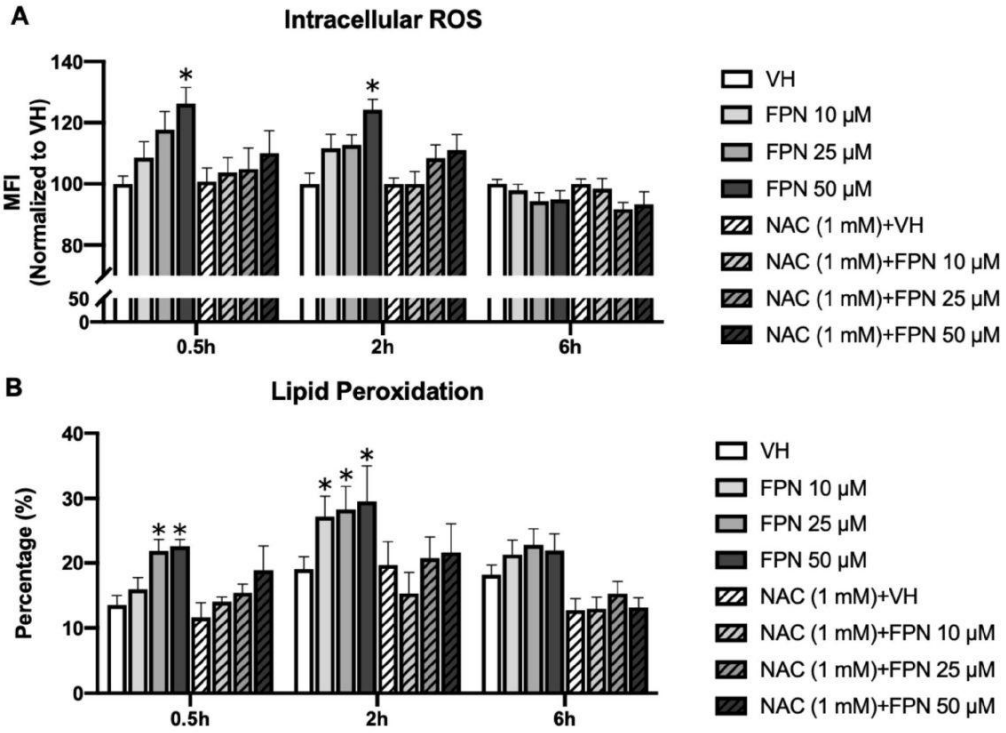

Figure 8. Induction of ROS and LPO levels by FPN. Primary thymocytes (5 × 106 cells/mL) were treated with 0.05% DMSO (VH) or FPN at different concentrations for 0.5, 2, and 6 h. ROS levels were measured using H_2-DCFDA staining, while LPO was assessed using C11-Bodipy581/591 staining. Data were expressed as the mean ± SEM of quadruplicate cultures and representative of four independent experiments. * $p < 0.05$ was significant compared to the VH group of each time point.

doi:10.6342/NTU202501708

3.3.5. Fipronil Exposure Elevates Lipid Peroxidation in Primary Thymocytes

To characterize the ROS induced by FPN, lipid peroxide (LPO) levels in FPN-treated primary thymocytes were quantified using the fluorescent probe C11-Bodipy$^{581/591}$. This sensor specifically detects LPO by undergoing oxidation in the presence of intracellular lipid peroxides, emitting a distinct bright green fluorescence signal that indicates oxidative stress. As shown in Figure 8B, a high concentration of FPN treatment resulted in a corresponding increase in lipid peroxidation levels at an early time point (0.5 h).

4. Discussion

Our sub-chronic in vivo and acute in vitro models provide the first investigation into the mechanisms underlying the immunotoxic effects of FPN on the thymus and primary thymocytes. While prior research has demonstrated that FPN induces oxidative stress and disrupts antioxidant defense mechanisms in various organs, its direct impacts on immune cells, particularly thymocytes, remain largely unexplored. Unlike hepatocytes, neurons, or renal cells, thymocytes are uniquely susceptible to oxidative stress due to their high turnover rate and critical role in T cell selection. Dysregulation of thymocyte apoptosis can have profound consequences on immune function, potentially increasing susceptibility to infections, altering immune tolerance, and predisposing individuals to autoimmune disorders. Our study reveals that even acute FPN exposure triggers oxidative stress-related apoptosis in thymocytes, which may contribute to persistent immune dysfunction.

FPN's toxic effects are not limited to a single organ system, impacting key regulators of systemic homeostasis such as the liver, thyroid, kidneys, and central nervous system. These multiorgan effects can have cascading consequences, potentially contributing to the development of immunotoxicity. Hepatic metabolism of FPN via cytochrome P450 enzymes leads to the formation of fipronil sulfone, a more persistent and biologically active metabolite. This bioactivation process can induce oxidative stress and disrupt detoxification pathways in the liver, ultimately influencing systemic immune responses [30,31]. FPN has also been shown to disrupt thyroid hormone homeostasis by modulating thyroid-stimulating hormone (TSH) and thyroid peroxidase activity [19]. Thyroid hormones play a crucial role in immune function, regulating T cell maturation and differentiation. Therefore, FPN-induced alterations in thyroid hormone levels could contribute to immune dysregulation. Furthermore, FPN acts as an antagonist of GABAA receptors, disrupting neuronal signaling and potentially leading to neurotoxicity [20,21]. Given the intricate interplay between the immune and nervous systems, mediated by cytokines and neurotransmitters, the neurotoxic effects of FPN could contribute to immune dysregulation.

Given FPN's widespread environmental persistence and presence as a residue on food products, its immunotoxic potential warrants further investigation. While previous research on FPN toxicity has mainly focused on active inflammatory responses and antigen-specific immune dysregulation [13,32], our prior findings demonstrated that FPN exposure severely impaired thymic development, disrupted IL-7 signaling, and led to significant thymic atrophy with marked reductions in thymocyte populations. To elucidate the underlying cellular and molecular mechanisms, we utilized both sub-chronic (young BALB/c mice) and acute (primary thymocyte cultures) models. These complementary approaches allowed us to dissect the immunotoxic effects of FPN at both the organismal and cellular levels, highlighting its potential risk in early-life exposure or acute poisoning events.

In our sub-chronic in vivo study, we opted for 4-week-old mice, a developmental stage characterized by active thymic function and ongoing T cell development. This age is particularly relevant for investigating potential immunotoxic effects, as the thymus plays a crucial role in immune system maturation. Furthermore, age-related differences in CYP enzyme activity can significantly impact the metabolism and toxicity of xenobiotics. Juvenile

doi:10.6342/NTU202501708

mice generally exhibit lower basal CYP activity compared to adults, potentially leading to slower FPN metabolism and prolonged systemic exposure [31]. This is particularly relevant given the link between CYP isozyme activity, oxidative stress, and apoptosis. Increased CYP-mediated metabolism can generate excessive ROS, potentially disrupting redox homeostasis and triggering apoptotic pathways. Considering the role of oxidative stress in FPN-induced immunotoxicity, metabolic differences between juvenile and adult mice could influence their susceptibility to FPN-induced immune dysregulation. Regarding dosage, our in vivo model employed a maximum FPN dose of 10 mg/kg. This is considerably lower than the estimated doses in reported cases of acute human FPN exposure (approximately 183–307 mg/kg) [33]. Even without considering interspecies differences in sensitivity and metabolic activity, our chosen dose allows for the investigation of potential immunotoxic effects at levels relevant to environmental or occupational exposures. Other in vitro studies using such neurons and hepatocytes have reported toxic effects of FPN within a concentration range of 30–200 μM [22]. The chosen concentrations of FPN in this in vitro study were within this range. While these concentrations may exceed those typically encountered in human exposure scenarios, they allow for the investigation of FPN's toxic mechanisms in a controlled setting. Although our study may not directly reflect the concentrations that cause toxicity under typical exposure conditions, we have confirmed the involvement of apoptotic mechanisms in FPN-induced immunotoxicity both in vivo and in vitro.

Our findings demonstrated that administration of FPN (1–10 mg/kg) over seven doses to young mice significantly decreased the mRNA expression of the anti-apoptotic genes *Bcl-2*, *Bcl-6*, and *Mcl-1* (Figures 2 and 3), indicating a disruption of anti-apoptotic mechanisms crucial for thymic homeostasis. Apoptosis plays a critical role in T cell biology, ensuring proper development, function, and immune homeostasis. During thymic development, thymocytes expressing nonfunctional or autoreactive T cell receptors (TCRs) are selectively eliminated via apoptosis, preventing the emergence of self-reactive cells that could induce autoimmunity. Furthermore, apoptosis regulates the contraction of expanded effector T cell populations following immune responses, thereby maintaining immune balance [34].

One major pathway leading to apoptosis is the intrinsic cell death pathway, controlled by Bcl-2 family members, which regulates mitochondrial membrane integrity. Both BCL-2 and MCL-1 are critical for T lymphocyte development and survival. BCL-2 production is tightly regulated during T-cell development to prevent abnormal apoptosis. Indeed, Bcl-2-deficient mice exhibit significant defects in T lymphocyte development, likely due to increased apoptosis [35,36]. In chimeric mice reconstituted with Bcl-$2^{-/-}$ adult bone marrow hematopoietic stem cells, the development of donor-derived lymphocytes is almost absent [37]. Interleukin-7 (IL-7) provides a crucial survival signal for lymphocyte precursors, and Bcl-2 is believed to function as a key anti-apoptotic molecule downstream of IL-7 signaling [38,39]. Early deletion of Mcl-1 during T lymphocyte development results in a blockade of T-lineage cells at the DN stage, and its deficiency also leads to apoptosis in mature T lymphocytes. Furthermore, IL-7 signaling significantly upregulates Mcl-1 expression, indicating that Mcl-1 is a critical pro-survival molecule downstream of IL-7 signaling [40]. Hematopoietic stem cells (HSCs) also require Mcl-1 for their survival, and stem cell factor (SCF) signaling markedly enhances Mcl-1 expression in mouse HSCs [41]. Thus, Mcl-1 is an essential anti-apoptotic protein in lymphocytes and HSCs.

Interestingly, we also observed a reduction in pro-apoptotic genes, including *Bnip3* and *Bim*, which underscores a complex interplay in the apoptotic pathways. Bim is a key mediator of negative selection in the thymus, facilitating the elimination of autoreactive or dysfunctional thymocytes. The downregulation of Bim mRNA in vivo suggests a potential impairment in the negative selection process during T cell lineage commitment, which

doi:10.6342/NTU202501708

may disrupt the progression of thymocytes beyond the double-positive (DP) stage [42]. This finding is consistent with our previous study using the same sub-chronic exposure model, where FPN treatment resulted in a significant accumulation of DP thymocytes within the CD4/CD8 subpopulations [14]. Meanwhile, *Bnip3* plays a dual role in regulating mitochondrial dynamics and T-cell homeostasis [43]. Beyond its established function in hypoxia-induced apoptosis, *Bnip3* modulates mitochondrial membrane potential and turnover through mitophagy, ensuring the removal of damaged mitochondria and maintaining cellular health [44,45]. Its reduced expression may represent an adaptive response to FPN-induced oxidative stress, preserving mitochondrial function under adverse conditions. Therefore, simultaneous deregulation of pro-apoptotic and anti-apoptotic gene expression may impair thymocyte development, potentially leading to long-term immune deficiencies. These findings highlight the need for further investigation into the molecular mechanisms driving FPN-induced immunotoxicity and its broader implications for immune system health.

Contrary to the results of the reduction of *Bim* in the thymus of FPN-treated mice, FPN directly induced the *Bim* mRNA expression in a dose-dependent manner in vitro. Bim is a pro-apoptotic BH3-only protein that plays a central role in intrinsic apoptosis, particularly during thymocyte selection [46,47]. Its upregulation in vitro suggests that FPN directly induces apoptotic stress within thymocytes, potentially through oxidative or mitochondrial pathways. This outcome aligns with the observed oxidative damage in the lipid peroxidation assays and reduced GSH levels, which likely contribute to apoptotic signaling activation. The upregulation of Bim in vitro may result from the rapid cytotoxic effects of FPN, leading to mitochondrial-driven apoptosis and oxidative stress. Alternatively, this increase may reflect an attempt to eliminate damaged or stressed thymocytes, preventing the persistence of compromised cells. However, the differences between the in vitro and in vivo findings suggest additional factors influencing Bim expression within the thymic microenvironment. In the sub-chronic model, the suppression of Bim expression could be a response to limit excessive thymocyte loss, given the overall toxicity of FPN on the thymus. Another possibility is that surviving thymocytes in vivo may have already undergone selection pressure, favoring cells that downregulate Bim to resist apoptosis. These findings highlight the complexity of thymocyte responses under toxicant exposure and suggest that multiple factors contribute to the regulation of Bim expression in different experimental settings.

These findings underscore the dynamic interplay between intrinsic thymocyte responses and extrinsic regulatory signals in maintaining immune homeostasis under FPN exposure. While acute exposure rapidly activates apoptosis via oxidative stress pathways, prolonged exposure may induce adaptive changes that modify apoptotic sensitivity. Further investigations into thymic niche signaling and epigenetic regulation of pro-apoptotic genes will be crucial in understanding how FPN shapes thymocyte fate and immune system development over time.

As intrinsic apoptotic genes were altered during FPN exposure, we further evaluated the kinetic changes of mitochondrial damage and redox factors associated with FPN-induced thymocyte apoptosis. Under normal conditions, mitochondria convert approximately 1–2% of the total oxygen consumed into superoxide anions and other reactive oxygen species (ROS). However, exceeding ROS production may significantly induce intrinsic apoptosis during toxicant exposure or unhealthy cellular conditions. Consequently, mitochondria serve as the primary source of ROS generation within cells [48]. The detailed mechanisms of FPN-induced mitochondrial toxicity were investigated by assessing mitochondrial metabolic activity, mitochondrial membrane potential, and markers of oxidative damage, including ROS levels, lipid peroxidation, and intracellular GSH, in isolated

doi:10.6342/NTU202501708

primary thymocytes. The MTT assay demonstrated a significant reduction in the viability of primary thymocytes (Figure 5A), indicating that FPN treatment adversely affected mitochondrial metabolic activity. To further investigate the underlying mechanisms, we evaluated apoptosis and mitochondrial function using Annexin V/PI staining, JC-1 staining, intracellular calcium, and cellular glutathione levels, respectively. Our results revealed a dose- and time-dependent increase in the proportion of depolarized thymocytes at higher concentrations of FPN (Figure 7A). This depolarization reflected a disruption in mitochondrial membrane potential, a critical early event in the apoptotic process. Mitochondrial dysfunction is a hallmark of apoptosis and an initiator of the intrinsic apoptotic pathway. The opening of the mitochondrial permeability transition pore results in transmembrane potential depolarization, release of apoptogenic factors, and loss of oxidative phosphorylation, ultimately leading to cell death [49,50]. The observed decrease in intracellular calcium levels might be linked to mitochondrial dysfunction, as indicated by the progressive increase in MMP (Figure 7B). Mitochondria regulate intracellular calcium homeostasis by sequestering calcium through the mitochondrial calcium uniporter (MCU). However, excessive MMP elevation can impair calcium uptake by increasing the electrochemical gradient beyond the optimal range for MCU activity, leading to reduced mitochondrial calcium sequestration and lower cytosolic calcium levels [51,52]. This calcium depletion disrupts mitochondrial bioenergetics and compromises ATP production, further exacerbating oxidative stress and apoptosis. Given the essential role of calcium in mitochondrial bioenergetics and cell survival, the interplay between FPN-induced MMP alterations and calcium dysregulation may represent a key event in the immunotoxic effects observed in thymocytes.

Our results demonstrated a significant increase in DCFDA-detected ROS levels at an earlier time point, indicating the rapid induction of oxidative stress following treatment (Figure 8A). This suggests that ROS accumulation serves as an initial trigger for intracellular stress responses, potentially leading to mitochondrial dysfunction, membrane potential collapse, and activation of cell death pathways. The early surge in ROS highlights its pivotal role as a mediator in the cytotoxic effects observed, providing crucial insight into the oxidative stress-driven mechanisms underlying the experimental treatment. Further investigation into antioxidant defense systems and mitochondrial function is warranted to elucidate the downstream effects of this early oxidative response. The broad range of non-target toxicity of FPN is primarily attributed to oxidative stress, which arises when there is an insufficient antioxidant capacity or an accumulation of free radicals [22]. Numerous in vivo and in vitro studies have reported that the generation of ROS or RNS exerts a significant impact on oxidative stress and related toxicities induced by FPN [6,53–56]. Therefore, the scavenging ability of antioxidants is an effective indicator for assessing oxidative damage from oxidative stimuli. Glutathione (GSH) is the predominant intracellular antioxidant that neutralizes hydroxyl radicals (•OH) and protects against oxidative damage [57]. Variations in GSH levels can significantly affect cellular responses to oxidative stress [58,59].

Our results demonstrate that FPN exposure disrupts intracellular oxidative balance, as reflected by a decline in GSH levels and an increase in lipid peroxidation. The CMFDA assay revealed a significant reduction in GSH levels in the high-dose FPN groups, beginning at the 2-h time point (Figure 7C). This decline corresponds to an early ROS surge, indicating that oxidative stress overwhelmed the cellular redox balance, resulting in the depletion of glutathione reserves. Similarly, the C11-Bodipy$^{581/591}$ assay indicated a pronounced increase in lipid peroxidation at 0.5 and 2 h post-exposure in the high-dose FPN groups (Figure 8B). Lipid peroxidation represents the oxidative degradation of polyunsaturated fatty acids in cellular membranes, causing membrane instability, loss of integrity, and

doi:10.6342/NTU202501708

subsequent cellular damage [60–62]. These findings suggest that ROS production was elevated by FPN and further affected cellular lipids, thus amplifying oxidative stress.

In the in vitro experiments, we observed time-dependent changes in apoptosis, MMP, and GSH levels in the VH-treated primary thymocytes. Specifically, apoptosis and MMP increased over time, and GSH levels progressively declined. These baseline fluctuations are consistent with the inherent sensitivity of primary thymocytes to in vitro culture conditions. Once isolated from the thymus, thymocytes rapidly undergo apoptosis due to the loss of essential survival signals from thymic stromal cells, cytokines, and other regulatory factors. Additionally, isolation itself induces oxidative stress, as cells are subjected to mechanical and enzymatic processing, shifts in oxygen tension, and changes in nutrient composition. The thymic microenvironment plays a crucial role in maintaining redox homeostasis, and its disruption leads to increased ROS production and antioxidant depletion, as previously reported in studies on oxidative stress following tissue dissociation. Thymic stromal cells, which provide key redox regulatory factors, are absent in vitro, potentially causing elevated ROS levels and compensatory mitochondrial hyperactivity, reflected in MMP changes. Furthermore, the oxygen tension in standard culture conditions is higher than that within the thymus, which may further exacerbate oxidative stress. However, we considered that baseline oxidative stress in VH-treated cells could potentially sensitize them to additional stressors. To address this, we have clarified that, while in vitro conditions contribute to some degree of apoptosis and redox imbalance, the differential effects observed in FPN-treated cells compared with VH-treated cells demonstrated a direct toxic effect of FPN on thymocytes.

Collectively, these findings firmly establish oxidative stress as a critical mechanism mediating FPN-induced immunotoxicity. The observed depletion of GSH directly impairs cellular capacity to neutralize ROS, while the concomitant increase in lipid peroxidation signifies oxidative damage to cellular membranes. These disruptions likely act synergistically, compromising thymocyte survival and disrupting immune homeostasis. This provides a compelling mechanistic explanation for the observed reductions in thymic gene expression and dysregulation of apoptosis. Critically, co-treatment with the antioxidant N-acetylcysteine (NAC) significantly attenuated the deleterious effects of FPN on thymocyte viability and mitochondrial function. As a well-established precursor of GSH, NAC effectively replenishes intracellular GSH levels, thereby mitigating oxidative stress and its downstream consequences.

In this study, NAC co-administration markedly reduced both the FPN-induced surge in ROS and the elevated levels of lipid peroxidation. Furthermore, NAC preserved mitochondrial membrane potential, as evidenced by JC-1 staining, and significantly decreased the proportion of apoptotic cells, as determined by Annexin V/PI staining. These findings strongly support the conclusion that NAC effectively prevents FPN-induced mitochondrial dysfunction and activation of apoptotic pathways. The protective effects of NAC underscore the central role of oxidative stress in FPN-mediated immunotoxicity and highlight the therapeutic potential of antioxidants in mitigating pesticide-induced immune dysfunction.

5. Conclusions

The present study demonstrated that fipronil (FPN) exposure disrupted thymic homeostasis by dysregulating key apoptotic and oxidative stress pathways. In vivo, FPN significantly reduced the expression of both anti-apoptotic (*Bcl-2*, *Bcl-6*, *Mcl-1*) and pro-apoptotic (*Bnip3*, *Bim*) genes in thymic tissue, indicating broad transcriptional disruption of apoptotic signaling. In vitro experiments further revealed the direct immunotoxic effects of FPN on mitochondrial dysfunction, characterized by reduced metabolic activity, decreased mitochondrial membrane potential ($\Delta\psi m$), depleted calcium levels, elevated ROS levels,

doi:10.6342/NTU202501708

depleted GSH levels, and increased lipid peroxidation (LPO). Notably, in vitro *Bim* expression was significantly increased, suggesting a direct pro-apoptotic response by FPN exposure. Taken together, these findings highlight the critical interplay between oxidative damage and apoptosis in FPN-induced thymocyte toxicity, underscoring the immunotoxic potential of FPN on thymocyte survival and thymus function.

Author Contributions: J.-F.K. and C.-C.W. conceived and designed the experiments, analyzed the data, and wrote the manuscript. J.-F.K. performed the experiments and drafted the manuscript. Y.-P.H. designed and performed the experiments and assisted in analyzing the data. Y.-D.W. and H.-P.W. assisted in the experimental operations. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Ministry of Science and Technology (Taipei, Taiwan) under Grant MOST 106-2320-B-037-002, MOST 107-2320-B-002-065, and NSTC-113-2313-B-002-002-. The funders had no role in the design of the collection, analysis, and interpretation of data.

Institutional Review Board Statement: All experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC Approval No: NTU108-EL-00026) and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was carried out in compliance with the ARRIVE 2.0 guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original data employed or analyzed in this present study can be obtained from the corresponding author upon making a reasonable request.

Acknowledgments: We thank Han-You Lin for providing the service of Agilent Technologies® Mx3005P qPCR system, Pei-Shiue Tsai for supplying the Fluo-4 AM calcium indicators, and Chuang Ching Hua for assistance in the execution of the experiment, all of them from the School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan.

Conflicts of Interest: The authors declare no competing financial interests.

Abbreviations

FPN	Fipronil
ConA	Concanavalin A
PMA/Iono	Phorbol 12-myristate 13-acetate/Ionomycin
NAC	N-acetylcysteine
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
MMP	Mitochondrial membrane potential
GSH	Glutathione
LPO	Lipid peroxidation

References

1. Kim, Y.A.; Yoon, Y.S.; Kim, H.S.; Jeon, S.J.; Cole, E.; Lee, J.; Kho, Y.; Cho, Y.H. Distribution of Fipronil in Humans, and Adverse Health Outcomes of in Utero Fipronil Sulfone Exposure in Newborns. *Int. J. Hyg. Environ. Health* **2019**, *222*, 524–532. [CrossRef]
2. Mohamed, F.; Senarathna, L.; Percy, A.; Abeyewardene, M.; Eaglesham, G.; Cheng, R.; Azher, S.; Hittarage, A.; Dissanayake, W.; Sheriff, M.R.; et al. Acute Human Self-Poisoning with the N-Phenylpyrazole Insecticide Fipronil–A GABAA-Gated Chloride Channel Blocker. *J. Toxicol. Clin. Toxicol.* **2004**, *42*, 955–963. [CrossRef]
3. Cam, M.; Durieu, E.; Bodin, M.; Manousopoulou, A.; Koslowski, S.; Vasylieva, N.; Barnych, B.; Hammock, B.D.; Bohl, B.; Koch, P.; et al. Induction of Amyloid-B42 Production by Fipronil and Other Pyrazole Insecticides. *J. Alzheimer's Dis.* **2018**, *62*, 1663–1681. [CrossRef] [PubMed]
4. Environmental Fate and Toxicology of Fipronil. Available online: https://www.jstage.jst.go.jp/article/jpestics/32/3/32_3_189/_article?form=MG0AV3 (accessed on 24 January 2025).

doi:10.6342/NTU202501708

5. Stehr, C.M.; Linbo, T.L.; Incardona, J.P.; Scholz, N.L. The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae. *Toxicol. Sci.* **2006**, *92*, 270–278. [CrossRef]
6. Khan, S.; Jan, M.H.; Kumar, D.; Telang, A.G. Firpronil Induced Spermotoxicity Is Associated with Oxidative Stress, DNA Damage and Apoptosis in Male Rats. *Pestic. Biochem. Physiol.* **2015**, *124*, 8–14. [CrossRef] [PubMed]
7. Yu, F.; Wang, Z.; Ju, B.; Wang, Y.; Wang, J.; Bai, D. Apoptotic Effect of Organophosphorus Insecticide Chlorpyrifos on Mouse Retina in Vivo via Oxidative Stress and Protection of Combination of Vitamins C and E. *Exp. Toxicol. Pathol.* **2008**, *59*, 415–423. [CrossRef] [PubMed]
8. Mossa, A.-T.H.; Swelam, E.S.; Mohafrash, S.M.M. Sub-Chronic Exposure to Fipronil Induced Oxidative Stress, Biochemical and Histopathological Changes in the Liver and Kidney of Male Albino Rats. *Toxicol. Rep.* **2015**, *2*, 775–784. [CrossRef]
9. Tingle, C.C.D.; Rother, J.A.; Dewhurst, C.F.; Lauer, S.; King, W.J. Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns. In *Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews*; Ware, G.W., Ed.; Reviews of Environmental Contamination and Toxicology; Springer: New York, NY, USA, 2003; pp. 1–66. ISBN 978-1-4899-7283-5.
10. Wu, J.; Lu, J.; Lu, H.; Lin, Y.; Chris Wilson, P. Occurrence and Ecological Risks from Fipronil in Aquatic Environments Located within Residential Landscapes. *Sci. Total Environ.* **2015**, *518–519*, 139–147. [CrossRef]
11. Ratra, G.S.; Casida, J.E. GABA Receptor Subunit Composition Relative to Insecticide Potency and Selectivity. *Toxicol. Lett.* **2001**, *122*, 215–222. [CrossRef]
12. Cole, L.M.; Nicholson, R.A.; Casida, J.E. Action of Phenylpyrazole Insecticides at the GABA-Gated Chloride Channel. *Pestic. Biochem. Physiol.* **1993**, *46*, 47–54. [CrossRef]
13. Kuo, J.-F.; Cheng, Y.-H.; Tung, C.-W.; Wang, C.-C. Fipronil Disturbs the Antigen-Specific Immune Responses and GABAergic Gene Expression in the Ovalbumin-Immunized BALB/c Mice. *BMC Vet. Res.* **2024**, *20*, 30. [CrossRef] [PubMed]
14. Kuo, J.-F.; Wu, H.-Y.; Tung, C.-W.; Huang, W.-H.; Lin, C.-S.; Wang, C.-C. Induction of Thymus Atrophy and Disruption of Thymocyte Development by Fipronil through Dysregulation of IL-7-Associated Genes. *Chem. Res. Toxicol.* **2024**, *37*, 1488–1500. [CrossRef] [PubMed]
15. Kawamoto, Y.; Ayaki, T.; Urushitani, M.; Ito, H.; Takahashi, R. Activated Caspase-9 Immunoreactivity in Glial and Neuronal Cytoplasmic Inclusions in Multiple System Atrophy. *Neurosci. Lett.* **2016**, *628*, 207–212. [CrossRef] [PubMed]
16. Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]
17. Murali, A.K.; Mehrotra, S. Apoptosis—An Ubiquitous T Cell Immunomodulator. *J. Clin. Cell Immunol.* **2011**, *2* (Suppl. S3), 1–10. [CrossRef]
18. Czabotar, P.E.; Lessene, G.; Strasser, A.; Adams, J.M. Control of Apoptosis by the BCL-2 Protein Family: Implications for Physiology and Therapy. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 49–63. [CrossRef]
19. Bano, F.; Mohanty, B. Thyroid Disrupting Pesticides Mancozeb and Fipronil in Mixture Caused Oxidative Damage and Genotoxicity in Lymphoid Organs of Mice. *Environ. Toxicol. Pharmacol.* **2020**, *79*, 103408. [CrossRef]
20. Vidau, C.; González-Polo, R.A.; Niso-Santano, M.; Gómez-Sánchez, R.; Bravo-San Pedro, J.M.; Pizarro-Estrella, E.; Blasco, R.; Brunet, J.-L.; Belzunces, L.P.; Fuentes, J.M. Fipronil Is a Powerful Uncoupler of Oxidative Phosphorylation That Triggers Apoptosis in Human Neuronal Cell Line SHSY5Y. *NeuroToxicology* **2011**, *32*, 935–943. [CrossRef]
21. Awad, M.A.; Ahmed, Z.S.O.; AbuBakr, H.O.; Elbargeesy, G.A.E.-F.H.; Moussa, M.H.G. Fipronil Induced Oxidative Stress in Neural Tissue of Albino Rat with Subsequent Apoptosis and Tissue Reactivity. *Acta Histochem.* **2021**, *123*, 151764. [CrossRef]
22. Wang, X.; Martínez, M.A.; Wu, Q.; Ares, I.; Martínez-Larrañaga, M.R.; Anadón, A.; Yuan, Z. Fipronil Insecticide Toxicology: Oxidative Stress and Metabolism. *Crit. Rev. Toxicol.* **2016**, *46*, 876–899. [CrossRef]
23. Weidinger, A.; Kozlov, A.V. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress Versus Signal Transduction. *Biomolecules* **2015**, *5*, 472–484. [CrossRef]
24. Badgujar, P.C.; Chandratre, G.A.; Pawar, N.N.; Telang, A.G.; Kurade, N.P. Fipronil Induced Oxidative Stress Involves Alterations in SOD1 and Catalase Gene Expression in Male Mice Liver: Protection by Vitamins E and C. *Environ. Toxicol.* **2016**, *31*, 1147–1158. [CrossRef]
25. Badgujar, P.C.; Pawar, N.N.; Chandratre, G.A.; Telang, A.G.; Sharma, A.K. Fipronil Induced Oxidative Stress in Kidney and Brain of Mice: Protective Effect of Vitamin E and Vitamin C. *Pestic. Biochem. Physiol.* **2015**, *118*, 10–18. [CrossRef] [PubMed]
26. Wang, R.; Green, D.R. Metabolic Checkpoints in Activated T Cells. *Nat. Immunol.* **2012**, *13*, 907–915. [CrossRef] [PubMed]
27. Pearce, E.L.; Pearce, E.J. Metabolic Pathways in Immune Cell Activation and Quiescence. *Immunity* **2013**, *38*, 633–643. [CrossRef]
28. Sena, L.A.; Li, S.; Jairaman, A.; Prakriya, M.; Ezponda, T.; Hildeman, D.A.; Wang, C.-R.; Schumacker, P.T.; Licht, J.D.; Perlman, H.; et al. Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. *Immunity* **2013**, *38*, 225–236. [CrossRef] [PubMed]
29. Peng, H.-Y.; Lucavs, J.; Ballard, D.; Das, J.K.; Kumar, A.; Wang, L.; Ren, Y.; Xiong, X.; Song, J. Metabolic Reprogramming and Reactive Oxygen Species in T Cell Immunity. *Front. Immunol.* **2021**, *12*, 652687. [CrossRef]

doi:10.6342/NTU202501708

30. Das, P.C.; Cao, Y.; Cherrington, N.; Hodgson, E.; Rose, R.L. Fipronil Induces CYP Isoforms and Cytotoxicity in Human Hepatocytes. *Chem. Biol. Interact.* **2006**, *164*, 200–214. [CrossRef]
31. Caballero, M.V.; Ares, I.; Martínez, M.; Martínez-Larrañaga, M.R.; Anadón, A.; Martínez, M.A. Fipronil Induces CYP Isoforms in Rats. *Food Chem. Toxicol.* **2015**, *83*, 215–221. [CrossRef]
32. Aldayel, T.S.; Abdel-Rahman, H.G.; Gad EL-Hak, H.N.; Abdelrazek, H.M.A.; Mohamed, R.M.; El-Sayed, R.M. Assessment of Modulatory Activity of Uncaria Tomentosa Extract against Fipronil Immunotoxicity in Male Rats. *Ecotoxicol. Environ. Saf.* **2021**, *224*, 112674. [CrossRef]
33. Chen, D.; Li, J.; Zhao, Y.; Wu, Y. Human Exposure of Fipronil Insecticide and the Associated Health Risk. *J. Agric. Food Chem.* **2022**, *70*, 63–71. [CrossRef]
34. Zhang, N.; Hartig, H.; Dzhagalov, I.; Draper, D.; He, Y.W. The Role of Apoptosis in the Development and Function of T Lymphocytes. *Cell Res.* **2005**, *15*, 749–769. [CrossRef] [PubMed]
35. Veis, D.J.; Sorenson, C.M.; Shutter, J.R.; Korsmeyer, S.J. Bcl-2-Deficient Mice Demonstrate Fulminant Lymphoid Apoptosis, Polycystic Kidneys, and Hypopigmented Hair. *Cell* **1993**, *75*, 229–240. [CrossRef] [PubMed]
36. Nakayama, K.; Nakayama, K.; Negishi, I.; Kuida, K.; Sawa, H.; Loh, D.Y. Targeted Disruption of Bcl-2 Alpha Beta in Mice: Occurrence of Gray Hair, Polycystic Kidney Disease, and Lymphocytopenia. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3700–3704. [CrossRef] [PubMed]
37. Matsuzaki, Y.; Nakayama, K.; Nakayama, K.; Tomita, T.; Isoda, M.; Loh, D.Y.; Nakauchi, H. Role of Bcl-2 in the Development of Lymphoid Cells from the Hematopoietic Stem Cell. *Blood* **1997**, *89*, 853–862. [CrossRef]
38. Akashi, K.; Kondo, M.; von Freeden-Jeffry, U.; Murray, R.; Weissman, I.L. Bcl-2 Rescues T Lymphopoiesis in Interleukin-7 Receptor–Deficient Mice. *Cell* **1997**, *89*, 1033–1041. [CrossRef]
39. Maraskovsky, E.; O'Reilly, L.A.; Teepe, M.; Corcoran, L.M.; Peschon, J.J.; Strasser, A. Bcl-2 Can Rescue T Lymphocyte Development in Interleukin-7 Receptor–Deficient Mice but Not in Mutant *Rag-1*$^{-/-}$ Mice. *Cell* **1997**, *89*, 1011–1019. [CrossRef]
40. Opferman, J.T.; Letai, A.; Beard, C.; Sorcinelli, M.D.; Ong, C.C.; Korsmeyer, S.J. Development and Maintenance of B and T Lymphocytes Requires Antiapoptotic MCL-1. *Nature* **2003**, *426*, 671–676. [CrossRef]
41. Opferman, J.T.; Iwasaki, H.; Ong, C.C.; Suh, H.; Mizuno, S.; Akashi, K.; Korsmeyer, S.J. Obligate Role of Anti-Apoptotic MCL-1 in the Survival of Hematopoietic Stem Cells. *Science* **2005**, *307*, 1101–1104. [CrossRef]
42. Wang, J.; He, N.; Zhang, N.; Quan, D.; Zhang, S.; Zhang, C.; Yu, R.T.; Atkins, A.R.; Zhu, R.; Yang, C.; et al. NCoR1 Restrains Thymic Negative Selection by Repressing Bim Expression to Spare Thymocytes Undergoing Positive Selection. *Nat. Commun.* **2017**, *8*, 959. [CrossRef]
43. Wan, J.; Martinvalet, D.; Ji, X.; Lois, C.; Kaech, S.M.; Von Andrian, U.H.; Lieberman, J.; Ahmed, R.; Manjunath, N. The Bcl-2 Family pro-Apoptotic Molecule, BNIP3 Regulates Activation-Induced Cell Death of Effector Cytotoxic T Lymphocytes. *Immunology* **2003**, *110*, 10–17. [CrossRef] [PubMed]
44. Rikka, S.; Quinsay, M.N.; Thomas, R.L.; Kubli, D.A.; Zhang, X.; Murphy, A.N.; Gustafsson, Å.B. Bnip3 Impairs Mitochondrial Bioenergetics and Stimulates Mitochondrial Turnover. *Cell Death Differ.* **2011**, *18*, 721–731. [CrossRef] [PubMed]
45. Gustafsson, Å.B. Bnip3 as a Dual Regulator of Mitochondrial Turnover and Cell Death in the Myocardium. *Pediatr. Cardiol.* **2011**, *32*, 267–274. [CrossRef]
46. Fischer, S.F.; Bouillet, P.; O'Donnell, K.; Light, A.; Tarlinton, D.M.; Strasser, A. Proapoptotic BH3-Only Protein Bim Is Essential for Developmentally Programmed Death of Germinal Center-Derived Memory B Cells and Antibody-Forming Cells. *Blood* **2007**, *110*, 3978–3984. [CrossRef]
47. Mérino, D.; Giam, M.; Hughes, P.D.; Siggs, O.M.; Heger, K.; O'Reilly, L.A.; Adams, J.M.; Strasser, A.; Lee, E.F.; Fairlie, W.D.; et al. The Role of BH3-Only Protein Bim Extends beyond Inhibiting Bcl-2–like Prosurvival Proteins. *J. Cell Biol.* **2009**, *186*, 355. [CrossRef] [PubMed]
48. Shaki, F.; Hosseini, M.-J.; Ghazi-Khansari, M.; Pourahmad, J. Toxicity of Depleted Uranium on Isolated Rat Kidney Mitochondria. *Biochim. Biophys. Acta (BBA) Gen. Subj.* **2012**, *1820*, 1940–1950. [CrossRef]
49. Ly, J.D.; Grubb, D.R.; Lawen, A. The Mitochondrial Membrane Potential (Δψm) in Apoptosis: An Update. *Apoptosis* **2003**, *8*, 115–128. [CrossRef]
50. Wang, C.; Youle, R.J. The Role of Mitochondria in Apoptosis. *Annu. Rev. Genet.* **2009**, *43*, 95–118. [CrossRef]
51. Bhosale, G.; Sharpe, J.A.; Sundier, S.Y.; Duchen, M.R. Calcium Signaling as a Mediator of Cell Energy Demand and a Trigger to Cell Death. *Ann. N. Y. Acad. Sci.* **2015**, *1350*, 107–116. [CrossRef]
52. Pathak, T.; Trebak, M. Mitochondrial Ca^{2+} Signaling. *Pharmacol. Ther.* **2018**, *192*, 112–123. [CrossRef]
53. Aboul-Enein, F.; Rauschka, H.; Kornek, B.; Stadelmann, C.; Stefferl, A.; Brück, W.; Lucchinetti, C.; Schmidbauer, M.; Jellinger, K.; Lassmann, H. Preferential Loss of Myelin-Associated Glycoprotein Reflects Hypoxia-Like White Matter Damage in Stroke and Inflammatory Brain Diseases. *J. Neuropathol. Exp. Neurol.* **2003**, *62*, 25–33. [CrossRef] [PubMed]
54. Ki, Y.-W.; Lee, J.E.; Park, J.H.; Shin, I.C.; Koh, H.C. Reactive Oxygen Species and Mitogen-Activated Protein Kinase Induce Apoptotic Death of SH-SY5Y Cells in Response to Fipronil. *Toxicol. Lett.* **2012**, *211*, 18–28. [CrossRef] [PubMed]

doi:10.6342/NTU202501708

55. Park, J.H.; Park, Y.S.; Lee, J.-B.; Park, K.-H.; Paik, M.; Jeong, M.; Koh, H.C. Meloxicam Inhibits Fipronil-Induced Apoptosis via Modulation of the Oxidative Stress and Inflammatory Response in SH-SY5Y Cells. *J. Appl. Toxicol.* **2016**, *36*, 10–23. [CrossRef]
56. Romero, A.; Ramos, E.; Ares, I.; Castellano, V.; Martínez, M.; Martínez-Larrañaga, M.R.; Anadón, A.; Martínez, M.A. Fipronil Sulfone Induced Higher Cytotoxicity than Fipronil in SH-SY5Y Cells: Protection by Antioxidants. *Toxicol. Lett.* **2016**, *252*, 42–49. [CrossRef]
57. Yadav, A.; Mishra, P.C. Modeling the Activity of Glutathione as a Hydroxyl Radical Scavenger Considering Its Neutral Non-Zwitterionic Form. *J. Mol. Model.* **2012**, *19*, 767–777. [CrossRef]
58. Aydin, B. Effects of Thiacloprid, Deltamethrin and Their Combination on Oxidative Stress in Lymphoid Organs, Polymorphonuclear Leukocytes and Plasma of Rats. *Pestic. Biochem. Physiol.* **2011**, *100*, 165–171. [CrossRef]
59. Wang, X.; Li, Y.; Zhong, S.; Zhang, H.; Wang, X.; Qi, P.; Xu, H. Oxidative Injury Is Involved in Fipronil-Induced G2/M Phase Arrest and Apoptosis in *Spodoptera frugiperda* (Sf9) Cell Line. *Pestic. Biochem. Physiol.* **2013**, *105*, 122–130. [CrossRef]
60. der Paal, J.V.; Neyts, E.C.; Verlackt, C.C.W.; Bogaerts, A. Effect of Lipid Peroxidation on Membrane Permeability of Cancer and Normal Cells Subjected to Oxidative Stress. *Chem. Sci.* **2015**, *7*, 489–498. [CrossRef]
61. Yang, H.; Zhou, M.; Li, H.; Wei, T.; Tang, C.; Zhou, Y.; Long, X. Effects of Low-Level Lipid Peroxidation on the Permeability of Nitroaromatic Molecules across a Membrane: A Computational Study. *ACS Omega* **2020**, *5*, 4798–4806. [CrossRef]
62. Zheng, Y.; Sun, J.; Luo, Z.; Li, Y.; Huang, Y. Emerging Mechanisms of Lipid Peroxidation in Regulated Cell Death and Its Physiological Implications. *Cell Death Dis.* **2024**, *15*, 859. [CrossRef]

doi:10.6342/NTU202501708