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茶多酚對甲基乙二醛誘導人類神經細胞毒性之保護功效

The Protective Effect of Tea Polyphenol against Methylglyoxal-induced Neurotoxicity

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國立臺灣大學碩士學位論文
口試委員會審定書

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The Protective Effect of Tea Polyphenol against Methylglyoxal-induced Neurotoxicity

本論文係黃孜立君（學號 R03641036）在國立臺灣大學食品科技研究所完成之碩士學位論文，於民國 105 年 7 月 20 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

（簽名）

（指導教授）

系主任、所長 （簽名）

謝誌

時光流逝總是令人不知所措，一眨眼碩士生涯即將步入尾聲，隨著論文的付梓更有一種完成的成就與離別的惆悵交織的複雜感受。首先感謝恩師 潘敏雄及李銘仁老師的悉心指導，在研究過程當中不斷給予協助與關心，當我面臨抉擇時老師們適時的給予指引，如此我才能夠在未知的領域當中自由翱翔，藉此理解研究的精髓並擴展了我的視野。此外感謝 何其儻教授在實驗架構建立上給予許多嶄新的想法與更開闊的眼界，以及口試委員何元順、陳宏彰教授協助我口試並給予我許多建議使我的論文更趨完整。

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中文摘要

甲基乙二醛 (methylglyoxal, MG) 為一種高反應活性的雙羰基醛類化合物，已有許多研究證實 MG 造成神經損傷進一步導致神經退化性疾病的發生。茶葉是一種傳統的飲品，流行病學研究指出，長期飲茶能有效降低罹患神經退化性疾病的風險。文獻提出綠茶中的主要活性物質:表沒食子兒茶素沒食子酸酯「(-)-epigallocatechin-3-gallate, EGCG」以及烏龍茶經發酵後產生的 EGCG 二聚物:聚酯型兒茶素 A (theasinensin A, TSA) 具有抗氧化等功效，然而其在神經保護之功效與分子機制仍不清楚。因此，本篇研究擬以人類神經瘤細胞 SH-SY5Y 探討茶多酚 EGCG 以及 TSA 對於 MG 誘導的神經毒性之保護作用及其分子機轉。本研究顯示，EGCG 與 TSA 對於 MG 誘導的神經毒性具有相當的保護效果。EGCG 與 TSA 皆可透過阻斷 MG 誘導之活性氧物質 (reactive oxygen species, ROS) 產生以及維持胞內 GSH 含量，藉此減少 MG 誘導的氧化壓力。透過 caspase 3 活化及其基質 PARP 降解可知 EGCG 能抑制 MG 誘導之細胞凋亡現象，然而 TSA 抑制 MG 誘導細胞凋亡現象並不顯著。透過 acridine orange (AO) 染色於共軛焦顯微鏡下觀察、AO 染色後透過流式細胞儀測量螢光強度以及 LC3 蛋白質的表現判斷 MG 在 TSA 存在下會促使細胞走向自噬作用進而保護神經細胞。在 MG 誘導神經毒性之情形下，EGCG 抑制 p53、MAPK 訊息傳遞路徑進而抑制細胞凋亡，另一方面 TSA 透過抑制 Akt 訊息傳遞路徑達到促進細胞自噬作用之功效。綜合上述發現在 MG 誘導之神經細胞死亡中 EGCG 和 TSA 扮演不同角色與功能，EGCG 透過抑制 MG 誘導的細胞凋亡而 TSA 則是透過誘發細胞自噬進而保護細胞免於 MG 誘導的細胞毒性。我們期望此研究成果未來能提供茶多酚開發為預防 MG 誘導神經相關疾病發展之重要依據。

關鍵字：甲基乙二醛、表沒食子兒茶素沒食子酸酯、聚酯型兒茶素 A、細胞凋亡、細胞自噬、氧化壓力

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Abstract

Methylglyoxal (MG) is a highly reactive dicarbonyl aldehyde. MG has been proved to be toxic to neuron and may be the reason of many neurodegenerative diseases. Tea is a traditional drinking and previous studies have observed that chronic tea consumption may reduce the risk of neurodegeneration. One of the most well-known tea catechin, (-)-epigallocatechin-3-gallate (EGCG), and theasinensin A (TSA), the dimer of EGCG had been found to have many bioactivities, such as anti-oxidation. However, the effect of the neuroprotective effect of tea polyphenol remains unclear. The aim of this study is to investigate the neuroprotective effects and the molecular mechanism of tea polyphenol against MG-induced toxicity in SH-SY5Y cell model. The cell viability assay demonstrated that EGCG and TSA treatment protected cells from MG-induced neurotoxicity. Besides, evidences from flow cytometry showed that EGCG and TSA inhibited MG-induced oxidative stress via reducing MG-induced reactive oxygen species (ROS) generation and induce intracellular GSH level. Through activation of caspase 3 and cleavage-PARP, EGCG would inhibit MG-induced apoptosis but TSA did not inhibit MG-induced apoptosis. The result of acridine orange (AO) stain, detecting AO fluoresce intensity and the level of LC3, showed that TSA would induce autophagy in MG-treated neuron cell. EGCG inhibited p53 and MAPK pathway to increase cell viability via western blotting analysis; on the other hand, TSA inhibited Akt pathway to induce autophagy. In conclusion, both EGCG and TSA has the ability to protect cell from MG-induced cytotoxicity via different mechanisms. We expect that EGCG and TSA could be reagents to protect neurodegenerative diseases.

Keywords： Methylglyoxal (MG)、(-)-Epigallocatechin-3-gallate (EGCG)、Theasinensin A (TSA)、Apoptosis、Autophagy、Oxidative stress

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圖目錄

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縮寫表

AGEs	advanced glycation end products
Akt	protein kinase B
ANOVA	analysis of variance
AO	acridine orange
Apaf-1	apoptotic protease-activating factor-1
AVO	acidic vesicular organelles
Bax	bcl-2-associated x protein
Bcl-2	B cell lymphoma-2
Bid	BH3 interacting-domain death agonist
BSA	bovine serum albumin
Caspase	cysteine-dependent aspartate specific protease
CMFDA	5-chloromethylfluorescein diacetate
DCFH	dichlorofluorescin
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DiOC6	3,3′-dihexyloxacarbocyanine iodide
DMSO	dimethyl sulfoxide
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin 3- gallate
ERK	extracellular signal-regulated protein kinases
FBS	fetal bovine serum

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GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
HO-1	heme oxygenase-1
JNK	c-jun n-terminal kinases
MAPK	mitogen-activated protein kinases
MG	methylglyoxal
MMP	mitochondria membrane potential
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PARP	poly ADP ribose polymerase
PCD	programmed cell death
PI3K	phosphoinositide 3-kinase
ROS	reactive oxygen species
SOD	superoxide dismutase
TSA	theasinensin A

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第一章 前言

甲基乙二醛 (methylglyoxal, MG) 屬於活性羰基物質 (reactive carbonyl species , RCS)，可在體內自行生成或是藉由梅納反應在體外產生。在體內合成中，MG 是糖解作用的副產物，另外也能藉由多元醇路徑產生 MG，內生性的 MG 約占 0.1%的糖解總產物 (Allaman et al., 2015)。外源性來源主要由食物中的梅納反應 (Maillard reaction) 產生，梅納反應是食品加工過程中會產生香味及顏色的非酵素褐變反應，反應方式相當複雜，主要由還原醣與胺基酸反應活化一系列的化學變化，MG 是其中一種產物 (Wang and Ho, 2012)。MG 廣泛分布於高度梅納反應的食物與飲料當中，如蛋糕、飲料。

神經退化性疾病是一種造成神經細胞型態功能改變或是神經細胞死亡的疾病，大多數的神經退化性疾病患者集中在老年人口。隨著全球人口數攀升，神經退化性疾病的發生率也隨之提升。先前的研究發現，神經損傷與糖尿病有相當程度的關聯性 (Sun et al., 2012)，研究者推測與高血糖環境產生過多的 RCS，此物質可直接在體內產生氧化壓力以及與胺基酸反應產生許多具有細胞毒性的糖化終產物 (Advanced glycation end-product , AGES) (Rabbani and Thornalley, 2015) 進而導致神經損傷。其中，MG 是最大量的且擁有與胺基酸結合最高活性的 RCS，因此研究者推測 MG 在帕金森氏症的形成中扮演相當重要的一環。然而在神經退化性疾病治療上並沒有很有效的治療方法，因此，如何預防神經退化性疾病儼然成為一個國際趨勢。

茶葉是大東亞地區主要的飲品之一也是全世界最廣為飲用之飲料。茶葉中含量最多同時也是最有效的植化素-兒茶素(catechin) 已被廣泛研究 (Qiao et al., 2014)，兒茶素是茶多酚中最重要的一種，約佔茶多酚含量的 75%到 80% (Qiao et al., 2014)，也是茶的苦澀味的來源之一。兒茶素主要分為四種：表兒茶素(epicatechin,

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EC)、表沒食子兒茶素(Epigallocatechin, EGC)、表兒茶素沒食子酸酯(epicatechin gallate, ECG)和表沒食子兒茶素沒食子酸酯(epigallocatechin gallate, EGCG)。兒茶素在發酵過程中透過氧化還原作用產生發酵茶中特殊多酚類: 聚酯型兒茶素 (Lee et al., 2008, Li et al., 2013)，而具有相當生理功效的茶多酚 EGCG 經過發酵後產生的二聚物-聚酯型兒茶素 A (theasinensin A, TSA) 是烏龍茶中特殊的植化素，已被研究具有抗菌 (Hatano et al., 2003)、抗發炎 (Pan et al., 2000)、抗氧化 (Hou et al., 2005)、降血脂 (Abe et al., 2000)等功效，因此認為 TSA 具有神經保護的潛力。

如何預防神經退化性疾病為現今重要的課題，若能改善日常飲食就擁有預防神經退化性疾病的功效，將對暴露在神經退化性疾病風險下的民眾有正面影響。然而茶多酚抑制帕金森氏症之功效及其分子機制並不清楚，因此為了解茶多酚之功效，細胞實驗將利用西方墨點法測量抗凋亡訊息傳導路徑蛋白質表現量及流式細胞儀測量抗氧化與細胞存活情形。綜合以上，本研究的主旨是藉由細胞實驗證實茶多酚 EGCG 以及 TSA 是否能抑制 MG 所產生的神經毒性及其分子機制。

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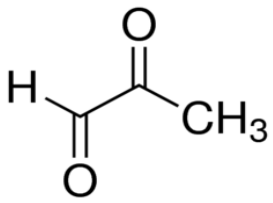
第二章 文獻回顧

第一節　甲基乙二醛 (methylglyoxal, MG)

(一) 結構

MG 示性式 $CH_3C(O)CHO$，分子量為 72.06 的有機化合物 (附圖 1)，具有兩個羰基、一個酮基、一個醛基，為一種高反應活性雙羰基醛類化合物 (highly reactive dicarbonyl aldehyde)，已有許多研究指名其對於人體有害處 (Kalapos, 2008, Rabbani and Thornalley, 2015)。



(http://www.sigmaaldrich.com/catalog/product/sigma/m0252?lang=en&region=TW)

附圖 1 MG 的結構式

(二) 內生性與外生性來源

MG 有內生性與外生性來源，細胞可藉由醣類、蛋白質與脂質三大營養素代謝產生 MG，其中以醣類代謝產生之 MG 為內生性主要來源。內生性的 MG 約占 0.1% 的糖解總產物 (附圖 2)。細胞藉由糖解作用 (glycolysis reaction) 與多元醇路徑 (polyol pathway) (Chakraborty et al., 2014)，代謝果糖後產生相同的中間產物甘油醛 3-磷酸 (glyceraldehyde-3- phosphate, GA3P) 與二羥丙酮磷酸 (dihydroxyacetone phosphate, PEP)，此二化合物進行非酵素反應後產生 MG (Allaman et al., 2015)。而蛋白質來源則可由蘇胺酸代謝中間產物 3-aminoacetone 產生，另外亦能由脂質過氧化產生。

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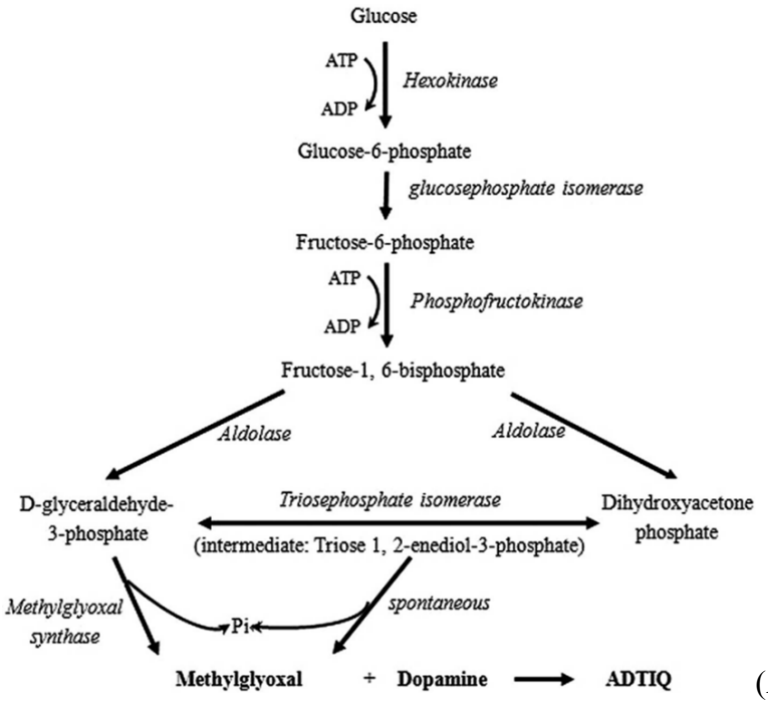

(Allaman et al., 2015)

附圖 2 MG 體內合成路徑

外生性來源主要是經由梅納反應產生。梅納反應是食品加工中產生香味及色澤的非酵素褐變反應，反應方式相當複雜，主要由還原糖與胺基酸反應活化一系列的化學變化，MG 是其中一種產物 (Eskin et al., 2013, Wang et al., 2009)。食物中的葡萄糖經由自氧化產生葡萄糖醛酮 (glucosone) 經由電子重組產生 1,2-enol 或是 2,3-enol，羥醛縮合反應 (retro-aldol condensation) 後產生 1-脫氧葡萄糖醛酮 (1-deoxyglucosone, 1-DG) 與 3-脫氧葡萄糖醛酮 (3-deoxyglucosone, 3-DG)，MG 由 3-脫氧葡萄糖醛酮斷裂產生 (附圖 3)。但除了上述路徑之外，MG 也可能由希夫鹼 (Schiff base) 產生 (Wang and Ho, 2012)。MG 廣泛分布於高度梅納反應的食物與飲料當中，其中在含糖量較高的食物較容易產生葡萄糖的自氧化，如蛋糕、飲料、蜂蜜、咖啡 (Lo et al., 2008, Wang and Ho, 2012)。因此，不論是體內產生或是飲食中攝取得來人們每天都會接觸到 MG。

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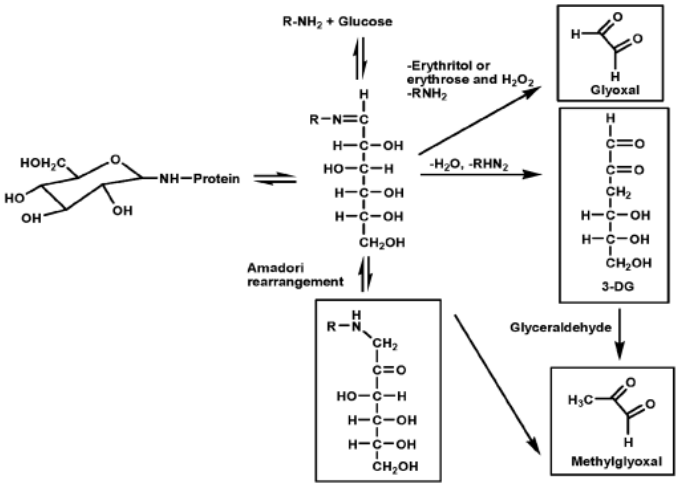

(Wang and Ho, 2012)

附圖 3 MG 梅納反應產生途徑

(三) 蛋白質修飾作用與糖化終產物之形成

MG 在體內很容易與 DNA、脂質及蛋白質結合產生有細胞毒性的糖化終產物 (advanced glycation end products, AGES) (附圖 4) (Rabbani and Thornalley, 2015)。這些糖化終產物會破壞正常蛋白質結構，使蛋白質交疊連接 (cross-link) 變性失去功能，研究已證實糖化終產物是糖尿病併發症一個相當重要的毒性物質。

MG 修飾所產生的糖化終產物有很多種，修飾離胺酸 (lysine) 會產生 N_{ε}-(1-carboxyethyl)lysine (CEL) 和 MG-derived lysine dimer (MOLD)，修飾精胺酸 (arginine) 會產生 hydroimidazolone N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl) ornithine (MGH1) 和 argpyrimidine (AP) (Chakraborty et al., 2014, Rabbani and Thornalley, 2015, Shipanova et al., 1997)。

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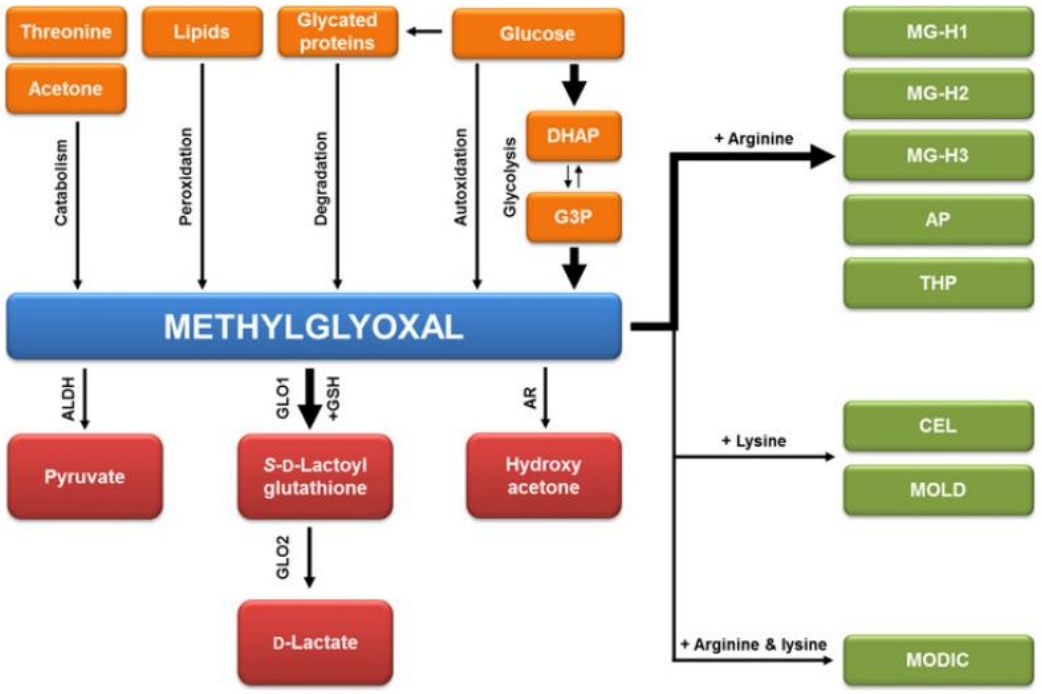

(Rabbani and Thornalley, 2015)

附圖 4 MG 與糖化終產物的生成

(四) MG 所造成的細胞損傷

MG 不只是糖化終產物的前驅物 (precursor)，更會直接體內產生氧化壓力，氧化壓力在發炎、細胞凋亡和細胞分化中扮演重要角色。MG 在不同的細胞，如神經細胞 (de Arriba et al., 2007) 與心肌細胞 (Vulesevic et al., 2014) 都會增加活性氧 (reactive oxygen species, ROS)、超氧陰離子、氫氧自由基、過氧化氫、過氧亞硝酸及促發炎細胞激素。MG 除了直接增加自由基的生成，亦會透過降低抗氧化物及酵素活性而增加體內氧化壓力進而導致細胞的死亡。

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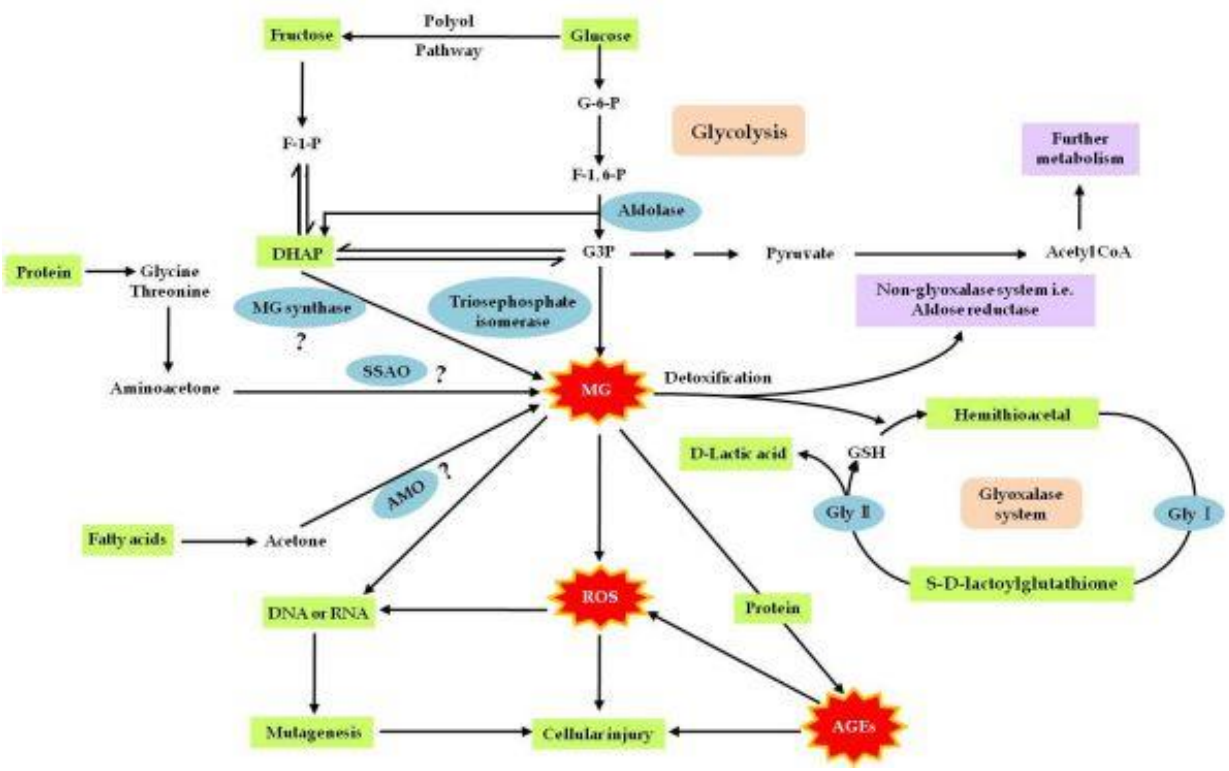

(Hossain et al., 2011)

附圖 5 MG 產生 ROS 與解毒路徑

(五) 對生物體的傷害

在先前研究中指出，MG 會造成葡萄糖吸收受損 (de Arriba et al., 2007)、抗氧化壓力失調 (Rabbani and Thornalley, 2015)、產生糖化終產物 (Chakraborty et al., 2014) 且與許多疾病相關，如糖尿病 (Tikellis et al., 2014)、動脈粥狀硬化 (Ahmed et al., 2014)、高血壓 (Rabbani and Thornalley, 2015)、神經受損 (Bennett et al., 2014, Zeier et al., 2003) 等。大部分 MG 造成的都是長期的傷害，它對於人體的許多器官都有不良的影響，特別是眼睛、肝臟、神經、血管。

1. 血管病變

在體內高血糖的情形下，血管內環境處在高度糖化狀態，血管內壁蛋白質以

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及血球都有可能受到 MG 攻擊產生氧化壓力與糖化終產物，導致蛋白質變性失去功能或是死亡 (Ahmed et al., 2014, Vulesevic et al., 2014, Wortmann et al., 2014)。

2. 視網膜病變

視網膜病變乃因視網膜受損，使血管破裂阻塞，液體由血管滲入水晶體，導致病患視覺模糊或是暫時失明的現象。糖尿病患者的水晶體內糖含量比較高，導致細胞走向多元醇路徑，產生更多的 MG，造成細胞的死亡 (Berner et al., 2012)。且視網膜的血管多且細小，MG 對於視網膜血管的損害更為顯著。實驗發現高濃度的 MG 會導致體外培養之視網膜細胞生長停滯及死亡，進而證明 MG 與視網膜病變相關 (Berner et al., 2012, Fosmark et al.)。

3. 肝臟疾病

已有研究發現 MG 會導致肝發炎 (Leclercq et al., 2007)，在肝細胞模式發現 MG 不只會產生氧化壓力，還會促進分化與促進發炎，導致肝炎更為顯著 (Leclercq et al., 2007)。

4. 神經退化性疾病

神經退化是一個廣泛的名詞，許多疾病可歸類為神經退化性疾病:像是阿茲海默症以及帕金森氏症 (Przedborski et al., 2003)。根據統計，在 2005 年神經退化性疾病約占全球負擔疾病的 6.3% (World health organization, 2006)。隨著老年人口的增加，罹患神經退化性疾病的人口也隨之增加。

阿茲海默症與帕金森氏症都是退化性神經疾病，阿茲海默症主要的病理特徵在於皮質與海馬迴神經細胞產生斑塊或是糾結，導致神經細胞的死亡，產生症狀如癡呆、口齒不清。研究指出阿茲海默症病患的腦脊髓液中 MG 含量顯著高於健康人 (Angeloni et al., 2014)。且餵食過多的 MG 的小鼠在老年時易產生較多的運動

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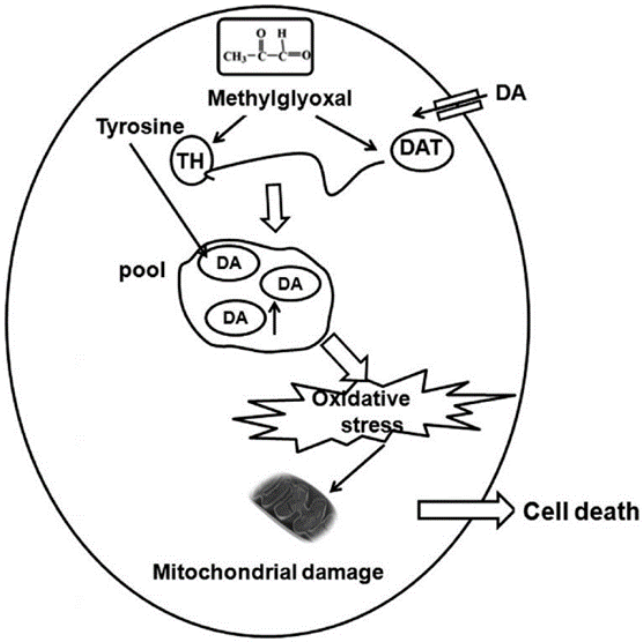
記憶能力缺失 (Angeloni et al., 2014)。

另外，帕金森氏症的病因為腦部黑質多巴胺生成細胞大量死亡導致身體控制能力失調，目前發現會導致多巴胺生成細胞死亡的原因相當多，包括遺傳性基因缺陷、環境毒素影響等等。遺傳性帕金森氏症是 DJ-1 基因缺陷導致抗氧化酵素無法而成，而無法抵擋 MG 所產生的氧化壓力 (Blesa et al., 2012)。2012 年台灣發表的文獻中指出:糖尿病患增加 1.3 倍風險罹患帕金森氏症 (Sun et al., 2012)，因此研究者認為高血糖會產生神經毒性物質，進而造成神經損傷，而 MG 被認為是誘發帕金森氏症之重要的神經毒性物質 (Xie et al., 2014)。在 2014 年的研究發現 MG 會使細胞內的多巴胺增加，造成神經細胞的氧化壓力上升進而導致神經細胞的死亡 (附圖 6) (Xie et al., 2014)。



(Xie et al., 2014)

附圖 6 MG 導致細胞內氧化壓力上升

此外，MG 除了會促進細胞內多巴胺生成量增加，還會與多巴胺結合生成 salsolinol 類毒性物質 (Song et al., 2014, Xie et al., 2015)。

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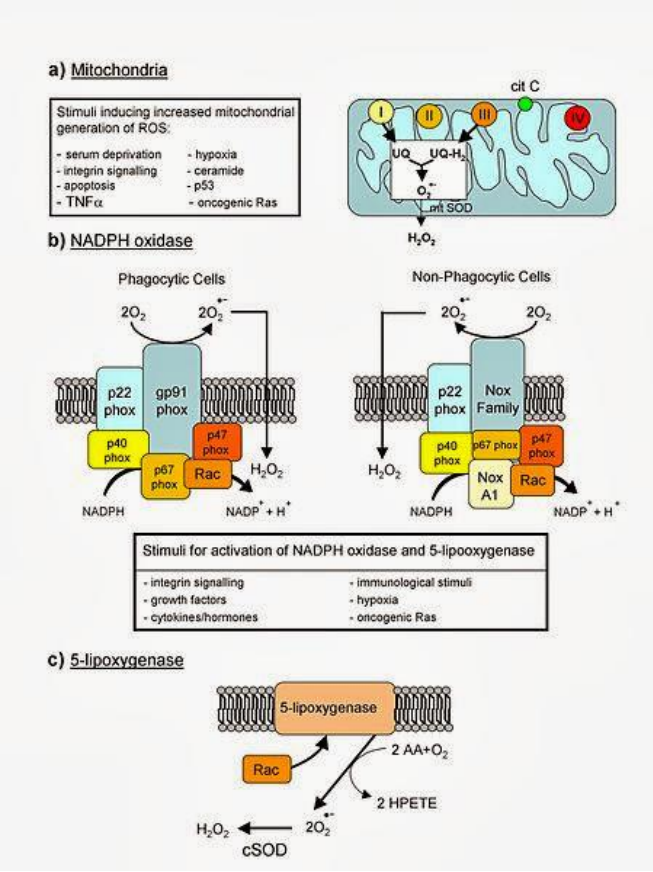
第二節　活性氧分子 (reactive oxygen species, ROS)

(一) 活性氧分子與氧化壓力 (oxidative stress)

ROS 是具有一個或多個未配對電子團的含氧分子，因此其具有相當高的反應活性能與其他分子結合，包含超氧陰離子自由基 ($O^{2.-}$)、單線態氧 ($^1O_2$)、過氧化氫 (H_2O_2) 和高活性的羥基自由基 (•OH) (Bayr, 2005)。在正常情況下，ROS 會透過一般代謝途徑產生 (附圖 7)，例如粒線體電子傳遞鏈 (electron transport chain)。然而，當細胞內 ROS 濃度太高時，ROS 的高活性會造成 DNA 的損害、蛋白質變性以及脂質的過氧化，造成細胞結構或是功能的損害，進而引起衰老、退化性疾病、心血管疾病、神經變性疾病。因此，如何調節體內 ROS 含量對於細胞的存活來說是相當重要的。



(Novo and Parola, 2008)

附圖 7 ROS 主要生成來源

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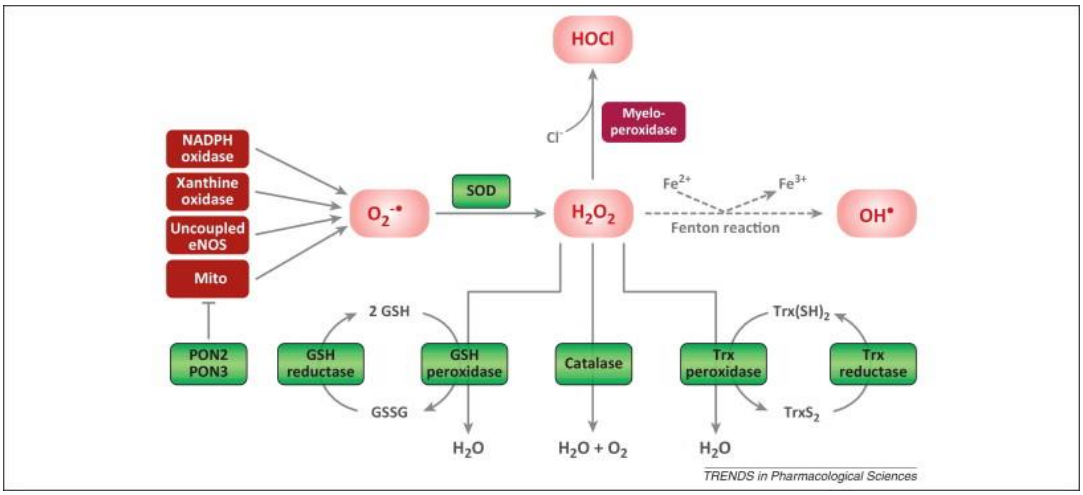
(二) 抗氧化防禦系統

為了抵抗 ROS 產生的氧化壓力，細胞內有一套對抗氧化壓力的方式，使 ROS 轉化成毒性較小或無毒的分子，使細胞免於氧化壓力的傷害。抗氧化防禦系統包含酵素型防禦系統及非酵素型防禦系統 (附圖 8)。酵素型防禦系統，如超氧化物歧化酶 (superoxide dismutase, SOD)、氫氧化酶 (catalase, CAT)、穀胱甘肽 (glutathione, GSH) 系統。非酵素型防禦系統包含維生素 E (α-tocopherol) 和抗壞血酸 (ascorbic acid) (Yadav et al., 2005)。



(Yadav et al., 2005)

附圖 8 透過抗氧化防禦機制以清除活性氧分子

其中 GSH 系統廣泛分布於各種型態的細胞中，更因為其能將酵素型與非酵素型抗氧化系統串聯的特色，在抗氧化防禦系統當中扮演舉足輕重的腳色 (附圖 9)。GSH 由穀氨酸 (glutamate)、半胱氨酸 (cysteine)及甘氨酸 (glycine) 所構成，位於粒線體的 GSH peroxidase 經過氧化氫還原成水與氧時協同作用使兩個 GSH 分子氧化為 glutathione disulfide (GSSG)，再透過 GSH-reductase 與 NADPH 的協助，可使 GSSG 再次回收生成 GSH，非酵素抗氧化系統如維生素 A、E、抗壞血酸也會協助 GSH 的回收。

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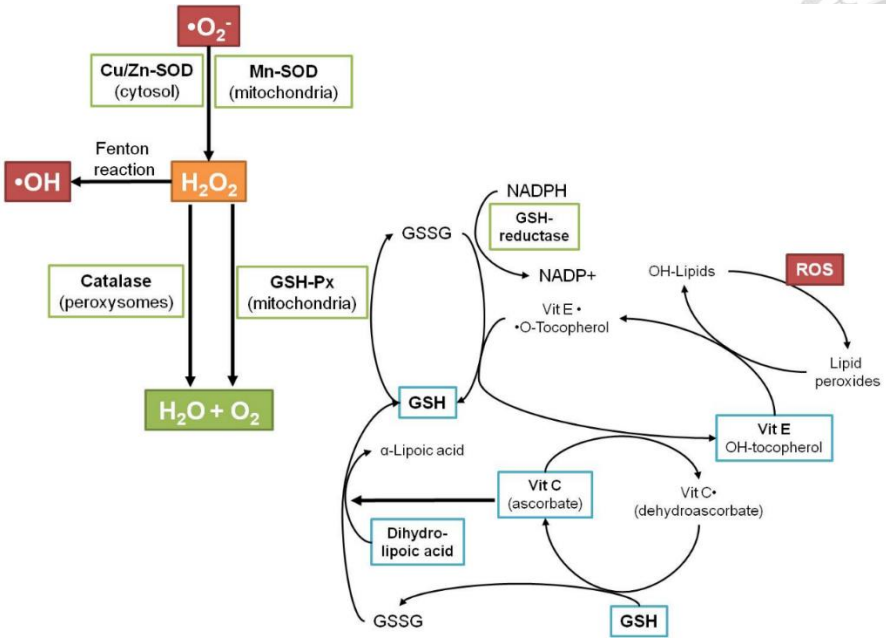

(Fernández-Mejía, 2013)

附圖 9 GSH 抗氧化系統

活性氧分子和抗氧化防禦系統在細胞中必須保持平衡以維持正常的細胞功能，當活性氧分子和抗氧化防禦系統之間不平衡則產生氧化壓力，氧化壓力的存在被認為是涉及癌症、帕金森氏症、阿茲海默症、動脈粥樣硬化等多種疾病的發展(Zhang and Gordon, 2004)。

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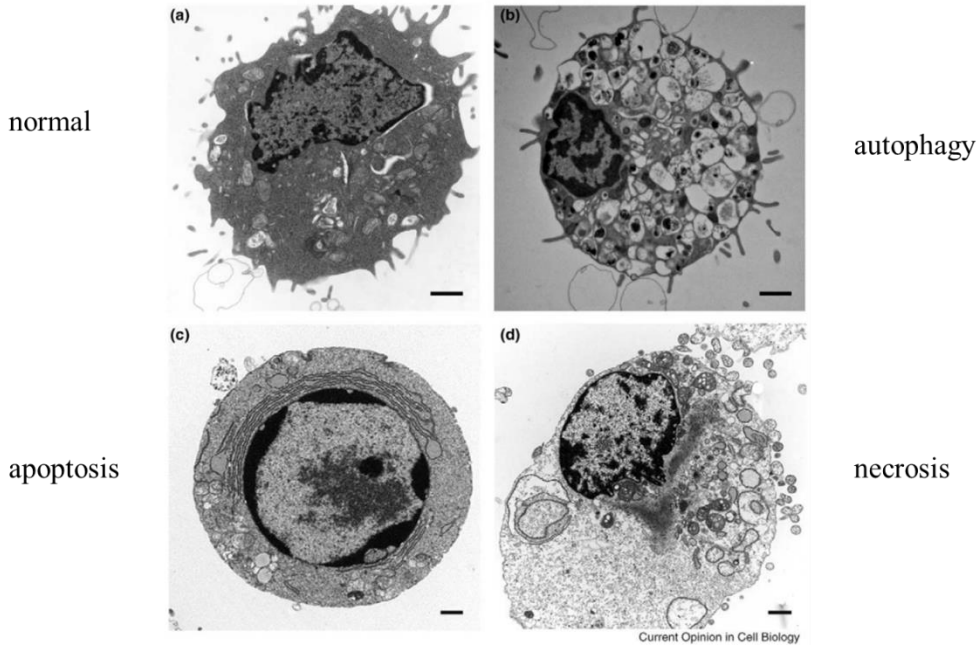
第三節　細胞死亡的途徑及保護機制

細胞死亡的方式主要分為細胞凋亡、細胞自噬、細胞壞死，這三種細胞死亡的方式，細胞呈現不同的形態 (附圖 10)，其誘導機制與調控機制以及對於生物體的影響也不盡相同因此能夠透過細胞型態初步區分細胞死亡型態 (附圖 11)。本論文就兩大類細胞程序性死亡：細胞凋亡以及細胞自噬做詳細的研究。



(Edinger and Thompson, 2004)

附圖 10 不同細胞死亡的型態比較

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Type of cell death	Morphological changes			Biochemical features	Common detection methods
	Nucleus	*Cell membrane*	*Cytoplasm*		
Apoptosis	Chromatin condensation; nuclear fragmentation; DNA laddering	Blebbing	Fragmentation (formation of apoptotic bodies)	Caspase-dependent	Electron microscopy; TUNEL staining; annexin staining; caspase-activity assays; DNA-fragmentation assays; detection of increased number of cells in subG1/G0; detection of changes in mitochondrial membrane potential
Autophagy	Partial chromatin condensation; no DNA laddering	Blebbing	Increased number of autophagic vesicles	Caspase-independent; increased lysosomal activity	Electron microscopy; protein-degradation assays; assays for marker-protein translocation to autophagic membranes; MDC staining
Mitotic catastrophe	Multiple micronuclei; nuclear fragmentation	–	–	Caspase-independent (at early stage) abnormal CDK1/cyclin B activation	Electron microscopy; assays for mitotic markers (MPM2); TUNEL staining
Necrosis	Clumping and random degradation of nuclear DNA	Swelling; rupture	Increased vacuolation; organelle degeneration; mitochondrial swelling	–	Electron microscopy; nuclear staining (usually negative); detection of inflammation and damage in surrounding tissues
Senescence	Distinct heterochromatic structure (senescence-associated heterochromatic foci)	–	Flattening and increased granularity	SA-β-gal activity	Electron microscopy; SA-β-gal staining; growth-arrest assays; assays for increased p53, INK4A and ARF levels (usually increased); assays for RB phosphorylation (usually hypophosphorylated); assays for metalloproteinase activity (usually upregulated)

CDK1, cycline-dependent kinase 1; MDC, monodansylcadaverine; MPM2, mitotic phosphoprotein 2; SA-β-gal, senescence-associated β-galactosidase; RB, retinoblastoma protein.

(Okada and Mak, 2004)

附圖 11 不同細胞死亡型態與檢測方法比較

(一) 細胞凋亡 (apoptosis)

1. 介紹

細胞凋亡又稱第一型細胞程式性死亡 (programmed cell death I) 是生物維持正常生理運作所需的細胞死亡調控方式，可以在不引起發炎反應的情形下去除生物體破損、衰老或是不需要的細胞，例如胚胎發育或是表皮細胞維持衰老細胞與新生細胞恆定。然而若是細胞凋亡失去調控將會與許多生理疾病有密切關聯，例如神經退化、免疫失調等疾病的發生。

細胞凋亡的發生包含以下步驟 (附圖 12)，正常細胞受到刺激後，細胞開始皺縮 (cell shrink) 且染色體凝集 (chromatin condensation)，接著胞膜外翻起泡 (membrane blebbing)、細胞核片斷化 (nuclear collapse)，最後形成凋亡小體

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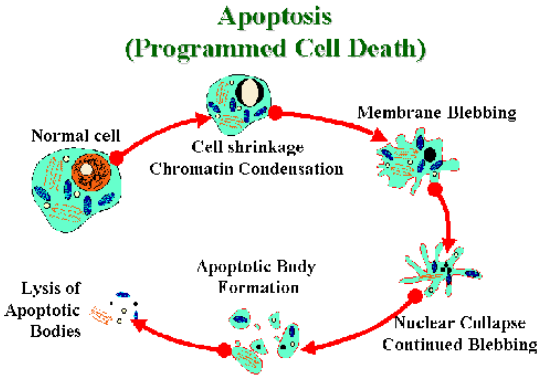
(apoptosis body formation)。巨噬細胞會經由外翻的磷脂質：磷脂醯絲氨酸(phosphatidylserine, PS) 辨識凋亡小體的位置並將其吞噬 (phagocytosis)，因此細胞凋亡並不會引起發炎反應。

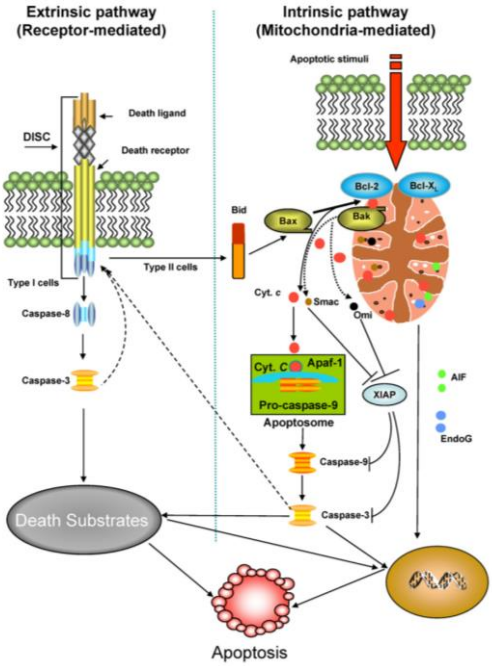


(Sameer, 2009)

附圖 12 細胞凋亡的過程

2. 細胞凋亡路徑

細胞凋亡可分為外在路徑 (extrinsic pathway, or receptor mediated pathway) 以及內在路徑 (intrinsic pathway, or mitochondria-mediated pathway) (附圖 13)。



(Fulda and Debatin, 2006)

附圖 13 細胞凋亡內在路徑與外在路徑

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外在路徑是因為細胞表面的死亡受器 (death receptor) 與細胞外配體 (ligand) 結合進而活化下游凋亡路徑而引發細胞凋亡。死亡受器為 (tumor necrosis factor, TNF) 受體家族之一，像是 TNF receptor 1 (TNFRI)，TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1)，相對應的配體分別為 TNFα、lymphotoxin-α 和 TRAIL。當配體與受器結合後活化下游的 caspase-8，caspase-8 進而活化下游 caspase-3 進而導致細胞凋亡或是由 caspase-8 剪切 Bid，進一步刺激粒線體活化。

內在路徑活化經由多種刺激，如細胞內 ROS 不平衡、內質網壓力，導致 Bcl2 家族蛋白活化，使粒線體外膜通透性增加進而釋放細胞色素 c (cytochrome c) 並活化下游相關凋亡蛋白，最終導致細胞凋亡。內在路徑又可依照有無 caspase 參與分為 caspase-dependent pathway 以及 caspase-independent pathway。caspase-dependent pathway 是利用調控粒線體通透性使細胞色素 c 釋放並活化下游 apoptotic protease-activating factor-1 (Apaf-1)，在 dATP 協助下 Apaf-1 與 caspase-9 結合形成 apoptosome 進而活化下游 caspase-3 導致細胞凋亡。caspase-independent pathway 透過調控粒線體膜通透性，使粒線體間質的凋亡相關蛋白釋出，例如 apoptosis-inducing factor (AIF)，endonuclease G (EndoG)，這些凋亡蛋白導致 DNA 降解最終使細胞死亡。

(二) 細胞自噬 (autophagy)

1. 介紹

細胞自噬為細胞受到氧化壓力、內質網壓力、飢餓等刺激誘導的路徑，細胞型態上會在細胞內產生一個個的小泡，這些小泡為自噬溶小體 (autolysosome)，內部富含許多酵素，能夠分解細胞內未正常折疊的蛋白質或是老舊胞器，分解後的代謝物作為能量或回收利用。細胞自噬的過程有三個階段：起始期、延長期與成熟期 (附圖 14)。當細胞受到刺激時，位於內質網的 Beclin 1/VPS34 蛋白複合體促使細胞內形成獨立的雙層膜構造-吞噬泡 (phagophore)，此為起始期。接著延長期

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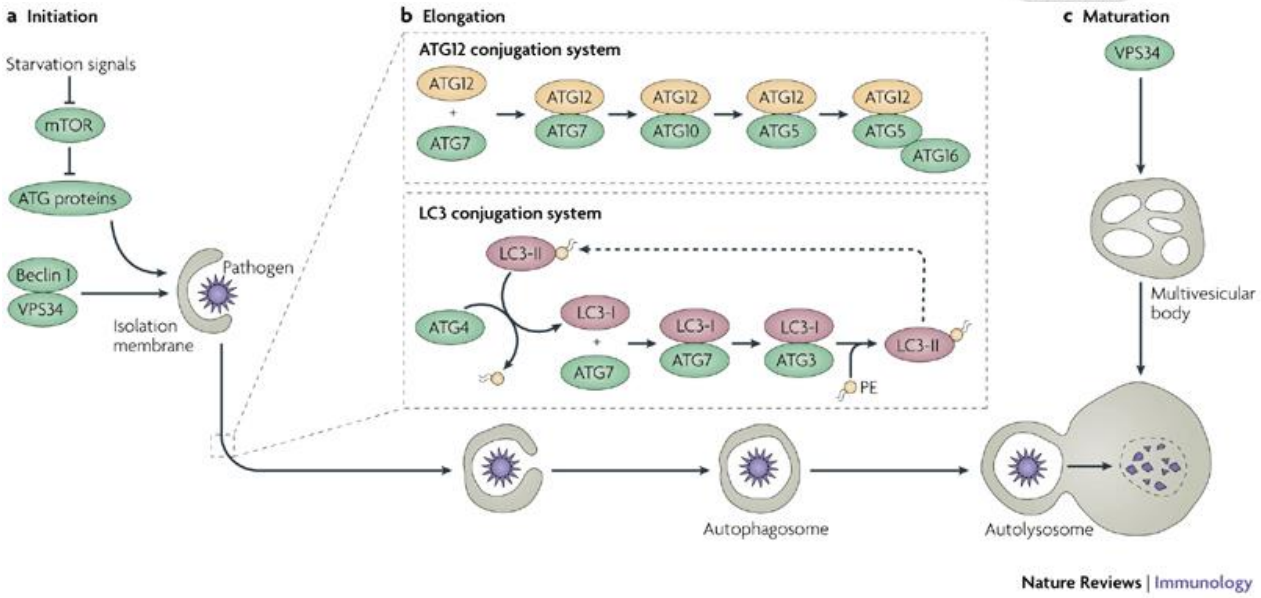
時活化 ATG 聚合系統與 LC3 聚合系統將吞噬泡經過一系列蛋白修飾使吞噬泡延長而生成自噬體 (autophagosome)。最後成熟期時 VPS34 促使自噬體與溶酶體 (lysosome) 融合生成酸性的自噬溶小體，使物質能在自噬溶小體中進行分解。



(Klionsky and Emr, 2000)

附圖 14 自噬作用的三個階段

2. 細胞自噬路徑

細胞自噬可分為三類：巨型自噬 (macroautophagy)、微型自噬 (microautophagy)、伴蛋白調節自噬 (chaperone-mediated autophagy, CMA) (附圖 15)。巨型自噬會生成雙層膜構造的自噬體，是細胞自噬當中最重要的路徑，主要應用在自噬老舊胞器。相反的，微型自噬並沒有繁瑣的誘導路徑，目標蛋白直接由溶酶體吞噬。最後，伴蛋白調節自噬是目標蛋白由具有 KFERQ motif 的胜肽標示後，由 Hsp70 伴蛋白辨認後將蛋白帶入自噬溶小體中進行分解。

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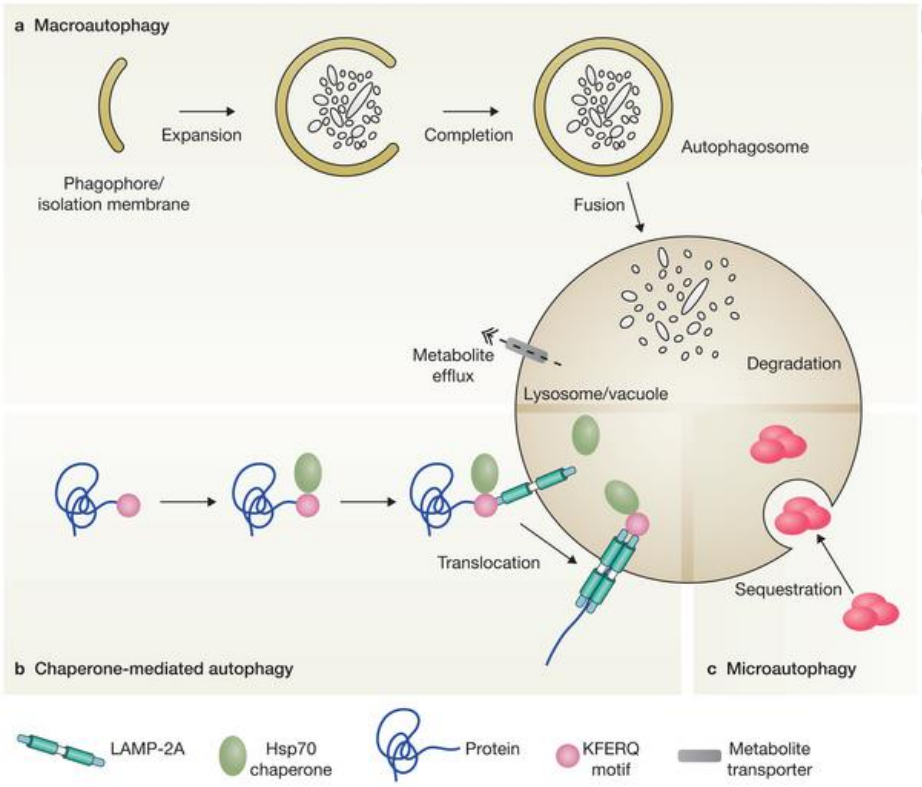

(Boya et al., 2013)

附圖 15 三種細胞自噬的模式

細胞自噬的作用可依據其分解細胞的程度分為細胞保護型以及細胞死亡型細胞自噬 (附圖 16)。首先因為自噬溶小體將胞內物質分解作用產生胺基酸及游離脂肪酸，而這些物質能提供粒線體作為能量使用或提供蛋白質合成所需的胺基酸，或是藉由除去對細胞有害的胞器進而提升細胞存活率，因此細胞自噬被認為具有調控細胞生長的功能。然而，細胞自噬也能透過自我拆解 (self-cannibalization) 及誘發細胞凋亡進而引發細胞死亡，因而細胞自噬具有雙重的功能。

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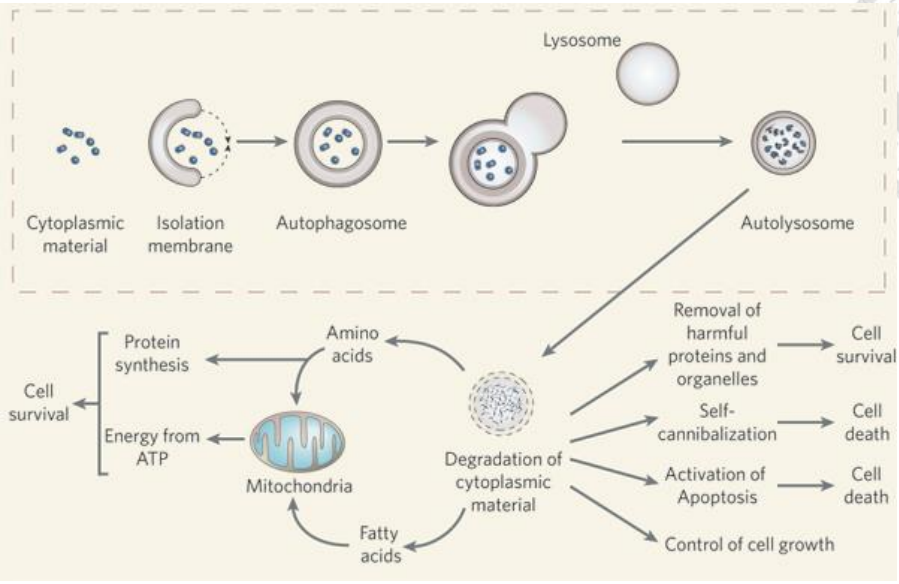

(Levine, 2007)

附圖 16 細胞自噬在細胞中扮演的角色

(三) 細胞凋亡與細胞自噬之間的交互作用

細胞凋亡與細胞自噬皆屬於程序性細胞死亡的一環，兩者的誘導路徑與細胞型態截然不同，然而他們的調控方式卻有著緊密且錯綜複雜的關係，目前有越來越多的研究證實細胞凋亡與細胞自噬間存在合作 (cooperate)，對抗 (antagonize) 或協助 (assist) 的關係 (Nikoletopoulou et al., 2013)。在合作關係下，細胞凋亡與細胞自噬皆會引起細胞死亡。在對抗關係下，細胞凋亡促進細胞死亡然而細胞自噬卻保護細胞避免細胞死亡。在協助關係下，細胞自噬並不直接導致細胞死亡，而是透過誘發細胞凋亡或是提供能量的方式促進細胞死亡 (附圖 17)。

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Table 1 Comparison of the two types of programmed cell death

	Type I apoptotic	*Type II autophagic*
Nucleus	Chromatin condensation Pyknosis of nucleus DNA laddering and nuclear fragmentation	Partial chromatin condensation Sometimes pyknosis of nucleus Nucleus intact until late stages No DNA laddering
Cytoplasm	Cytoplasmic condensation Ribosome loss from RER Fragmentation to apoptotic bodies Lysosomal protease release to cytosol may be involved Mitochondrial permeability transition is often involved Caspases are active	Increased autophagic vesicle number Increased autolysosome number Increased lysosomal activity Enlarged Golgi, sometimes dilatation of ER Mitochondrial permeability transition may be involved Caspase-independent
Cell membrane	Blebbing	Blebbing
Corps clearence	Heterophagy by other cells	Late and occasional heterophagy by other cells
Detection methods	Electron microscopy Nuclear/cellular fragmenation detection Caspase activation tests Caspase substrate cleavage tests DNA laddering detection TUNEL staining Increase in sub G1 cell population assessed by FACS analysis Annexin V staining	Electron microscopy Test of increased long-lived protein degradation Tests of increased lysosomal activity (MDC, acridine orange or lysotracker staining, etc) Test of increased cytoplasmic sequestration (LDH or sucrose sequestration tests) Detection of LC3 recruitment to autophagic membranes (protein band shift or change in intracellular localization)

(Gozuacik and Kimchi, 2004)

附圖 17 細胞凋亡與細胞自噬的比較

1. Bcl-2家族成員 (Bcl-2 family)

Bcl-2 家族成員是一群來自同源基因的蛋白質，主要功能是掌管粒線體外膜的穿透性 (mitochondrial outer membrane permeabilization, MOMP)， Bcl-2 家族成員在細胞凋亡與細胞自噬的調控當中扮演重要角色。依據其結構不同可分為抗凋亡蛋白 (anti-apoptotic proteins)，例如 Bcl-2, Bcl-xL, Mcl-1，和促凋亡蛋白 (pro-apoptotic protein)，如 Bak, Bax, Bim (附圖 18)。然而，Bcl-2 同時也能促進細胞自噬的發生，Bcl-2、Bcl-xl 和 Beclin-1 結合活化下游路徑促使自噬溶小體的形成 (附圖 19)。

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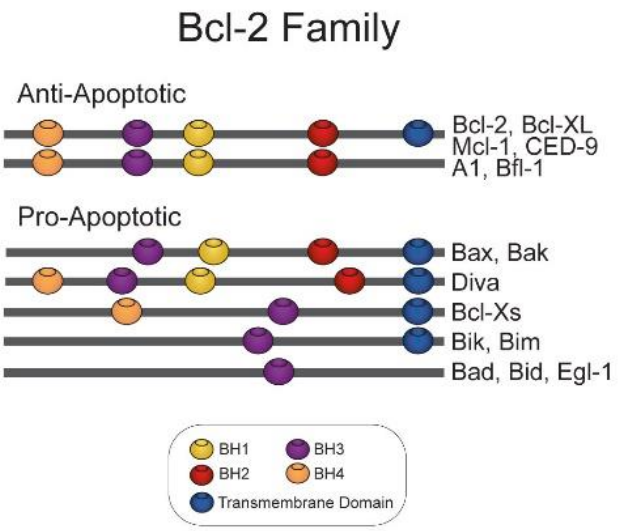
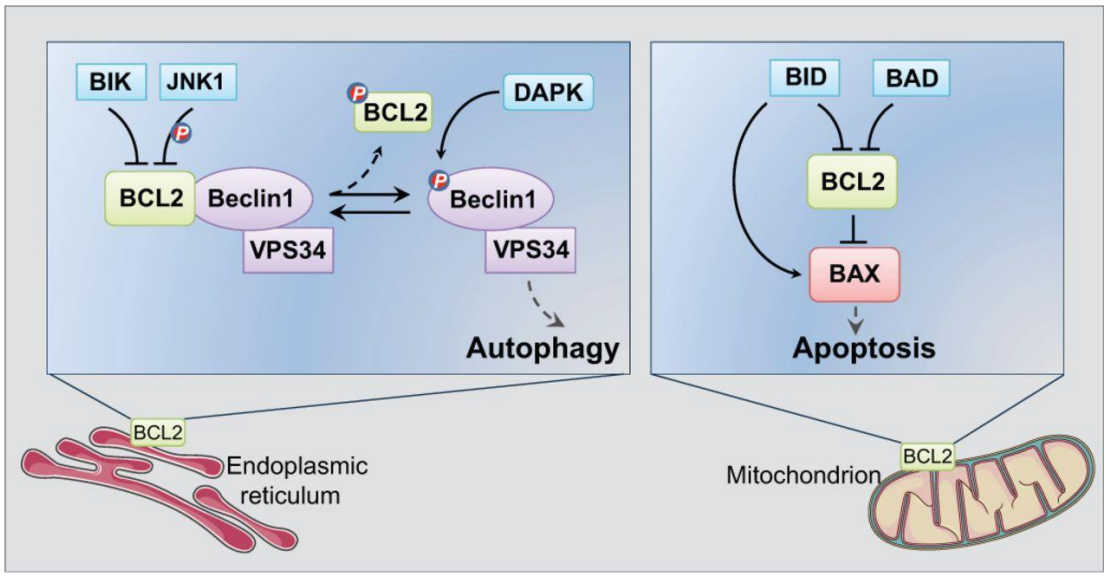


(https://en.wikipedia.org/wiki/Bcl-2_family)

附圖 18 Bcl-2 家族基因序列



(Levine et al., 2008)

附圖 19 Bcl-2 在細胞凋亡和自噬中扮演居中協調的角色

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1. Akt訊息傳遞路徑

Akt 訊息傳遞路徑主要功能是抑制細胞凋亡，透過抑制 caspase-3 活性而抑制細胞凋亡的發生，在細胞自噬當中 Akt 抑制 mTOR 活性進而抑制細胞自噬 (Wang et al., 2012)。

2. p53訊息傳遞路徑

p53 訊息傳遞路徑能在細胞凋亡與細胞自噬當中做居中調節的功用，p53 能促進細胞凋亡 (Ouyang et al., 2012)。在細胞自噬的調節，活化的 p53 能與 damage-regulated autophagy modulator (DRAM) 結合，活化下游引發巨型自噬的因子。然而細胞處在代謝壓力的狀態下，活化的 p53 反而會抑制自噬作用的發生。

3. MAPK訊息傳遞路徑

細胞在氧化壓力狀態下會誘發 MAPK 訊息傳遞路徑，ERK1/2 以及 JNK 是主要的 MAPK 路徑，ERK1/2 以及 JNK 誘發細胞凋亡的發生。

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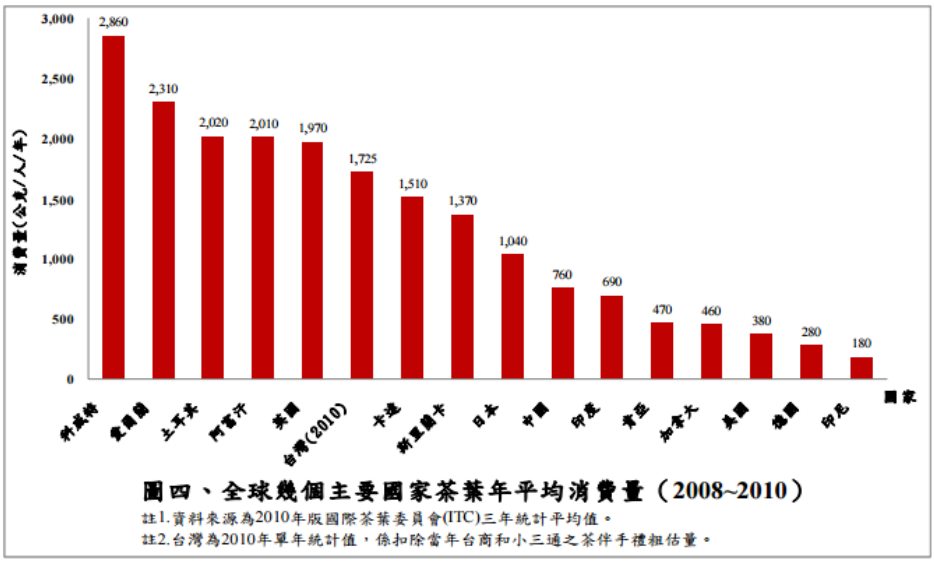
第四節 茶葉及其發酵產物介紹

(一) 茶葉

茶 (學名：*Camellia sinensis*)，屬山茶科山茶屬，為多年生常綠木本植物。茶葉廣泛分佈為各個國家，其葉片加工過後泡成的汁液即為茶湯為最多人飲用的飲品之一。

1.茶葉平均消費量

根據聯合國糧食及農業組織 (Food and Agriculture Organization of the United Nations, FAO) 2015 年的統計，2013 年全球茶葉產量為 506.4 萬公噸，主要生產國為中國大陸、印度、越南等東亞國家 (Chang, 2015)。由 FAO 統計 2013 年全球茶葉平均消費量為 484.2 萬公噸，另外根據 2012 年國際茶葉委員會統計，全球茶葉平均消費量台灣以人均消費量 1725 克位居第六，且近十年來台灣人均茶葉消費量也不斷上升 (張如華, 2012)，由此可見台灣人對茶葉的喜好。



圖四、全球幾個主要國家茶葉年平均消費量（2008~2010）

註1.資料來源為2010年版國際茶葉委員會(ITC)三年統計平均值。

註2.台灣為2010年單年統計值，係扣除當年台商和小三通之茶伴手禮粗估量。

(張如華, 2012)

附圖 20 台灣茶葉平均消費量

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2.成分分析

茶多酚 (tea polyphenols) 是從茶湯中萃取所得到的多酚類的總稱，為一種芳香烴。以黃烷醇類物質 (兒茶素) 佔最大比例，目前被認為是茶葉中最主要的活性成分。兒茶素含有:表兒茶素(Epicatechin EC)、表沒食子兒茶素(Epigallocatechin EGC)、表兒茶素沒食子酸酯(Epicatechin gallate ECG)和表沒食子兒茶素沒食子酸酯(Epigallocatechin gallate EGCG)，其中 EGCG 是含量最豐富的一種兒茶素也有最多研究證實其功效的物質，兒茶素萃取物中含量最多的是 EGCG 約占 68%，比次多的 EGC 多了將近 3.5 倍 (Zhu et al., 1997)。

3.生理活性

許多研究指出茶湯具有許多活性物質，抑制癌化和腫瘤生長、抗氧化和抗發炎等多種有益功效。此外茶葉能夠有效預防神經退化性疾病，根據流行病學的統計，飲中式與日式茶頻率較高的老年人罹患帕金森氏症的比例顯著性減少 (Tanaka et al., 2011)。另外在老化鼠實驗當中，給予不同濃度的綠茶對於神經退化性疾病具有保護效果 (Paulo Andrade and Assuncao, 2012)，此結果與流行病學實驗相呼應。

(二) 表沒食子兒茶素沒食子酸酯 ((-) – Epigallocatechin – 3 - gallate, EGCG）

EGCG 是茶葉中含量最多的兒茶素，據文獻指出每杯 (237 mL) 的綠茶和烏龍茶含有 30-130 mg 的 EGCG，而紅茶最多含有 70 mg 的 EGCG (Sang et al., 2005)，由以上數據推估每杯茶品所含 EGCG 濃度介於 200~1100 μM。因為 EGCG 的結構帶有許多 OH 基團，已有許多文獻證實 EGCG 具有許多生理活性，例如抗氧化(Tipoe et al., 2007)、減緩糖尿病 (Wolfram et al., 2006)，並能透過 Akt 等多種訊息傳導蛋

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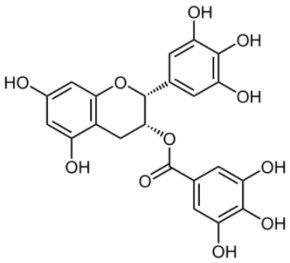
白的活化進而達到神經保護的效果 (Weinreb et al., 2004) 且對於AGEs誘導的神經損傷具有保護的功效 (Lee and Lee, 2007)。



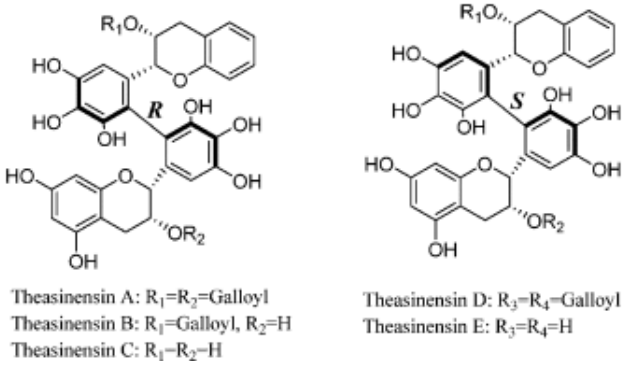
(https://en.wikipedia.org/wiki/Epigallocatechin_gallate)

附圖 21 EGCG 的結構

(三) 聚酯型兒茶素 A (Theasinensin A, TSA)

1.分類

theasinensin 是在 1984 年新發現的氧化黃烷醇 (flavanols)，包含有 theasinensins A, B, C, D, E, F 和 G 共七類，需透過兒茶素的氧化還原作用生成，綠茶中 theasinensin 含量 0.05%而烏龍茶中 0.65% (Stodt and Engelhardt, 2013)，因此 theasinensin 主要存在於發酵茶中，其中 TSA 在烏龍茶中含量是 theasinensins 中最多的 (Tanaka et al., 2010)。



(Weerawatanakorn et al., 2015)

附圖 22 Theasinensins 的結構

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Components of oolong tea beverage.

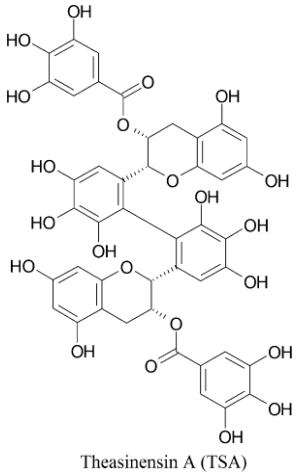
Compounds	Contents (mg/100 mL)
Catechin	1.65
Gallocatechin	6.68
Epigallocatechin	16.14
Epicatechin	5.08
Catechin gallate	0.6
Epicatechin gallate	5.73
Epigallocatechin gallate	25.73
Allocatechin gallate	1.85
Gallic acid	2.19
Caffeine	23.51
Polymerized	33.65
Total polyphenols	99.32

(Weerawatanakorn et al., 2015)

附圖 23 烏龍茶中富含的成分

2.已經研究的功效

TSA 是半發酵茶中的特殊物質，在茶葉的發酵過程當中，EGCG 經由 polyphenoloxidase 氧化或是自氧化 (auto-oxidation) 產生的 EGCG 二聚物，因此 TSA 的結構有較 EGCG 更多的 OH 抗氧化基團。先前研究指出 TSA 擁有的生理活性如抗菌 (Hatano *et al*., 2003)、抗高血壓(Miyata et al., 2013)、抗高血脂(Miyata et al., 2013) 與抗發炎 (Hisanaga *et a*l., 2014) 的功效，但是對於神經保護方面的研究十分稀少。



(http://www.genome.jp/db/pcidb/kna_kcfs_clst7s/29)

附圖 24 TSA 的結構

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Health benefits and proposed molecular mechanisms of theasinensins.

Bioactivity	Experimental model		Compound tested/control	Mechanism/biomarker
	In vitro	*In vivo*		
Antioxidant activity	Ferric thiocyanate assay		Theasinensins A–E/alpha-tocopherol	Decreasing lipid peroxidation
Anti-inflammation	LPS-activated murine macrophage RAW264.7 cells		Theasinensins A–E	Reducing gene expression of cyclooxygenase-2 (COX-2) and PGE_2
	LPS-activated murine macrophage RAW264.7 cells (a genome-wide microarray)		Theasinensin A	22,050 genes of inflammatory and immune response
	LPS-activated murine macrophage RAW264.7	Mouse paw edema model	Theasinensin A	Reducing the production of NO/iNOS, IL-12 (p70), TNF-α, and MCP-1
Anti-cancer	Human fibrosarcoma HT1080 cells		Theasinensin D	Suppressing invasion by reducing Gelatinase/Type IV Collagenases (MMP-2 and -9) activities
	Human histolytic lymphoma (U937) cell line and acute T cell leukemia (Jurkat) cell line		Theasinensin A	Inducing DNA fragmentation, and caspase activation
Hypoglycemic effect		KKAy mice and Sprague-Dawley rats	Theasinensin A	Regulation of serum glucose, lipid serum, hepatic fatty acid synthase activity
	Rat skeletal muscle cells		Theasinensin A and B	Increasing glucose uptake
	Alpha-glucosidase from rat intestinal acetone powder		Catechin, theaflavin, theasinensin A	Increasing alpha-glucosidase (AGH) inhibitory activity
Anti-microbial effect		MRSA (strains OM48, 505,584, and 623)	Theasinensin A and EGCG	Increasing antibiotic resistance
		HSV-1 and HSV-2	Theasinensin A, theaflavin, and EGCG	Enhancing protein aggregation

NBDG; 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose: herpes simplex virus (HSV)
MRSA; methicillin-resistant *Staphylococcus aureus.*

(Weerawatanakorn et al., 2015)

附圖 25 theasinensins 的活性功能

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第三章 實驗目的及架構

第一節 實驗目的

隨著世界人口平均壽命的增加，對於高齡人口容易罹患的神經退化性疾病的關注也逐漸提升。在先前流行病學的研究發現 MG，此種高反應活性化合物，與阿茲海默症或是帕金森氏症等神經退化性疾病有顯著性的關係。MG 是重要的糖化終產物前驅物且在體內容易產生氧化壓力，進而造成神經細胞損傷、失去功能。近年來對於神經保護的關注成為一個新興的議題，茶葉是一種傳統的飲品，流行病學研究指出長期飲用茶飲能有效減緩神經退化性疾病的風險，且近期研究指出兒茶素中的主要成分: EGCG 對於 AGEs 所引起的神經毒性具有保護作用，此外，烏龍茶中的特殊多酚類: TSA 雖有研究指出具抗氧化功效但目前對於神經保護的研究相當少。因此，本篇研究以人類神經細胞瘤細胞株 SH-SY5Y，探討茶多酚 EGCG 以及 TSA 對於 MG 誘導的神經毒性之保護作用及其分子機轉，以期能提供未來對於 MG 導致的腦神經損傷的理解之重要依據。

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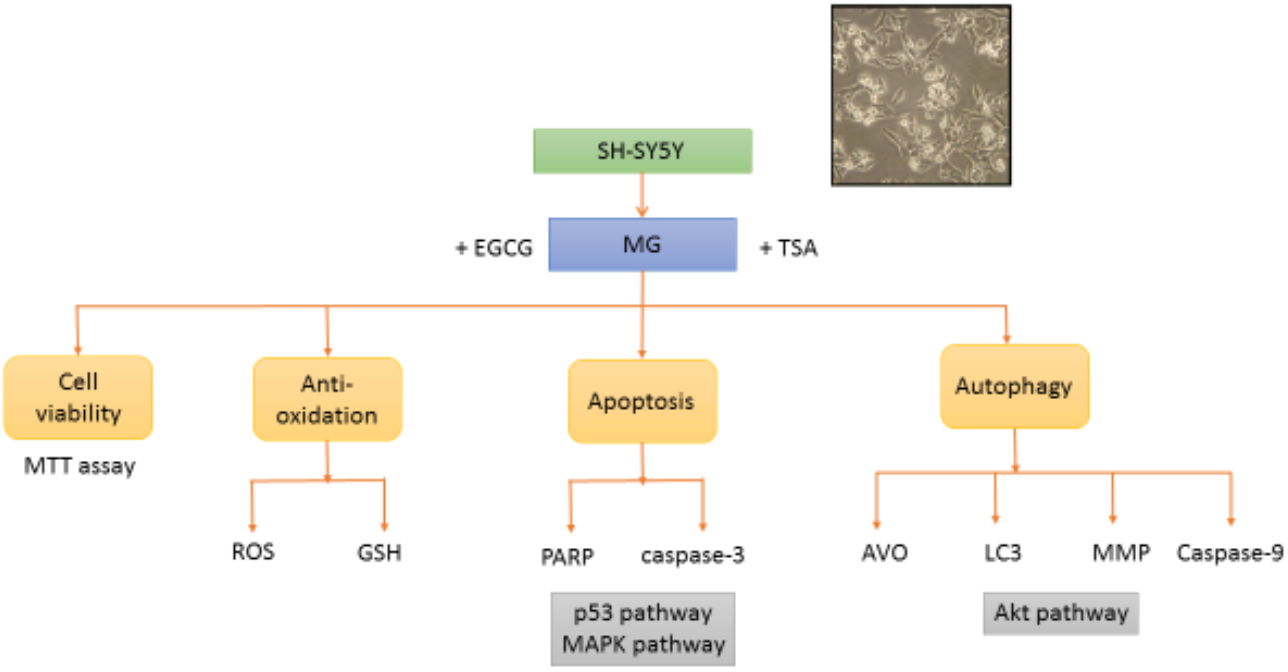
第二節　實驗架構



本研究主要研究物質是茶多酚 EGCG 與 TSA，TSA 的合成採用 Shii 等人的實驗方法改良以大量製備 TSA (Shii et al., 2011)。細胞實驗選用 SH-SY5Y 細胞株作為研究平台，探討 MG 與 EGCG、TSA 對於保護細胞免於 MG 誘導細胞死亡的分子機制。利用細胞存活率試驗確認茶多酚的神經保護效果，再透過流式細胞儀觀察是否有 ROS 生成以及 GSH 含量觀察茶多酚對於抑制 MG 誘導氧化壓力的能力。接著探討茶多酚對於 MG 誘導的細胞凋亡是否有抑制效果。最後透過西方墨點法確認茶多酚對於 MG 誘導細胞損傷保護效果的分子機轉。

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第四章 實驗材料及方法

第一節 實驗材料

(一) 樣品試劑

1. 下列產品購自 GIBCO®公司

Dulbecco's Modified Eagle Media: Nutrient Mixture F-12(DMEMF-12), Fetal bovine serum (FBS), Penicillin-Streptomycin, 0.5% Trypsin-EDTA (10X)

2. 下列產品購自 Millipore 公司

Western Chemiluminecsent HRP substrate (ECL), Polyvinylidene fluoride (PVDF) membrane, Ammonium persulfate (APS)

3. 下列產品購自 Merk 公司

Potassium chloride (KCl), Methanol

4. 下列產品購自 Sigma-Aldrich 公司

Methylglyoxal, Isopropanol, Thiazolyl blue tetrazolium bromide (MTT), Sodium orthovanadate (Na_3VO_4), Tris-base, Potassium dihydrogen phosphate (KH_2PO_4), Disodium dihydrogen phosphate (Na_2HPO_4), Glycine, Acridine orange (AO), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), Bovine serum albumin (BSA), Sodium hydroxide (NaOH), 3,3′-dihexyloxacarbocyanine iodide (DiOC6), 5-chloromethylfluorescein diacetate (CMFDA)

5. 下列產品購自 J.T. Baker®公司

Sodium pyrophosphate ($Na_4P_2O_7$), Sodium chloride (NaCl), Sodium dodecylsulfate (SDS), disodium salt ($Na_2O_8 \cdot 2H_2O$)

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6. 下列產品購自 Bio Basic 公司

Ethylene glycol tetraacetic acid (EGTA)

7. 下列產品購自日本和光純藥工業公司

Triton X-100

8. 下列產品購自日本林純藥工業公司

Glycerol, Tween 20

9. 下列產品購自 SHOWA 公司

Hydrochloric Acid (HCl)

10. 下列產品購自 Bio-Rad Laboratories 公司

Bio-Rad protein assay dye reagent

11. 下列產品購自 AMRESCO 公司

Acrylamide

12. 下列產品購自 BIO SHOP 公司

N,N,N',N'-Tetramethylethylene-Diamine (TEMED)

13. 下列產品購自 PROTECH 公司

Protein loading dye (6X)

14. 下列產品購自 Roche 公司

Protease inhibitor tablets

15. 下列產品購自 Fisher Chemical 公司

Dimethyl sulfoxide (DMSO)

16. 下列產品購自 Fluka AG – Chemische Fabrik, Switzerland

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Ethylenediaminetetraacetic acid (EDTA)

17. 下列產品購自 BIOMAN 公司

Protein marker, Agarose

18. 下列產品購自 Cell signaling 公司

Anti-PARP antibody, Anti-LC3 antibody, Anti-ERK antibody, Anti-p- ERK antibody, Anti-JNK antibody, Anti-p-JNK antibody, Anti-Akt antibody, Anti-p-Akt antibody, Anti-p53 antibody, Anti-p-p53 antibody

19. 下列產品購自 SANTA CRUZ 公司

Anti-β-actin antibody

20. 下列產品購自 McCormick Scientific

Parraffin

21. 下列產品購自 Bio SB

Mouse/Rabbit polydetector HRP/DAB detection system

22. 下列產品購自 Daintree Scientific

Reagent Alcohol 100%

doi:10.6342/NTU201603033

(二) 儀器設備

1. 細胞培養箱: Thermo
2. 無菌操作台: 造鑫
3. 倒立式顯微鏡: PLYMPUS
4. 離心機: Beckman Coulter
5. 桌上型冷凍離心機(小): HERMLE
6. 桌上型冷凍離心機(大): KUBOTA
7. 振盪器(Vortex): Scientific industries
8. 乾浴器: Major Science
9. 數位迴轉式震盪器: YIH DER
10. 烘箱: 今日
11. 電源供應器: Thermo、Major Science
12. 蛋白質轉漬槽: Hoefer
13. SDS-PAGE 電泳槽: Whatman Biometra TM
14. pH meter: Toledo
15. 高壓殺菌釜: 雲集
16. 水浴槽: 雲集
17. 電器攪拌器: IWAKI glass
18. 4℃冰箱: Collect
19. -20℃冰箱: Frigidaire
20. -80℃冰箱: 智勤
21. 影像處理系統: Bio top
22. ELISA reader: Bio Tek
23. 流式細胞儀: BD Bioscience

doi:10.6342/NTU201603033

24. Digital Dry Bath Incubator: GENEPURE TECHNOLOGY

25. 精密電子秤: Thermo

26. 電子秤: Ohaus

27. 純水製造機: ELGA

28. 微波爐: panasonic

29. 血球計數器: Horsham

30. Pipet aid: Drummond

31. 24、96 well: Corning

32. 10 cm Dish: Corning

doi:10.6342/NTU201603033

第二節 樣品製備

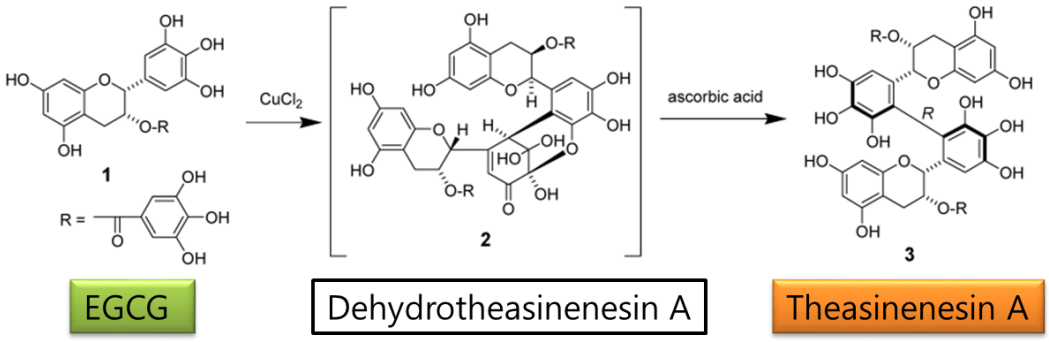
(一) 樣品來源

EGCG 由 Rutgers University 何其儻教授提供，其純度大於 99%。

TSA 自行合成，並透過 HPLC 分析檢測其純度大於 99%且產率為 37.47%。

(二) 合成原理

TSA 的合成參考 Shii 等人於 2011 年發表的文獻的實驗方法改良以大量製備 theasinensin A (Shii et al., 2011)。選用此方法的原因為多數的 theasinensin 合成方法並沒有結構選擇性，同時產生 TSA 以及 TSD。然而此方法藉由 EGCG 經由氯化亞銅 ($CuCl_2$) 及抗壞血酸 (ascorbic acid) 反應後能選擇性產生 TSA。EGCG 經由氯化亞銅加熱反應後產生結構不穩定的醌形式二聚物 (quinone dimer) – dehydrotheasinensin A，再加入 ascorbic acid 進行還原反應生成 TSA (附圖 26)。



附圖 26 TSA 合成原理

(三) 實驗步驟

1.TSA 的合成

取 2000 mL 30%甲醇的二次水溶液，加入 5 g EGCG 及 1.7 mg 二水合氯化亞銅配置成合成溶液，將合成溶液於常溫下攪拌反應 24 小時。隔天將溶液添加 5 g 抗

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壞血酸後置於水浴槽 85 ℃加熱 15 分鐘，合成步驟即完成。

2.分離純化

然而合成步驟當中添加許多化合物，因此需透過分離純化步驟將 TSA 純化。純化方式使用兩支管柱 HP20 以及 LH20，透過 HP20 管柱分離茶多酚與二水合氯化亞銅、抗壞血酸，再利用 LH20 分離 TSA 及 EGCG。

(1) 管柱: HP20

移動相梯度: 每種移動相攪拌均勻後以超音波震盪去除氣泡備用。

移動相 (二次水:甲醇)	**體積 (mL)**
100 : 0	1200
80 : 20	600
70 : 30	600
50 : 50	600-1200 (至 TSA 完全沖提並收集完成)
40 : 60	600
0: 100	600

步驟:

HP20 以甲醇活化 24 小時後二次水潤洗三次，將 HP20 分次充填入垂直架設的管柱，以二次水將 HP20 填壓緊實。將合成溶液抽氣過濾並減壓濃縮至 50 mL，待二次水至管柱交界面即可加入合成溶液，依照移動相梯度添加移動相沖提。沖提液每 50 mL 收集一管，每管沖提液取少量體積利用 TLC 初步分析其成分組成，將含有 TSA 的沖提液收集成一瓶並減壓濃縮備用。

(2) TLC 條件

展開液: 甲苯:乙酸乙酯:甲酸=1:7:1 之溶液

固定相: silica gel

步驟:

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將展開液倒入展開槽中放置一張濾紙，緊閉展開槽放置數分鐘待展開槽內達到氣液平衡。利用毛細管取少量沖提液作為樣品以及少量 EGCG 和 TSA 作為標準品，將樣品與標準品點在 TLC 片起始點處，確定樣品點溶液皆揮發後，利用鑷子把 TLC 片夾至展開槽平整放入，緊蓋展開槽等待展開液進行至 TLC 片頂端下方約 0.2 cm 處即可取出 TLC 片，待展開液揮發完全後，將 TLC 片置於紫外光下觀察吸光位置。

(3) 管柱: LH20

移動相梯度: 每種移動相攪拌均勻後以超音波震盪去除氣泡並減壓過濾後備用。

移動相　(二次水:甲醇)	**體積 (mL)**
50 : 50	600
40: 60	600
30: 70	600
20: 80	600-1200 (至 TSA 完全沖提並收集完成)
0: 100	600

步驟:

利用甲醇將 LH20 活化 24 小時後以二次水潤洗三次，將管柱垂至架設並分次充填 LH20，並以二次水將 LH20 填壓緊實。待二次水至管柱交界面即可加入 HP20 純化後的 TSA 沖提液，依照移動相梯度添加移動相沖提。同樣使用 TLC 初步分析沖提液成分組成，將含有 TSA 的沖提液收集成一瓶並減壓濃縮。濃縮完成的沖提液倒入保鮮盒中鋪平置於-20℃冰箱待凝固後，以鋁箔覆蓋後放置冷凍乾燥機冷凍乾燥 overnight，此紅棕色粉末即為 TSA 合成物。

3.HPLC 分析

樣品製備: 將合成好的 TSA 利用水溶解配置成 1000 ppm 的溶液待用。

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管柱: Cosmosil C18-MS (5μm, 4.6 i.d x 250mm)

移動相: A 液為甲醇:甲酸:二次水=20:0.3:79.7 之溶液，B 液為 100 % 乙腈(acetonitrile)，所有移動相皆混和均勻去氣備用

梯度:

時間 (min)	**A 液 (%)**	**B 液 (%)**
0-11	100	0
11-15	90	10
15-20	80	20
20-25	75	25
25-28	72	28
28-29	60	40
29-35	50	50
35-40	20	80

流速: 1 mL/min

偵測波長: 280 nm

(四) 製備

配製 100 mM TSA 以及 EGCG，溶於 DMSO 中，作為 Stock solution，再依各個實驗的需求稀釋成適當的濃度。

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第三節 細胞培養

(一) 細胞株

SH-SY5Y 細胞株為人類神經瘤細胞，屬於多巴胺生成細胞，多用在帕金森氏症研究細胞模式，SH-SY5Y 同時包含貼附型及懸浮型，倍增時間 (doubling time) 為 48 小時，正常情況下會團聚成堆。。其突變基因 ALK, FOXD4L1, HLA-DRB1, NBPF10, NBPF14, PABPC3, TEKT4。

細胞來源由台大醫院臨床醫學所神經部李銘仁醫師提供。

(二) 試劑配置

培養基:使用市售 DMEM-F12 medium 粉包，每公升添加 2.48 克 sodium bicarbonate、1 mM sodium pyruvate 及 1 % 抗生素溶於 900 毫升二次水後使用 0.22 μm 濾杯過膜，不含血清的 medium 放於 4℃冰箱中存放，於使用前再添加 10 % FBS。

10x PBS: 將二次水定容至 1 L 並調整 pH 至 7.4 滅菌後即可使用。

Potassium chloride (KCl)	2 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.4 g
di-sodium hydrogen phosphate (Na_2HPO_4)	14.4 g
Sodium chloride (NaCl)	80 g

(三) 解凍

將 SH-SY5Y 人類神經瘤細胞株由液態氮桶中取出後隨即放入 37 ℃ 水浴槽將其解凍，解凍之細胞株培養至含有新鮮 DMEM-F12 medium (含 1 % 抗生素、10

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% FBS 及 1 mM sodium pyruvate) 之 culture dish 中，放置於細胞培養箱 (37 ℃、5 % CO_2) 中培養，隔日移除上清液並添加新鮮的 medium 以去除 DMSO。

(四) 繼代

待細胞長至約八分滿時，吸除 medium，加入 5 mL 1x PBS 清洗細胞，吸除 PBS 隨後加入 1 毫升 1x trypsin 吸除 1x trypsin 後反應 1~2 分鐘，待細胞被切下後，以 medium 中和並收集至 50ml 離心管中離心 1000 rpm 5 分鐘，去除含有 trypsin 之上清液，加入新鮮 medium 均勻打散細胞，以 1:5 比例繼代培養至新的 dish 中，並每 2 至 3 天換一次新鮮 medium。

(五) 凍管

待細胞長至約八分滿時，切下細胞將其懸浮於 FBS : DMSO = 9 : 1 之溶液當中，每 1 個冷凍小管取 1 毫升溶液冰至-80℃冰箱一天後再凍入液態氮中儲存。

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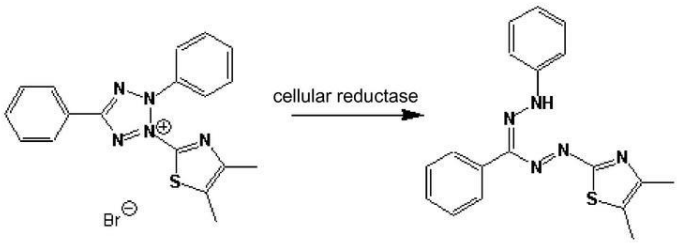
第四節 細胞存活率分析 (MTT assay)

(一) 試劑

0.2% MTT solution：取 0.1 g MTT 粉末溶於 50 mL 1x PBS 中並使用 0.22 μm 過膜後避光儲存於 4℃冰箱。

(二) 原理

MTT assay 是一種測量細胞存活率的實驗。實驗原理為透過 MTT 是一種 tetrazolium 類物質，能接受氫離子的此種特性，當活細胞粒線體的呼吸鏈的琥珀酸脫氫酶 (Succinate dehydrogenase, SDH) 和細胞色素 C 對 MTT 產生還原作用，MTT 環狀結構打開生成 formazan 呈紫色結晶狀，利用 DMSO 溶解結晶後側得吸光值代表 MTT 還原的多寡 (附圖 27)。因為當細胞死亡後酵素活性也隨之消失，因此能藉由紫色結晶產生多寡推測細胞存活率。



附圖 28 MTT 的作用原理

(三) 實驗步驟

將 SH-SY5Y 細胞濃度調整至 $5x10^5$ cell/ml，均勻打散後接種 1 mL/well 至 24 well 中，放入培養箱培養 12 小時後更換成無血清 medium。依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 3 小時。接著將 24 well

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離心 (2000 rpm，10 分鐘)，除去上清液後以 1x PBS 清洗，再離心 (2000 rpm，10 分鐘)且除去上清液後，加入 1 mL/well MTT 試劑 (以 0.2% MTT solution:無血清 medium =1:9 之比例稀釋) 後於培養箱培養 2 小時至 control 組有明顯紫色結晶生成，取出 24 well 以 3500 rpm, 10 分鐘離心後，去除上清液並加入 1 mL/well DMSO 溶解紫色結晶。最後利用 EILISA reader 在 570 nm 下偵測吸光值，以下列公式計算出相對應於控制組的細胞存活率。

存活率(%) = (各個濃度平均吸光值 / 控制組平均吸光值) x 100

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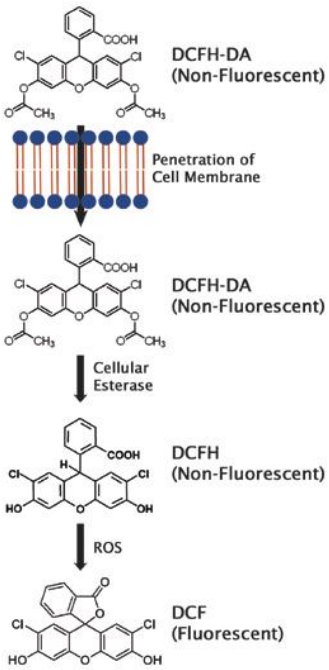
第五節 胞內 ROS 產生量分析

(一) 試劑

DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) stock solution: 10 mM DCFH-DA 溶於 DMSO 中

(二) 原理

DCFH-DA 是目前廣為運用在測量氧化壓力的螢光染劑。DCFH-DA 能夠自由通透細胞膜，DCFH-DA 進入細胞後能與細胞內的酯解酶 (esterase) 作用生成 DCFH (dichlorodihydrofluorescein)，DCFH 能被細胞中 ROS 氧化生成能發射出螢光的 DCF- 25 -(dichlorofluorescein)，因此可利用螢光強度推算細胞中 ROS 含量多寡 (附圖 29)。



(http://www.cellbiolabs.com/reactive-oxygen-species-ros-assay)

附圖 30 DCFH-DA 產生螢光的機制

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(三) 實驗步驟

將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 1 mL/well 至 24well 中，放入培養箱 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 30 分鐘。將每個 well 內的上清液吸起分別置入 eppendorf 中，以 200 μL 之 1x PBS 沖洗並將沖洗之 1x PBS 收集至 eppendorf，接著加入 200 μL 之 1x trypsin 放入培養箱中培養至細胞切除再收集細胞至 eppendorf 中。將 eppendorf 離心 (3000 rpm，10 分鐘) 後去除上清液，加入 1mL 1x PBS 清洗再離心 (3000 rpm，10 分鐘) 去除上清液，最後將細胞打散懸浮於 500 μL 之 1x PBS 加入 DCFH-DA (最終濃度 20 μM) 於培養箱中避光培養 30 分鐘，隨後將細胞懸浮於康氏管中以流式細胞儀分析螢光強度，數據以 Flow Jo 軟體進行分析。

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第六節 GSH 含量測定分析

(一) 試劑

CMFDA (5-chloromethylfluorescein diacetate) : 40 μM 溶於 1xPBS 中

(二) 原理

CMFDA 是一種能夠和細胞內 GSH 結合的探針，因此可透過螢光強度得知細胞內 GSH 含量。

(三) 實驗步驟

將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 1 mL/well 至 24well 中，放入培養箱 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 EGCG 或 TSA 後再加入 1.5 mM MG 培養 30 分鐘。將每個 well 內的上清液吸起分別置入 eppendorf 中，以 200 μL 之 1x PBS 沖洗並將沖洗之 1x PBS 收集至 eppendorf，接著加入 200 μL 之 1x trypsin 放入培養箱中培養至細胞切除再收集細胞至 eppendorf 中。將 eppendorf 離心 (3000 rpm，10 分鐘) 後去除上清液，加入 1mL 1x PBS 清洗再離心 (3000 rpm，10 分鐘) 去除上清液，最後將細胞打散懸浮於 200 μL 之 1x PBS 加入 CMFDA (最終濃度 40 nM) 於培養箱中避光培養 15 分鐘，隨後將細胞懸浮於康氏管中以流式細胞儀分析螢光強度，數據以 Flow Jo 軟體進行分析。

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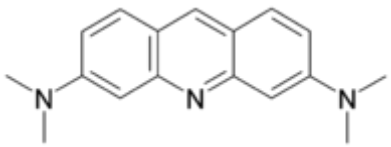
第七節 AO 染色分析

(一) 試劑

Acridine orange (AO) stock solution: 5 mg/mL AO 溶於 1x PBS 中

(二) 原理

藉由 AO 能夠穿透細胞膜且能在酸性下發出橘色螢光以及能和核酸結合的特性，不只能夠標定細胞自噬發生過程當中形成的 AVO (acidic vesicular organelles)，也能標定細胞核位置與有染色體凝集現象的凋亡現象 (附圖 31)。



(https://en.wikipedia.org/wiki/Acridine_orange)

附圖 32 AO 的分子結構

doi:10.6342/NTU201603033

Cell death pathway characteristics

	Apoptosis	Autophagy	Necrosis	Senescence	Mitotic catastrophe
Morphologic changes					
Cell membrane	Blebbing, membrane integrity maintained	Blebbing	Loss of membrane integrity	Flattening; increase in cell size	?
Nucleus	Chromatin condensation, DNA laddering, nuclear fragmentation	Partial chromatin condensation, no DNA laddering	Random DNA degradation	Accumulation of heterochromatin foci	Mis-segregation of chromosomes during cytokinesis; micronuclei
Cytoplasm	Condensed membrane-bound cellular fragments; depolymerization of cytoskeleton	Increased number of autophagic vesicles, degradation of Golgi, polyribosomes, and the ER	Swelling of cellular organelles	Granularity	?
Detection methods	Annexin V staining, DNA fragmentation assays, caspase activation	LC3 localization	Early permeability to vital dyes, release of intracellular contents	Senescence-associated β-galactosidase activity	Visualization of multinucleated cells
Release of cellular contents	Lysophosphatidylcholine	?	HMGB1, S100 molecules, purine metabolites, heat-shock proteins, uric acid, HDGF	?	?
Immunologic response	Suppressive, engulfment of cell carcass	?	Stimulatory, initiation of cell growth and tissue repair	?	?

(Ricci and Zong, 2006)

附圖 33 細胞型態觀察基準

(三) 實驗步驟

將蓋玻片浸泡 95%酒精瀝乾後過火殺菌，殺菌後的蓋玻片置於 6 cm dish 中再將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 5 mL/dish，放入培養箱 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 3 小時。將每個 dish 內的上清液吸起，以 1 mL 之 1x PBS 沖洗並吸除，接著加入最終濃度 1 μg/mL 之 AO 放入培養箱中培養 15 分鐘後，將蓋玻片取出並置入玻璃玻片上，於共軛焦顯微鏡下 (激發光 488 nm，發射光 500-540 nm (DNA)以及 566-606 nm (AVO)) 觀察細胞染色情形。

doi:10.6342/NTU201603033

第八節 細胞自噬分析

(一) 試劑

Acridine orange (AO) stock solution: 5 mg/mL AO 溶於 1x PBS 中

(二) 原理

AO 是一種橘色的螢光染劑，能夠穿透細胞膜且對細胞內 pH 值敏感。細胞自噬發生過程當中，隨著自噬溶酶體生成，為了使酵素水解蛋白質小泡內呈酸性，AO 在酸性環境下呈現橘色，此為 AVO，因此可以藉由 AVO 的生成作為自噬作用發生指標。

(三) 實驗步驟

將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 1 mL/well 至 24well 中，放入培養箱 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 3 小時。將每個 well 內的上清液吸起分別置入 eppendorf 中，以 200 μL 之 1x PBS 沖洗並將沖洗之 1x PBS 收集至 eppendorf，接著加入 200 μL 之 1x trypsin 放入培養箱中培養至細胞切除再收集細胞至 eppendorf 中。將 eppendorf 離心 (3000 rpm，10 分鐘) 後去除上清液，加入 1mL 1x PBS 清洗再離心 (3000 rpm，10 分鐘) 去除上清液，最後將細胞打散懸浮於 200 μL 之 1x PBS 加入 AO (最終濃度為 25 μg/mL) 於培養箱中避光培養 15 分鐘，隨後將細胞懸浮於康氏管中以流式細胞儀分析螢光強度，數據以 Flow Jo 軟體進行分析。

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第九節 蛋白質電泳 (SDS-PAGE) 與西方墨點法 (Western blotting)

(一) 試劑

1. Gold lysis buffer 配製

10 % (v/v) glycerol、1 % (v/v) TritonX-100、1 mM Sodium orthoranadate、1 mM EGTA 、10 mM NaF、1 mM Sodium pyrophosphate、20 mM Tris (pH7.9)、100 μM β-glycerophosphate、137 mM NaCl、5 mM EDTA。取上述配好的 Gold lysis buffer 50 ml 加入一錠市售 Protease inhibitor cocktail tablets 即為萃取蛋白質用之 Gold lysis buffer。

2. 5X Sample loading buffer

350 mM Tris-HCl (pH6.8)、12 % SDS、0.02 % bromophenol blue、35 % glycerol 、30 % 2-mercaptoethanol。

3. 10X SDS Running buffer

25 mM Tris-base 、250 mM glycine、 0.1 % SDS

3. 5X Transfer buffer

取 288 g 加入 60 g Tris-base 溶於 4 L 二次水即為五倍的 Transfer buffer stock solution。取 5 倍的 Transfer buffer stock solution 1L 加入 1LMethanol 再加入 3L 的二次水，即為 Transfer buffer working solution。

4. Blocking solution

20 mM Tris-base、125mM NaCl、0.2% Tween 20、1% BSA、0.1% Sodium azide。

5. TPBS

1X PBS 含 0.2 % Tween 20

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6. 一次抗體稀釋液

將一級抗體以 Blocking solution 稀釋約 1000 倍。

7. 二次抗體稀釋液

將二級抗體以 TPBS 稀釋約 5000 倍。

(二) 原理

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) 是膠體電泳的一種，利用 SDS 界面活性劑的特性，將蛋白質變性形成帶負電之長條狀蛋白質結構，再利用電壓的作用使蛋白質依據分子量大小分離。經過 SDS-PAGE 分離的蛋白質樣品，轉移至 PVDF 膜以非共價鍵形式吸附蛋白質，以方便後續實驗進行。以 PVDF 膜上的蛋白質作為抗原，利用能辨識抗原的特定抗體辨識蛋白質，隨後接上對應一級抗體辨識位的二級抗體與以及抗體結合，藉由二級抗體上的酵素 HRP 能與 ECL 反應液中的 luminol 反應產生冷光，經由照膠系統能分析特定蛋白的表現狀態。

(三) 實驗步驟

1. 蛋白質萃取

將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 10 mL 至 10 cm dish 中，放入培養箱培養 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 3 小時。將細胞刮下連同 medium 一同收集至 15 ml 離心管中，並以 2 ml 之 1x PBS 將細胞清洗後移入 15 ml 離心管中，清洗兩次後離心 (4℃，3500 rpm，10 分鐘)。除去上清液，加入 1ml1x PBS 將細胞轉移到 1.5 mL eppendorf 中再離心 (4℃，3500 rpm，10 分鐘)，沉澱的細胞以 100 μL 的 gold lysis buffer 打散細胞，置於冰上反應每 5 min Vortex 一次，共進行 12 次，最後於 4℃下離心 12000 rpm，30 分鐘，收取離心後的上清液即為

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總蛋白質。

2. 蛋白質定量

先製作蛋白質標準曲線，取 200 μl/well 之 1X Bio-Rad reagent 於 96 well 中，再以 BSA (2 mg/ml) 做標準曲線。接著每個樣品取出 0.4 μl 與 1X Bio-Rad reagent 混和均勻後避光反應 10 分鐘，再以 ELISA reader 測定 595nm 吸光值，利用標準曲線推估樣品中總蛋白濃度。

3. 蛋白質樣品前處理

依據蛋白質定量結果，從每個樣品中取出含有 50 μg 蛋白質之體積加入 0.5ml eppendorf 中，每個 eppendorf 加入 2-10 μl sample loading buffer，混勻後於乾浴器中以 100℃加熱 10 分鐘冷卻備用。

4. 配製 SDS-PAGE

依下列配方分別配製適當濃度之上膠 (separating gel) 與下膠 (stacking gel) 聚丙烯醯胺膠體：

下膠(15 ml)

試劑& Acrylamide 濃度	**8%**	**10%**	**12%**	**15%**
H_2O	7.9	7.15	6.4	5.27
40% acrylamide	3.0	3.75	4.5	5.63
1.5 M Tris - HCl (pH 8.8)	3.8	3.8	3.8	3.8
10 %SDS	0.15	0.15	0.15	0.15
10 % APS	0.15	0.15	0.15	0.15
TEMED	0.009	0.009	0.009	0.009

單位：ml

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上膠 (5 ml)

試劑及濃度	5% Acrylamide
H_2O	3.6
40% acrylamide	0.63
1.0 M Tris – HCl (pH 6.8)	0.63
10 %SDS	0.05
10 % APS	0.05
TEMED	0.005

單位：ml

將配好的下膠注入適當量於電泳玻璃片當中，以酒精壓平介面等待 1 小時至下膠凝固後倒除酒精，加入適當量的上膠並插入齒梳約 1 小時後待上膠凝固，移除齒梳、清洗膠體表面後，將膠片裝置再電泳槽上並注入足夠量的 running buffer，清除膠體底部氣泡後即可準備電泳。

5. 電泳分析

將定量並冷卻完成的樣品完全注入上膠的 well 中，上膠使用電壓 50 V，直到蛋白質進行到上膠與下交的介面即可將電壓調高至 100V，待電泳將所需分析蛋白質分離即完成電泳。

6. 轉漬

將適當大小的 PVDF 膜活化，以甲醇浸泡 10 分鐘，再以二次水清洗表面。取電泳夾由下往上依序疊放:海綿、3M paper、PVDF 膜、SDS-PAGE gel、3M paper、海綿，並浸泡於二次水和自來水 1:1 之溶液中，以塑膠棒趕除每一層間的氣泡後將電泳夾夾緊，之後放入 4℃冰箱的轉漬槽中，以 500 mA 電流轉漬 overnight。

7. 西方墨點法

轉漬完成的 PVDF 膜加入適當量之 blocking solution 於室溫下震盪 1 小時。接

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著回收 blocking solution 再加入一級抗體使其均勻覆蓋在膜上，於 4℃冰箱中均勻震盪 overnight。

隔日將一級抗體回收後取適量 TPBS 於室溫下搖晃清洗 10 分鐘，總共清洗三次。依照一級抗體的特性加入適合的二級抗體，二級抗體以 TPBS 配置稀釋 5000 倍後於室溫下震盪反應 1 小時。完成反應後，取適量 TPBS 於室溫下搖晃清洗 10 分鐘，總共清洗三次。最後倒除 TPBS 後使用 ECL 溶液 (ECL1 : ECL 2 = 1 : 1 混合) 淋在 PVDF 膜上反應製呈色，再利用照膠系統觀察目標蛋白的表現量變化，並利用 Image J 軟體進行定量分析。

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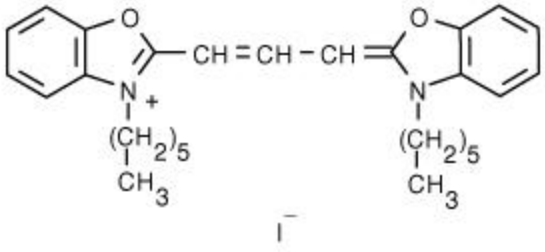
第十節 粒線體膜電位分析

(一) 試劑

DiOC6 (3,3'-dihexyloxacarbocyanine iodide) stock solution: 40 μM DiOC6 溶於 1x PBS 中

(二) 原理

DiOC6 是能夠染細胞內胞器的綠色螢光染劑，能夠穿透細胞膜。粒線體為雙層膜結構，在正常情況下外膜會帶些微的正電，而 DiOC6 對於陽離子具有染色能力，因此 DiOC6 的螢光強度高低即能表示粒線體膜電位的高低。



(https://www.thermofisher.com/order/catalog/product/D273)

附圖 34 DiOC6 的結構

(三) 實驗步驟

將 SH-SY5Y 以 $5x10^5$ cell/ml 細胞濃度接種 1 mL/well 至 24well 中，放入培養箱 12 小時後吸除 medium 更換成無血清 medium，依實驗設計處理不同濃度的 TSA 以及 EGCG，培養 1 小時後加入誘導劑 MG 培養 15 分鐘。將每個 well 內的上清液吸起分別置入 eppendorf 中，以 200 μL 之 1x PBS 沖洗並將沖洗之 1x PBS 收集至 eppendorf，接著加入 200 μL 之 1x trypsin 放入培養箱中培養至細胞切除再收集

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細胞至 eppendorf 中。將 eppendorf 離心 (3000 rpm，10 分鐘) 後去除上清液，加入 1mL 1x PBS 清洗再離心 (3000rpm，10 分鐘) 去除上清液，最後將細胞打散懸浮於 200 μL 之 1x PBS 加入 DiOC6 (最終濃度 40 nM) 於培養箱中避光培養 15 分鐘，隨後將細胞懸浮於康氏管中以流式細胞儀分析螢光強度，數據以 Flow Jo 軟體進行分析。

第十一節 統計分析

所有數據皆以 Microsoft Excel 軟體進行數據整理，並以 Sigmaplot 12.0 繪圖與統計分析，使用 one-way ANOVA 分析並用 Dunnett's multiple range test 事後檢定，統計標準為 p 值小於 0.05 具有統計學上顯著性差異。

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第五章 實驗結果

第一節 茶多酚對於 MG 誘導神經細胞株毒性之保護效果

先前研究指出,MG 會透過細胞凋亡的方式造成細胞死亡,因此透過 MTT assay 測量細胞存活率藉此觀察茶多酚對於 MG 誘導細胞死亡的保護效果。首先利用不同濃度的 MG 處理細胞 3 小時,觀察會使細胞半數死亡的劑量。由圖 1 結果可知,MG 處理確實會造成細胞死亡且隨著處理濃度增加具有劑量效應 (dose-dependent)。處理 1mM 的 MG 與控制組已具有顯著差異而處理 1.2mM 的 MG 已能使細胞存活率降至 80%以下。最後,1.5 mM 的 MG 為 54%的細胞存活率,大約是細胞半數致死濃度,因此後續的 MG 誘導細胞死亡的濃度選用 1.5mM。

接著確認茶多酚是否對細胞產生毒性。將茶多酚處理 24 小時後測量細胞存活率,結果顯示 (圖 2) 茶多酚處理 2.5、5、10、25、50、100 μM 都不會對細胞造成毒性。為了觀察茶多酚是否具有預防 MG 造成細胞死亡的效用,採用預處理 1 小時的茶多酚後 MG 處理 3 小時的方式觀察細胞存活率的變化。由圖 3 顯示,不論是 EGCG 或是 TSA 都能有效保護細胞免於 MG 造成的細胞損傷,EGCG 25-50 μM 是保護效果最佳的濃度,細胞存活率約在 80%,因此後續實驗選用 25-50 μM 的 EGCG 及 TSA 進行更深入的分子機制探討。

另外,在細胞型態的部分 (圖 3),處理 MG 後細胞漂浮情形嚴重,細胞由貼附的紡錘型變成圓形,且能觀察到凋亡小體的生成。在預處理 EGCG 後,可以看到正常型態的細胞增加。然而預處理 TSA 的組別細胞型態呈現貼附型泡狀,懸浮圓形細胞減少,且能夠觀察到許多空泡狀。

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第二節 茶多酚抑制 MG 誘導之胞內 ROS 含量

透過流式細胞儀分析細胞內部 ROS 生成量，將 MG 1.5 mM 處理細胞收集 15, 30, 60, 180 分鐘的胞內 ROS 生成量，確認誘導最多 ROS 的時間為 30 分鐘 (圖 4)。接著預處理茶多酚 1 小時再 MG 處理 30 分鐘後比較 EGCG 及 TSA 清除 ROS 能力。透過圖 5 的數據得知經 EGCG 和 TSA 處理的組別都能有效清除胞內 ROS，且 TSA 清除 ROS 能力較 EGCG 更好。

利用流式細胞儀偵測 GSH 的含量，GSH 是抗氧化相當重要的酵素，能夠去除細胞內 ROS 生成。由圖 6 數據得知，MG 組別較控制組明顯的下降至對照組的 59%，表示 MG 造成細胞內部 GSH 含量減少，而茶多酚的組別的 GSH 含量大多與控制組相差不大，因此推測茶多酚能夠減少氧化壓力的發生。

第三節 EGCG 抑制 MG 所誘導之細胞凋亡

最後收集預處理茶多酚 1 小時候 MG 處理三小時的細胞蛋白質，由西方墨點法分析 PARP (圖 7)。PARP 在細胞凋亡路徑當中會被剪切，因此利用此特性作為細胞凋亡指標蛋白。在預處理多種濃度的 EGCG 的組別能有效抑制 PARP 的活化且有效濃度與 MTT 實驗相符，同樣為 25 及 50 μM 的茶多酚處理能有最好的保護效果。然而預處理 TSA 組別並沒有看到抑制 PARP 剪切的功效。在圖 7 預處理 25 及 50 μM 的 EGCG 後處理 MG 3 小時的 cleavaged-caspase 3 蛋白表現，cleavaged-caspase 3 表現量減少，此一結果表示凋亡作用的減緩。

第四節 TSA 誘發細胞自噬保護 MG 所誘導之神經毒性

因為並沒有看到 TSA 具有顯著的抑制細胞凋亡相關蛋白的能力，因此推測 TSA 可能是透過不同的路徑保護細胞免於 MG 誘導的細胞傷害。透過 AO 染色能夠觀察到凝集的染色體、凋亡小體、酸性小泡，DNA 透過 AO 染色後呈現綠色而

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酸性小泡會呈現橘色。細胞處理方式是使用 25 和 50 μM 濃度的 EGCG 和 TSA 預處理 1 小時候 MG 處理 3 小時，之後將細胞使用 AO 染色再用共軛焦顯微鏡觀察此兩種螢光發射狀況。在 AO 染色過後 (圖 8)，呈現綠色小點的為細胞核染色體部分，由圖片白色實心箭頭所示，當染色體凝集現象發生，AO 會呈現更高的螢光量。由圖片可知，MG 組有許多染色體凝集的現象，而預處理 EGCG 能有效抑制細胞凋亡，且預處理高濃度的 EGCG 有些微自噬小體的的產生。但預處理 TSA 的組別則有許多橘色的自噬溶小體產生，如圖片白色虛線箭頭所示。

為了再次確認 TSA 的保護機制是否與自噬作用相關，因此將經過處理後的細胞透過 AO 染色偵測螢光強度以確認 TSA 是否透過自噬作用保護細胞。由量化圖圖 8 (B) 可知，預處理 TSA 的組別 AO 螢光強度有顯著上升，代表 AVO 的數量顯著提升，且高濃度 EGCG 預處理也會造成少數細胞走向細胞自噬但是並沒有顯著差異，而低濃度的 EGCG 並沒有顯著增加 AVO 的現象。

最後收集預處理茶多酚 1 小時候 MG 處理 3 小時的細胞蛋白質，由西方墨點法分析自噬作用指標蛋白 LC3 (圖 8)。另外，LC3 是自噬作用前端自噬小體生成當中相當重要的表現蛋白,LC3 I 與 PE 結合轉變成 LC3 II 藉此協助自噬小體生成。EGCG 預處理的組別並沒有很顯著的 LC3 II 蛋白質表現量提升但是 LC3 II 隨著 TSA 處理濃度增加，表現量也隨之增加。因此，在 MG 誘導細胞毒性之下，TSA 能促使細胞走向自噬進而保護細胞。

第五節 茶多酚抑制 MG 誘導神經毒性之分子機轉

在 p53 訊息傳遞路徑部分，p53 具有雙重功用，具有活化細胞凋亡及抑制細胞自噬。預處理 EGCG 後處理 MG 的處理組別能夠抑制 p53 的表現量進而抑制細胞凋亡，另一方面 TSA 預處理後也會抑制 p53 訊息傳遞路徑，進而活化細胞自噬的發生 (圖 9)。另一條與細胞自噬相關的訊息傳遞路徑: Akt 訊息傳遞路徑扮演著抑制細胞自噬的作用， TSA 處理組別顯著抑制 Akt 訊息傳遞路徑 (圖 10)。

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經由西方墨點法可知處理 1.5 mM MG 後能活化 JNK 和 ERK 訊息傳遞路徑，預處理 EGCG 及 TSA 皆能抑制 JNK 和 ERK 訊息傳遞路徑 (圖 11)。JNK 和 ERK 訊息傳遞路徑與促進細胞凋亡相關，EGCG 能夠透過抑制 ERK 和 JNK 訊息傳遞路徑有效抑制細胞凋亡的發生，然而 TSA 同時也會抑制 ERK 和 JNK 訊息傳遞路徑，MAPK 路徑在細胞凋亡和細胞自噬間所扮演的角色需要更進一步的研究。

粒線體膜電位利用 DiOC6 染劑用流式細胞儀的方式偵測螢光強度，由量化圖 (圖 12) 可知處理 MG 後粒線體膜電位顯著下降，然而 TSA 在膜電位也是有顯著性下降的。粒線體膜電位的下降與粒線體外膜通透性有關，其中 Bcl-2 能夠使粒線體膜電位維持在較高的膜電位。預處理 TSA 使 Bcl-2 表現量下降，此一結果與粒線體膜電位的下降結果相符 (圖 12)。caspase-9 需要裂解後具有活性，由圖 12 得知 TSA 使 cleavaged-caspase-9 表現增加。

粒線體膜電位的下降除了與細胞凋亡有關，自噬作用的誘發也需粒線體膜電位的下降而誘發，因此 TSA 組別的粒線體膜電位下降與下游自噬作用誘發相關。caspase-9 的活化能夠誘導下游自噬作用的發生，此一結果能夠解釋 TSA 誘導自噬作用的路徑。因此推測 TSA 所誘導的自噬作用是透過使膜電位降低並且活化下游的 caspasae-9。

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第六章 討論

MG 主要在體內主要由糖解作用代謝生成，是活性雙羰基化合物，已有許多研究指出 MG 與神經病變有相當大的關連性。在一個 2012 年台灣發表的文獻中指出: 糖尿病患增加 1.3 倍風險罹患帕金森氏症 (Sun et al., 2012)，因此研究者認為高血糖會產生神經毒性物質，進而造成神經損傷，而 MG 被認為是誘發神經退化性疾病之重要的神經毒性物質 (Xie et al., 2014)。目前已知茶葉中主要活性物質是茶多酚類，除了含量最多最廣為人知的 EGCG 外當中的 TSA 更是烏龍茶中特殊物質，近年來有許多對於茶葉中活性物質的研究。已有許多研究證實 EGCG 能抗氧化、神經保護而 TSA 具有抗氧化、抗發炎的功效，然而茶多酚對於 MG 造成的神經損傷的保護效果研究並不多，因此本實驗以 EGCG 及 TSA 作為實驗材料研究茶多酚的神經保護效果。

而不同的刺激會造成不同的細胞死亡型態，例如細胞凋亡會形成凋亡小體、染色體凝集，細胞自噬會產生細胞自噬溶小體，而細胞壞死會導致細胞脹裂，透過細胞型態觀察能初步分別細胞死亡型態 (Ricci and Zong, 2006)。在 MG 對於神經損傷的研究上，先前研究指出 MG 對於細胞死亡主要是透過產生 ROS，使粒線體膜電位下降、釋放 cytochrome c、活化 caspase-3 進而誘導細胞凋亡的路徑 (Heimfarth et al., 2013, Kimura et al., 2009)。本研究在細胞型態部分，MG 處理的組別在顯微鏡底下可以明顯觀察到細胞皺縮、還有凋亡小體的生成。此現象利用 AO 染色後，可更為清晰看到凝集的染色體。這些 MG 所造成的細胞傷害，透過預處理茶多酚後能有效預防細胞凋亡的發生，在細胞存活率實驗當中證實在 MG 誘導神經毒性之下 EGCG 和 TSA 都具有保護細胞的效果。

另外，透過流式細胞儀偵測胞內 ROS 生成發現 MG 處理後 30 分鐘 ROS 生成量達到頂峰，這些研究結果與先前的文獻結果相符合。處理茶多酚的組別能有效清除 ROS 且 TSA 的效果更勝 EGCG。此外，先前研究指出 TSA 與 EGCG 都具有

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抗氧化的功效 (Tipoe et al., 2007, Weerawatanakorn et al., 2015)，在本研究當中抗氧化酵素 GSH 表現量在處理茶多酚後能保持正常含量。其中，TSA 清除 ROS 的能力更勝 EGCG，推測與 TSA 結構較 EGCG 擁有更多的 OH 基，因此 TSA 清除 ROS 效果更好。

由細胞存活率實驗以及 AO 染色觀察證實，不論是 EGCG 或是 TSA 都能有效保護細胞免於 MG 造成的細胞損傷。EGCG 能夠抗凋亡使染色體凝集狀況稍微舒緩並且誘導少量自噬酸性小泡生成，並能透過抑制粒線體膜電位的下降進而抑制細胞凋亡的發生，能與先前研究 EGCG 對於 AGEs 產生傷害有保護作用相呼應 (Lee and Lee, 2007)，然而針對 MG 造成之細胞損傷能有保護作用是新的發現。至於 TSA 能夠誘發的保護路徑包括—抗發炎、抗氧化，關於其在細胞死亡當中扮演角色目前是鮮有文獻指出，在本研究中發現 TSA 處理組別因為 AO 染色觀察推測在 MG 誘導神經毒性下 TSA 誘導細胞走向自噬作用，透過 AO 染色、AO 螢光強度偵測以及西方墨點法 LC3 表現量再次確認，證實 TSA 能誘發細胞保護型自噬作用。

茶多酚抑制 MG 誘導細胞凋亡之分子機轉透過抑制 p53 訊息傳遞路徑。p53 屬於促進凋亡的訊息傳遞路徑 (Fridman and Lowe, 2003)。文獻指出處理 MG 誘發 p53 訊息傳遞路徑 (Zhou et al., 2015)，而在本研究中處理茶多酚後 p53 受到抑制表示茶多酚能夠抑制 p53 誘導的細胞凋亡。另一方面，TSA 抑制 MG 誘導細胞凋亡之分子機轉透過抑制 Akt 訊息傳遞路徑進而促進自噬作用的發生。MG 的處理會下調 Akt 訊息傳導路徑，處理 EGCG 後能些微上調 Akt 訊息傳遞路徑，這表示著 EGCG 能透過活化 MG 會活化 Akt 進而促進細胞存活，另一方面在 MG 誘導細胞毒性之下 TSA 則是顯著抑制 Akt 訊息傳遞路徑，先前研究指出活化的 Akt 訊息傳遞路徑會活化下游 mTOR 而 mTOR 會抑制自噬作用 (Wang et al., 2012)，因此推測 TSA 透過抑制 Akt 訊息傳遞路徑使 MG 處理的細胞走向自噬。

文獻指出 MG 會促進 MAPK 路徑的 JNK, ERK, 和 p38 (Guo et al., 2016)，因為

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MAPK 路徑會被 ROS 活化進而活化下游促凋亡因子 (Son et al., 2011, Zhou et al., 2015)。 在本研究中也發現經由西方墨點法得知處理 1.5 mM MG 後活化 JNK 和 ERK 訊息傳遞路徑，使細胞走向細胞凋亡。然而有趣的是處理 EGCG 和 TSA 皆能抑制 JNK 和 ERK 訊息傳遞路徑，2007 年的文獻指出 MAPK 路徑的活化可能與自噬作用成熟期的調控相關，若是抑制 MAPK 路徑會形成較大的自噬溶小體 (Corcelle et al., 2007)，這部分與 AO 染色當中觀察到的 TSA 預處理組別產生的自噬溶小體顆粒較大是吻合的，然而是否 MAPK 訊息傳遞路徑在細胞凋亡及細胞自噬當中扮演調節轉換的角色，而達到保護細胞的功效，這部分需要更多的研究證實。

接著探討粒線體膜電位與 caspase-9 與自噬作用間的關聯性。MG 透過抑制 Bcl-2 表現量 (Guo et al., 2016, Zhou et al., 2015) 進而導致粒線體膜電位下降。本研究利用西方墨點法證實粒線體外膜通透性相關蛋白: Bcl-2 的表現量減少有利於粒線體膜電位下降。利用 DiOC6 染劑用流式細胞儀的方式偵測螢光強度，觀察到處理 MG 後粒線體膜電位顯著下降。因此推測 TSA 所誘導的自噬作用是透過使膜電位降低並且活化下游的 caspasae-9 的路徑。在 2011 年的文獻中發現對乳癌細胞處理抗發炎藥物所誘發的細胞自噬途徑是透過活化 caspase-9 (Jeong et al., 2011)，在本篇研究中探討 TSA 抑制 MG 誘導神經毒性的分子機轉也發現此現象。此路徑是一條新發現的路徑能夠調節細胞凋亡與細胞自噬的轉換，然而 caspase-9 如何誘導自噬作用的機制並不明朗 (Han et al., 2014)。

綜合以上結果顯示，茶多酚能夠預防細胞免於 MG 的傷害，且透過兩個不同的路徑保護細胞，EGCG 透過抑制 MG 誘導的細胞凋亡達到神經保護的功效，而 TSA 則是在 MG 誘導神經毒性之下誘發自噬作用保護細胞。因為兩者結構有很大的不同，推測 EGCG 和 TSA 走向不同的分子機轉是因為多酚類的 OH 基立體構型的差異導致。

另本實驗是利用細胞模式研究 MG 造成神經損傷以及茶多酚的保護作用，在

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本研究中發現茶多酚具有保護功效，然而各個路徑間的交互關係相當複雜，需要更進一步的實驗研究，且並未有動物實驗驗證，故未來可探討在生物體當中是否也具有相同功效。

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第七章 結論

根據本實驗結果，我們對於茶多酚的功能性有的更進一步的了解，茶多酚 EGCG 和 TSA 對於 MG 誘導的神經毒性具有神經保護的功效。以 SHSY5Y 細胞株作為細胞模式的細胞存活率實驗發現 MG 處理 3 小時之下其半數抑制劑量為 1.5 mM 並具有劑量效應。在使用茶多酚預處理 1 小時候在處理 3 小時的 MG 後，細胞存活率有顯著的提升，表示茶多酚能夠保護細胞避免 MG 誘導之細胞死亡。為了釐清增加細胞存活率的原因，透過偵測胞內 ROS 的方式發現 EGCG 和 TSA 都具有清除胞內 ROS 的作用，且能透過維持胞內 GSH 含量的方式間接抑制 ROS 造成的氧化壓力。使用西方墨點法凋亡指標蛋白 PAPR 發現 EGCG 對於細胞凋亡具有抑制效果，然而 TSA 對於抑制細胞凋亡的效果並不是很顯著。因此接著探討 TSA 是否透過其他路徑抑制 MG 誘導的細胞毒性。以 AO 染色觀察細胞型態以及螢光強度並且使用西方墨點法測量自噬指標蛋白 LC3 的蛋白質表現量，而 TSA 會促使 SH-SY5Y 細胞中 LC3-I 轉變為 LC-3 II，這些結果顯示 TSA 參與細胞自噬溶小體的合成，使細胞走向細胞自噬。更進一步的分子機制探討發現，EGCG 透過 caspase-3 裂解，並抑制 MAPK、p53 訊息傳遞路徑進而抑制細胞凋亡。另一方面 TSA 則是透過引發粒線體膜電位下降、caspase-9 表現活性、抑制 MAPK、p53、Akt 訊息傳遞路徑進而誘發細胞自噬。綜合以上實驗數據顯示茶多酚 EGCG 及 TSA 對於 MG 誘導的細胞毒性具有保護功效，且兩者走向不同的保護機制，EGCG 透過抑制 MG 誘導的細胞凋亡，而 TSA 則是透過誘發自噬作用保護 MG 誘導的細胞毒性。期望此結果能提供證實茶多酚具有潛力來預防及減緩神經退化性疾病 (圖 13)。

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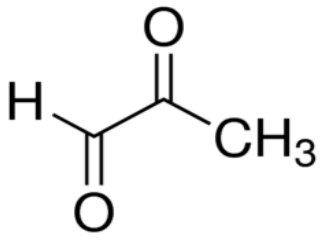
第八章 圖表

(A)



(B)

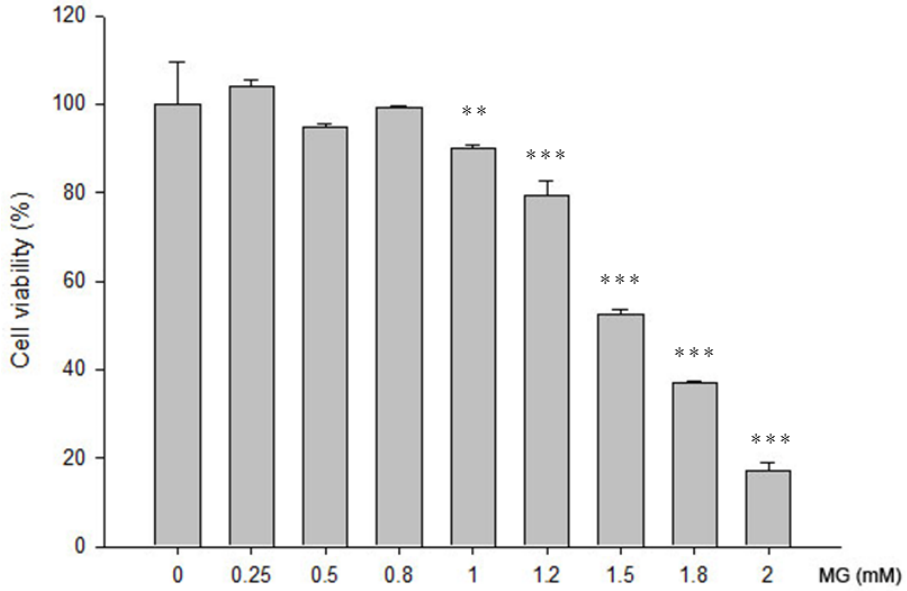


圖 1 、MG 結構與 MG 誘導細胞毒性對細胞存活率之影響

Figure 1 The structure of MG and effects of MG on cytotoxicity.

MG structure (A) and the cell viability of cells was determined by MTT assay. Cell were treated with 0.25, 0.5, 0.8, 1, 1.2, 1.5, 1.8, 2 mM of MG for 3 hours (B). * p<0.05 **p<0.01 ***p<0.001 compared with control group by one-way ANOVA and Dunnett's multiple range test.

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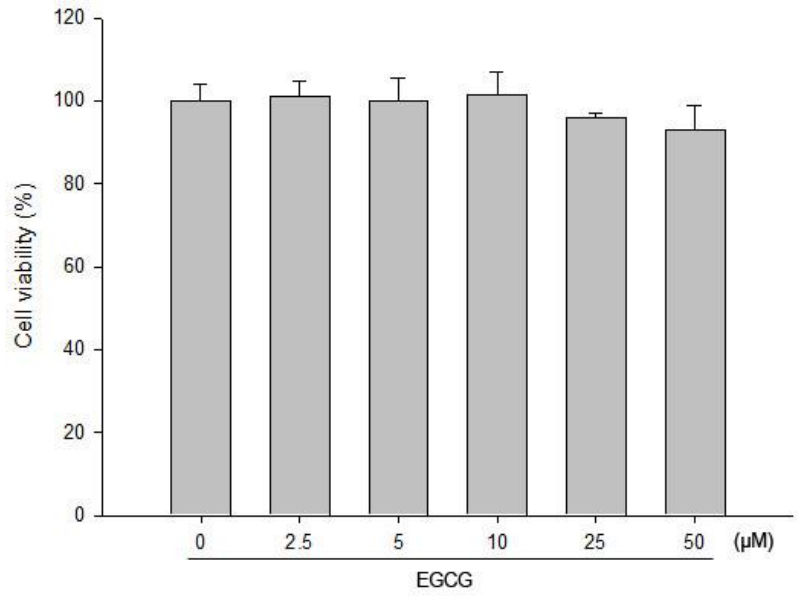
(A)



(B)

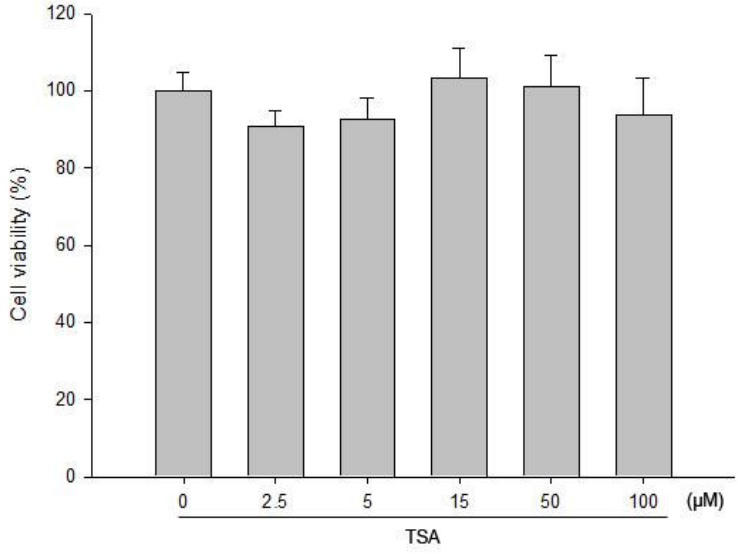


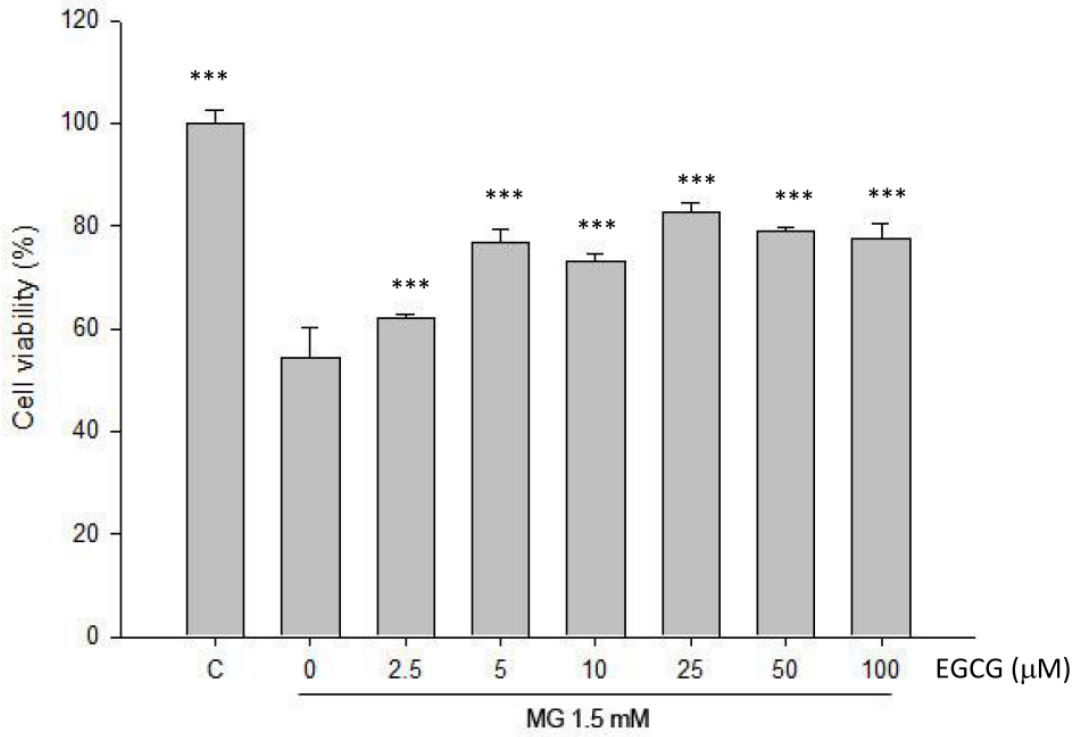
圖 2 、EGCG 及 TSA 對神經細胞存活率之影響

Figure 2 Effects of EGCG and TSA on neurotoxicity.

The cell viability of cells was determined by MTT assay. Cell were treated with 2.5, 5, 10, 25, 50 μM of EGCG (A) and 2.5, 5, 10, 25, 50, 100 μM of TSA (B) for 24 hours. * p<0.05 **p<0.01 ***p<0.001 compared with control group by one-way ANOVA and Dunnett's multiple range test.

doi:10.6342/NTU201603033

(A)



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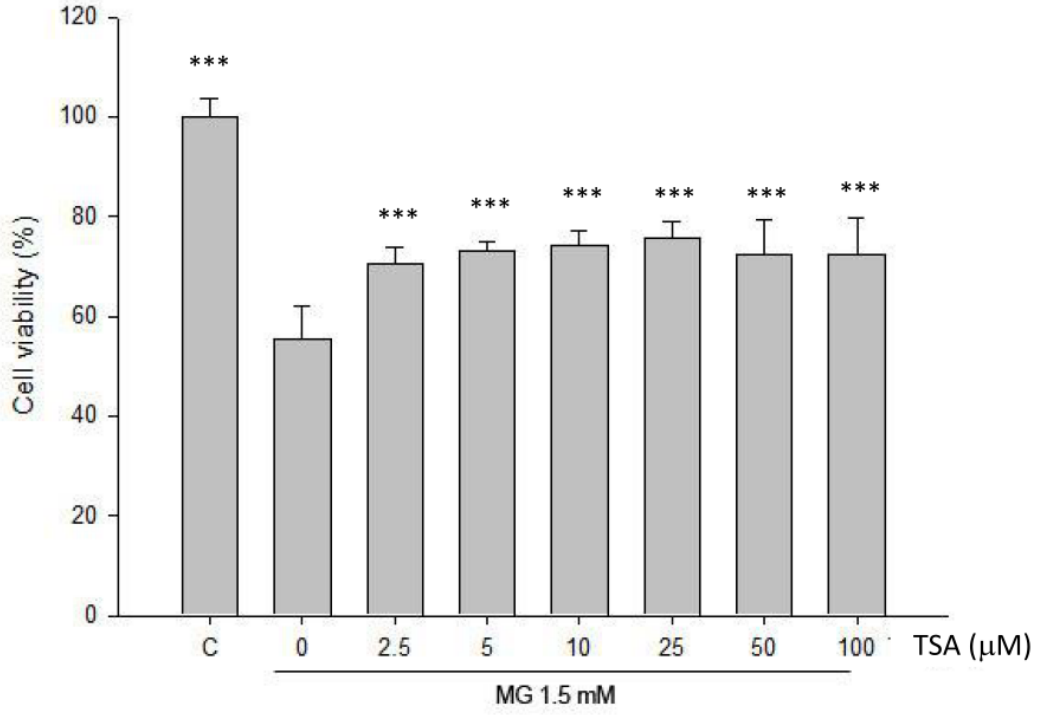

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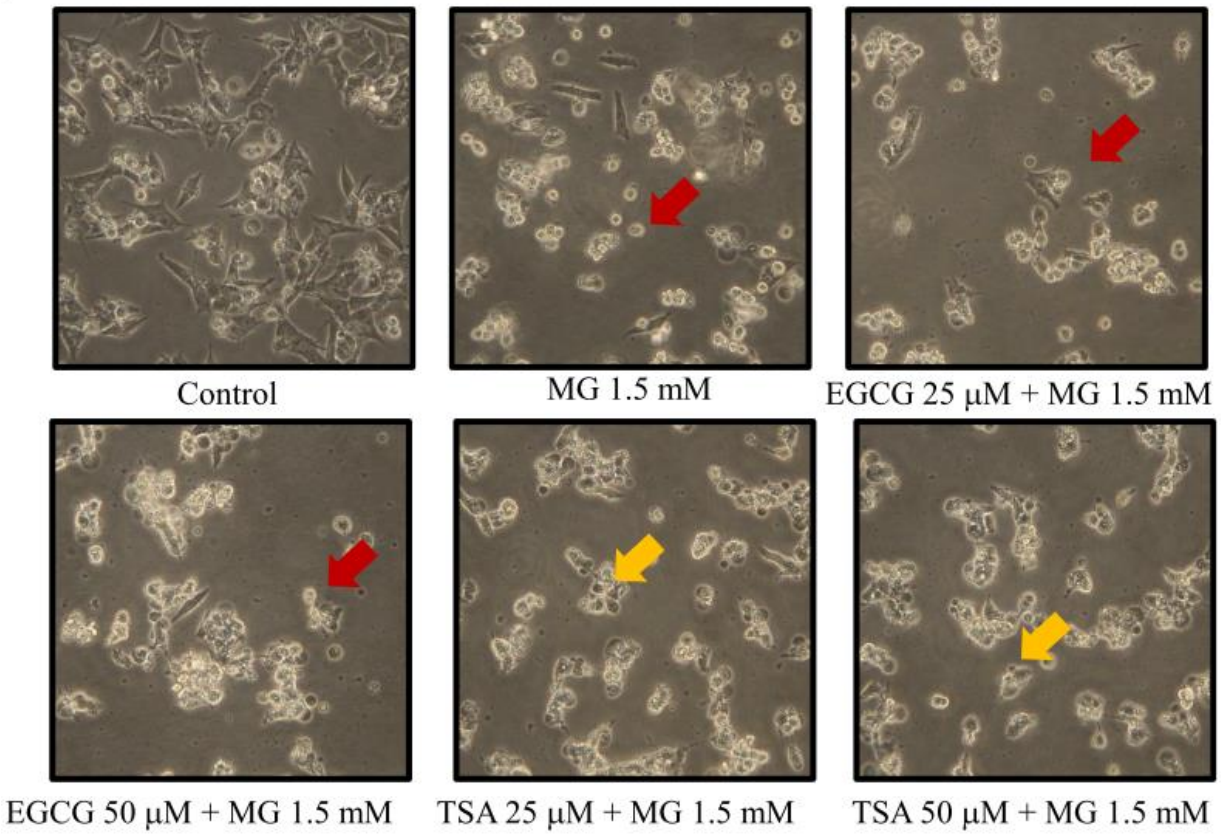


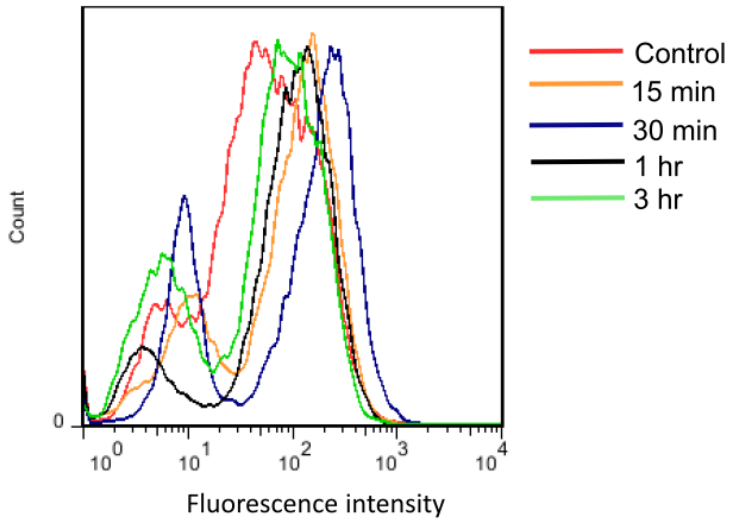
圖 3 、EGCG 及 TSA 抑制 MG 誘導神經細胞毒性

Figure 3 Effects of EGCG and TSA on MG-induced neurotoxicity.

The cell viability of cells was determined by MTT assay. Cell were pre-treated with different concentration of EGCG (A) and TSA (B) for an hour then treated MG for 3 hours. The cell morphology after treatment was observed via inverted microscope (C). Red arrow means shrink cells and yellow arrow means bubble type cell (magnification: 400x). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with MG treatment by one-way ANOVA and Dunnett's multiple range test.

doi:10.6342/NTU201603033

(A)



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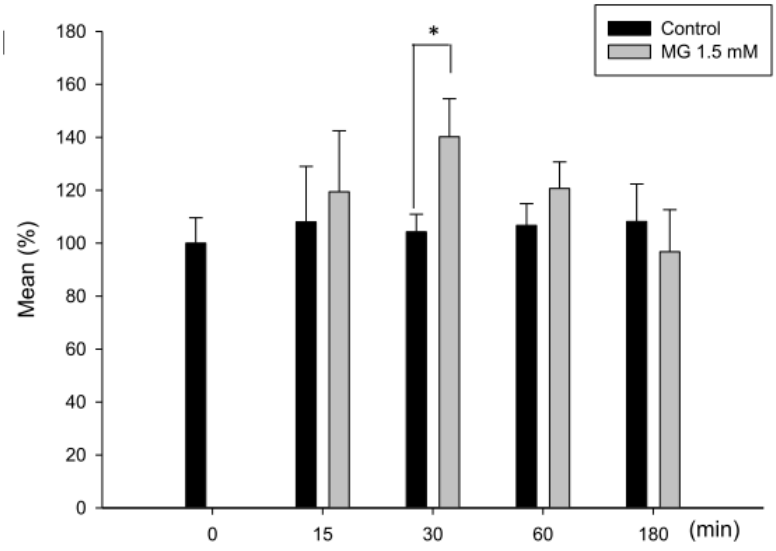


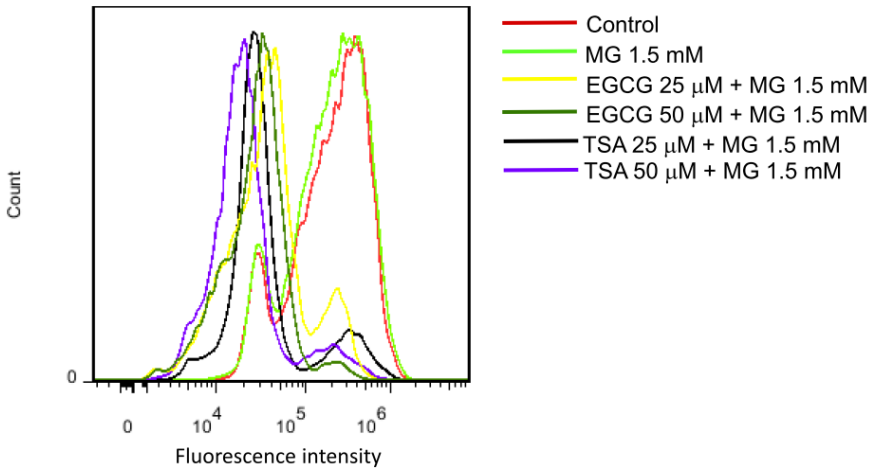
圖 4 、MG 誘導胞內 ROS 之生成

Figure 4 MG induced intracellular production of ROS in SH-SY5Y cell.

Cell were treated with 1.5 mM of MG for 0, 5, 15, 30, 60, 180 min followed by incubated DCFH-DA and analyzed by flow cytometry (A) (B). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with control group by student's *t*- test.

doi:10.6342/NTU201603033

(A)



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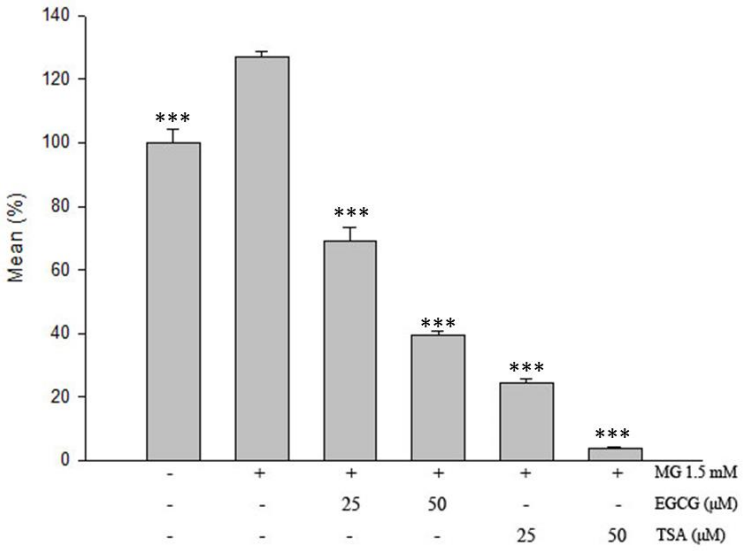


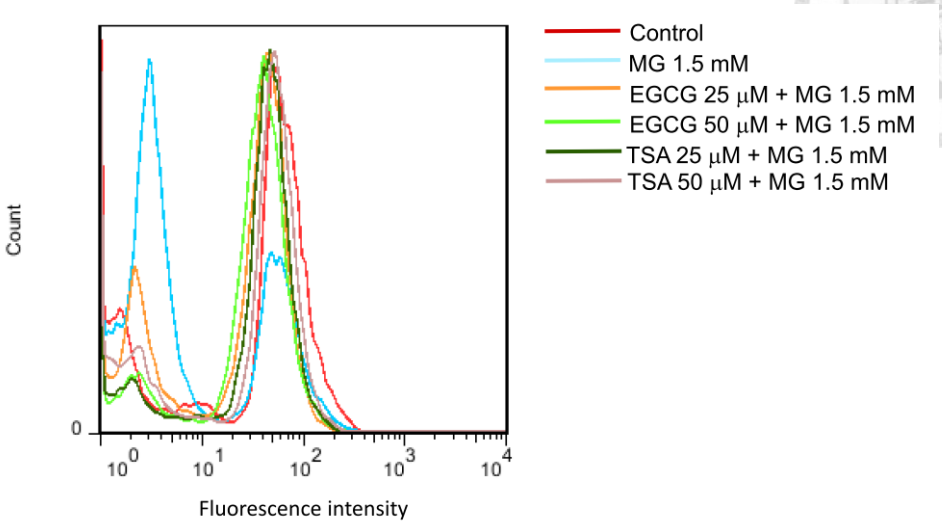
圖 5 、 EGCG 及 TSA 抑制 MG 誘導 ROS 之生成

Figure 5 EGCG and TSA inhibited MG-induced intracellular production of ROS in SH-SY5Y cell.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated DCFH-DA and analyzed by flow cytometry (A) (B). * $p<0.05$ **$p<0.01$ ***$p<0.001$ compared with MG-treated group by one-way ANOVA and Dunnett's multiple range test.

doi:10.6342/NTU201603033

(A)



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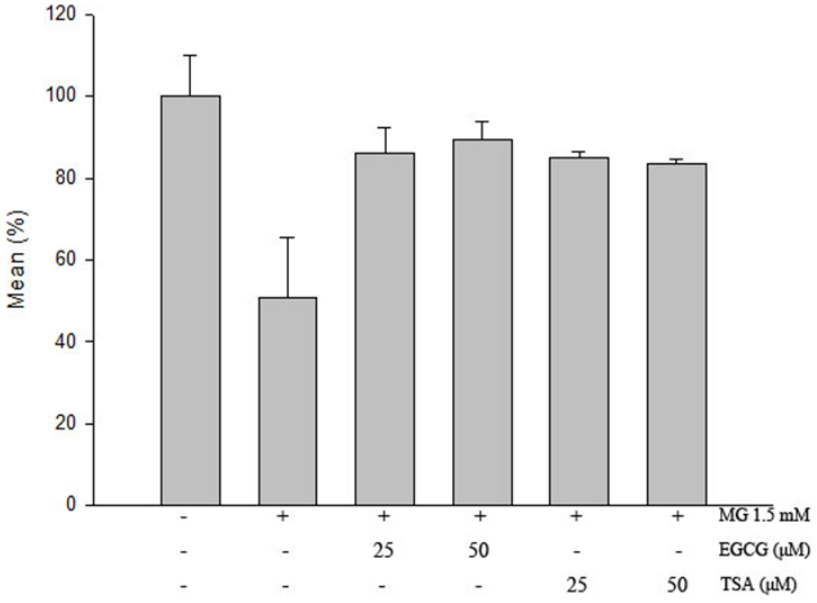

圖 6 、EGCG 及 TSA 對於 MG 抑制胞內 GSH 含量之影響

Figure 6 EGCG and TSA modulated MG-decreased intracellular level of GSH.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated CMFDA and analyzed by flow cytometry (A) (B). * $p<0.05$ **$p<0.01$ ***$p<0.001$ compared with MG-treated group by one-way ANOVA and Dunnett's multiple range test.

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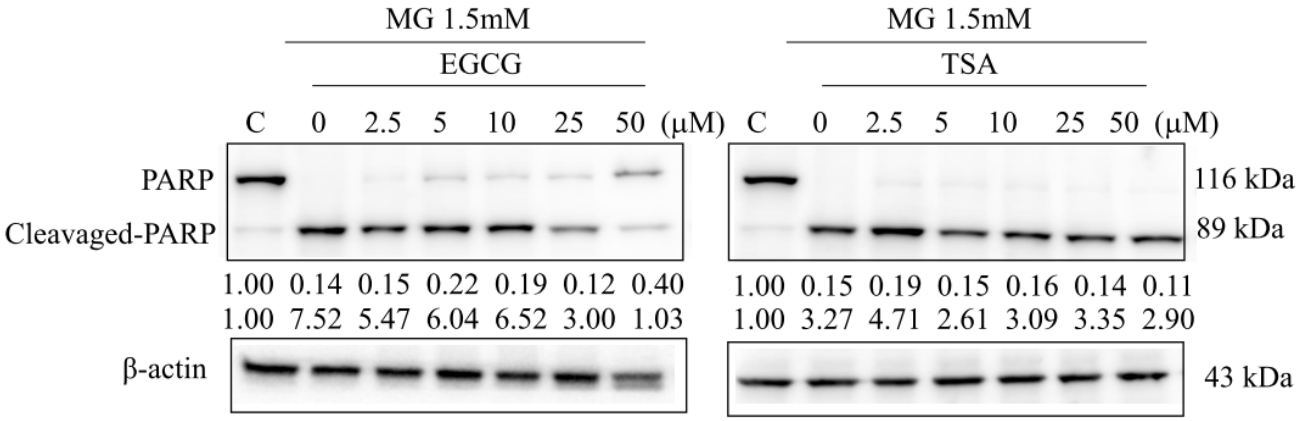
(A)



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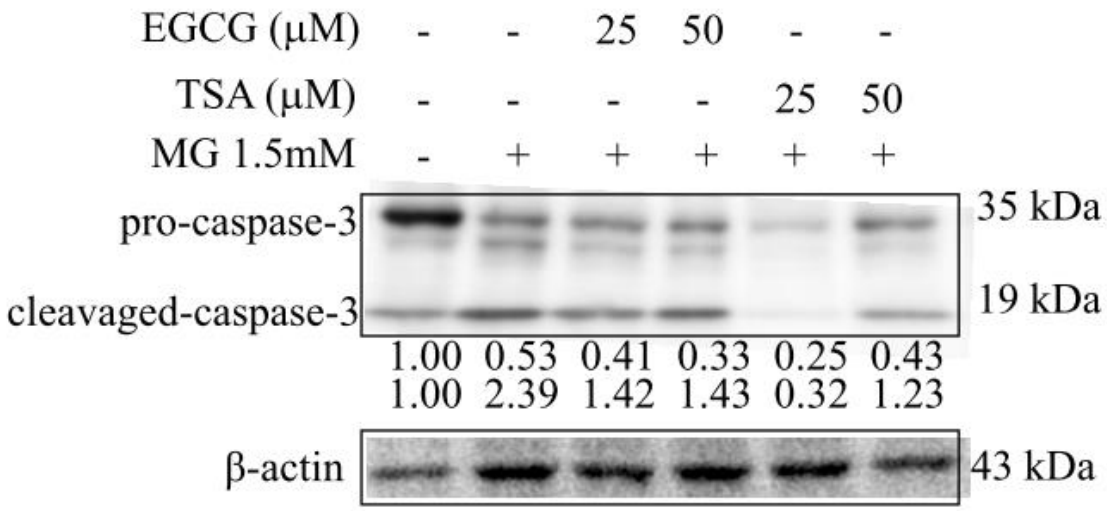


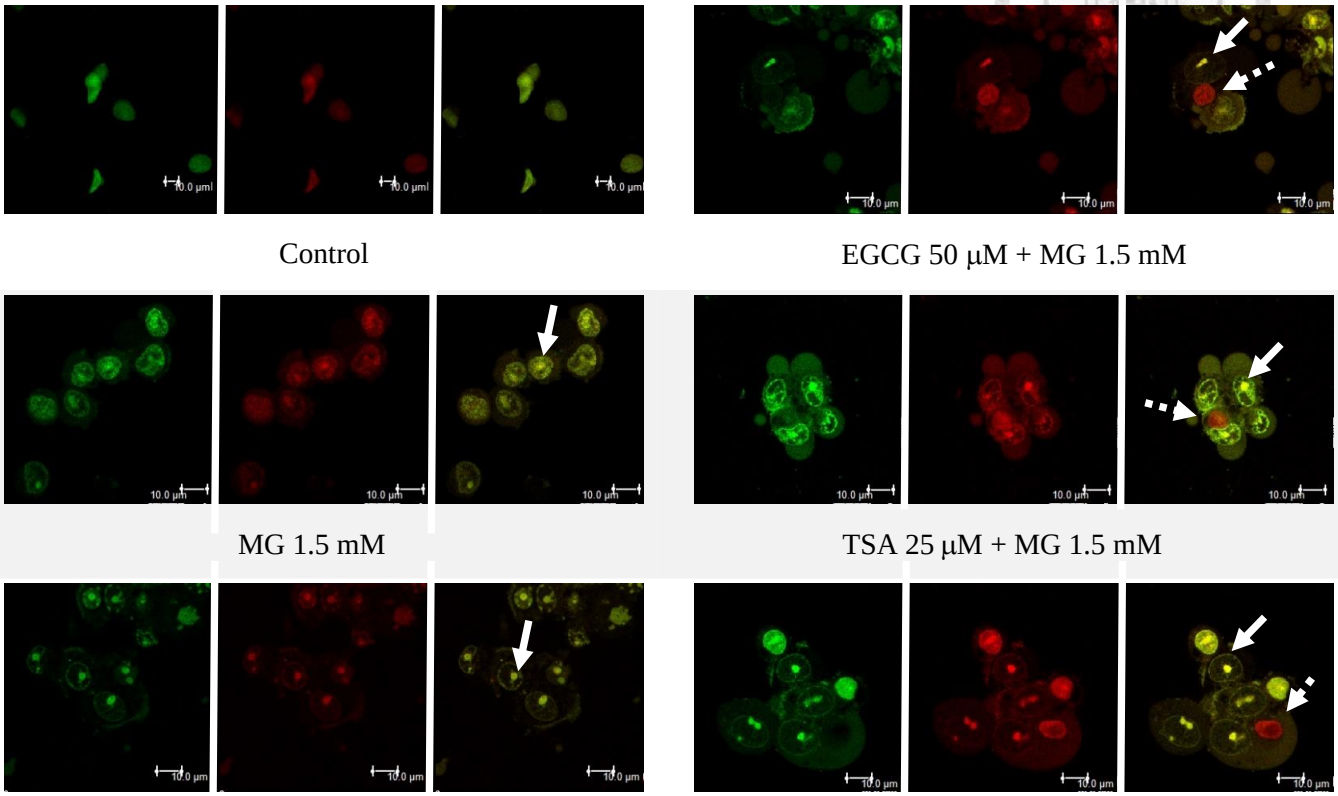
圖 7 、EGCG 及 TSA 對於 MG 誘導細胞凋亡之影響

Figure 7 Effects of EGCG and TSA on MG-induced apoptosis.

Cleavaged-PARP (A) and caspase-3 (B) protein levels in SH-SY5Ycell was analyzed by western blotting. The cells were treated with 0, 2.5, 5,10, 25, 50 μM of EGCG or TSA for 1 hours and then treated 1.5 mM of MG for 3 hours.

doi:10.6342/NTU201603033

(A)



Control

EGCG 50 μM + MG 1.5 mM

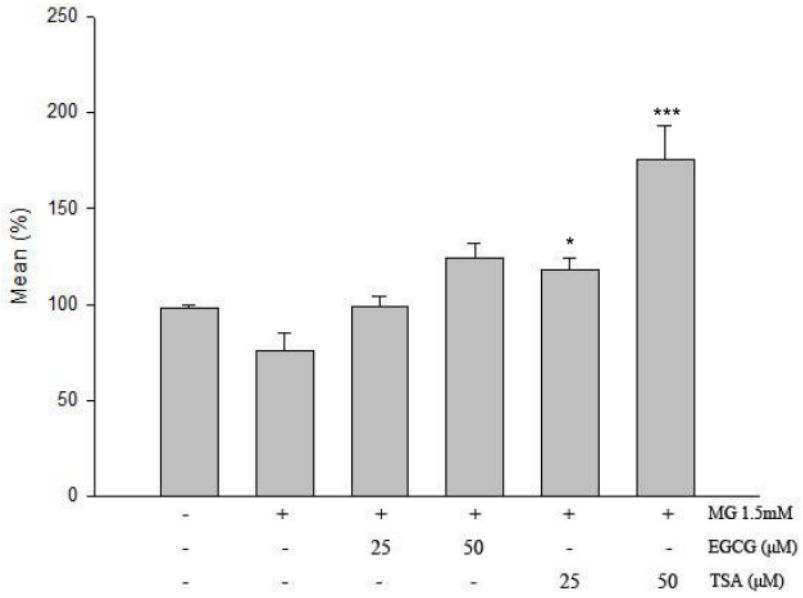
MG 1.5 mM

TSA 25 μM + MG 1.5 mM

EGCG 25 μM + MG 1.5 mM

TSA 50 μM + MG 1.5 mM

(B)



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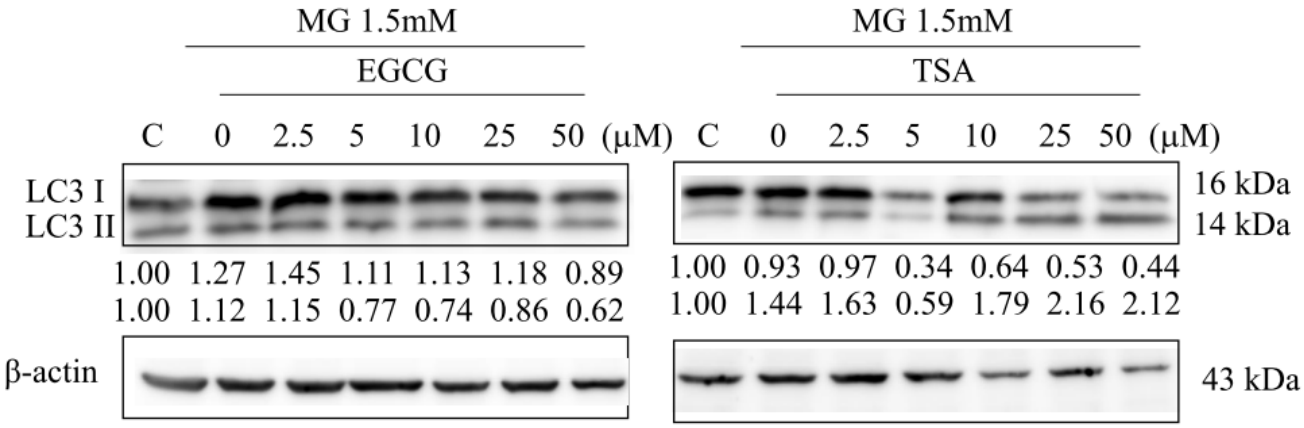


圖 8 、TSA 對 MG 處理 SH-SY5Y 細胞誘導自噬作用

Figure 8 TSA induced autophagy on MG-treated SH-SY5Y cell.

The cell morphology after pre-treated 25, 50 μM of tea polyphenol for an hour and 1.5 mM of MG treated for 3 hours (A). The cells were stained with AO as described in experimental procedures. White solid arrow means condensed chromosome and white dashed arrows means AVO. (Scale bar=10 μm) After incubation with 25, 50 μM of tea polyphenol for an hour and then treated 1.5 mM of MG for 3 hours, intracellular AVO value was analyzed by flow cytometry (B). The autophagy marker- LC3 II protein levels was analyzed by western blotting (C).

doi:10.6342/NTU201603033

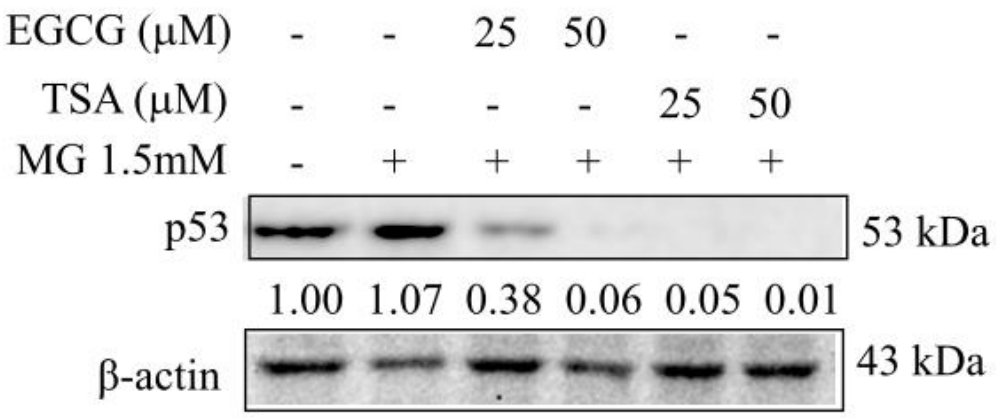


圖 9 、EGCG 及 TSA 調控 MG 誘導 p53 訊息傳遞路徑

Figure 9 Effect of EGCG and TSA on MG-induced p53 pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of p53 pathway protein was detected by western blotting.

doi:10.6342/NTU201603033

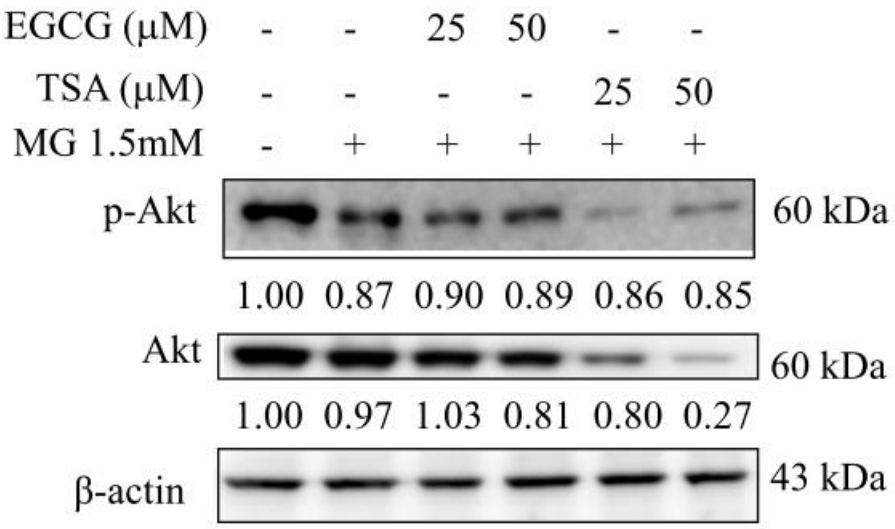


圖 10 、EGCG 及 TSA 調控 MG 誘導 Akt 訊息傳遞路徑

Figure 10 Effect of EGCG and TSA on MG down-regulated Akt pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of Akt pathway protein was detected by western blotting.

doi:10.6342/NTU201603033

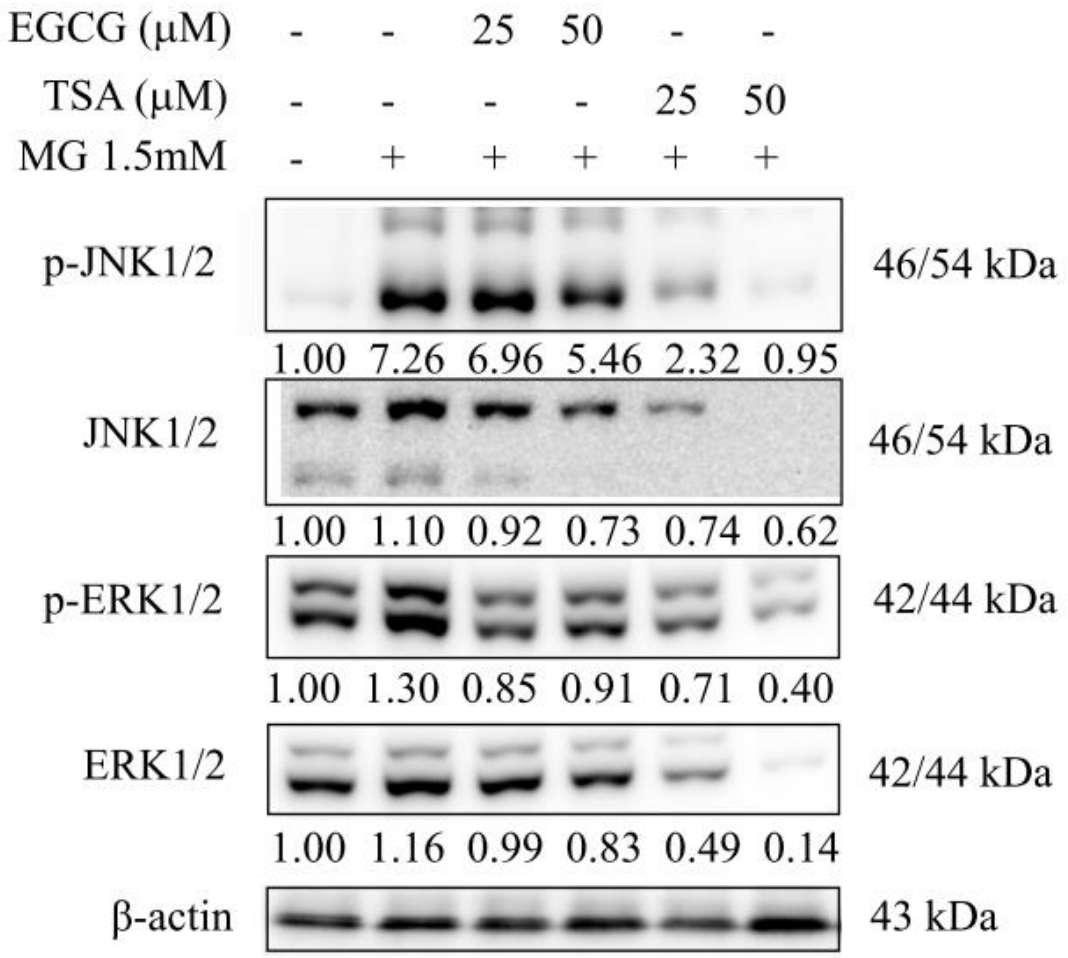


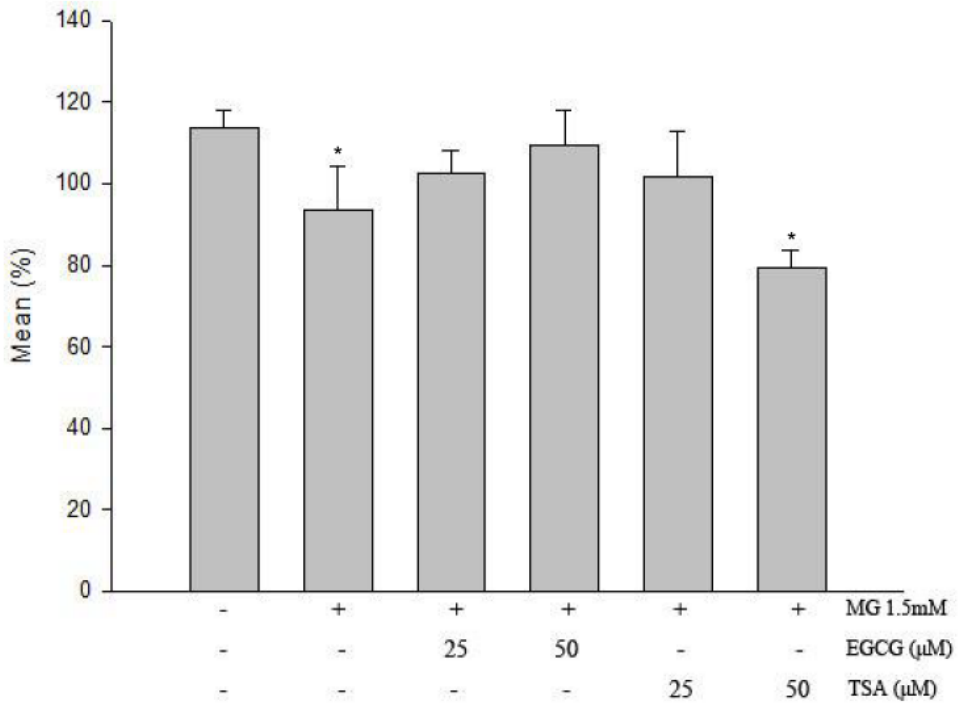
圖 11 、EGCG 及 TSA 調控 MG 誘導 ERK 及 JNK 訊息傳遞路徑

Figure 11 Effect of EGCG and TSA on MG-induced ERK and JNK pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of ERK and JNK pathway protein was detected by western blotting.

doi:10.6342/NTU201603033

(A)



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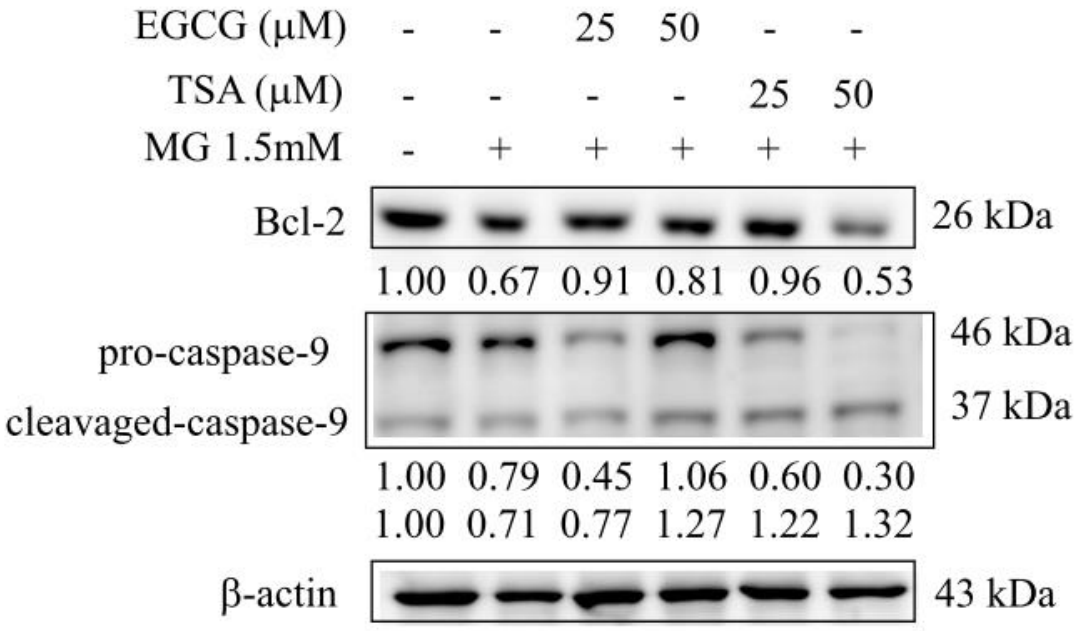

圖 12 、EGCG 及 TSA 對 MG 調控粒線體膜電位之影響

Figure 12 Effect of EGCG and TSA on MG-induced MMP alteration.

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Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated DiOC6 and analyzed by flow cytometry (A). For detecting the expression of Bcl-2 and cleavage-caspase-9 by western blotting, cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 3 hours (B). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with control group by one-way ANOVA and Dunnett's multiple range test.

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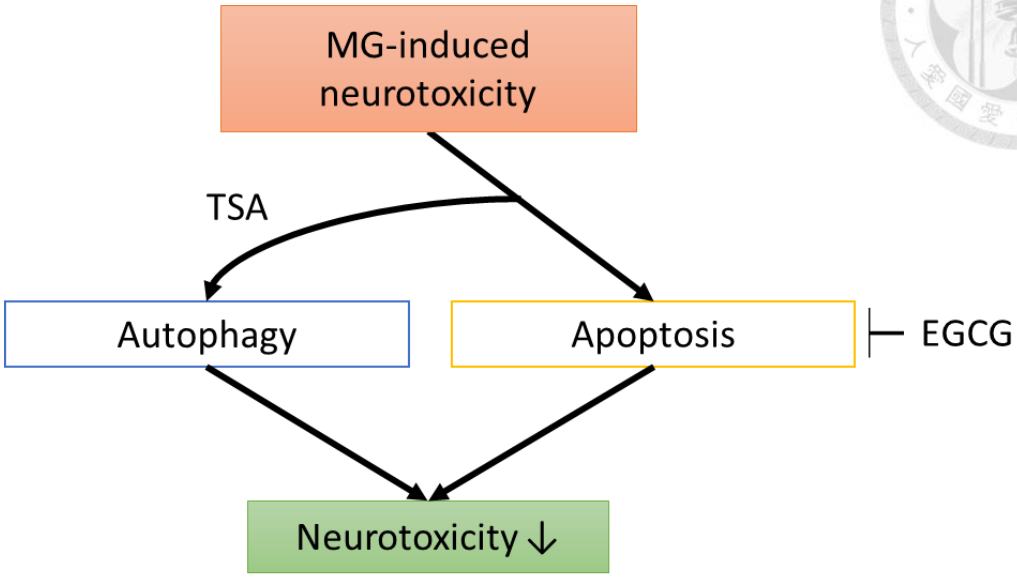
(A)



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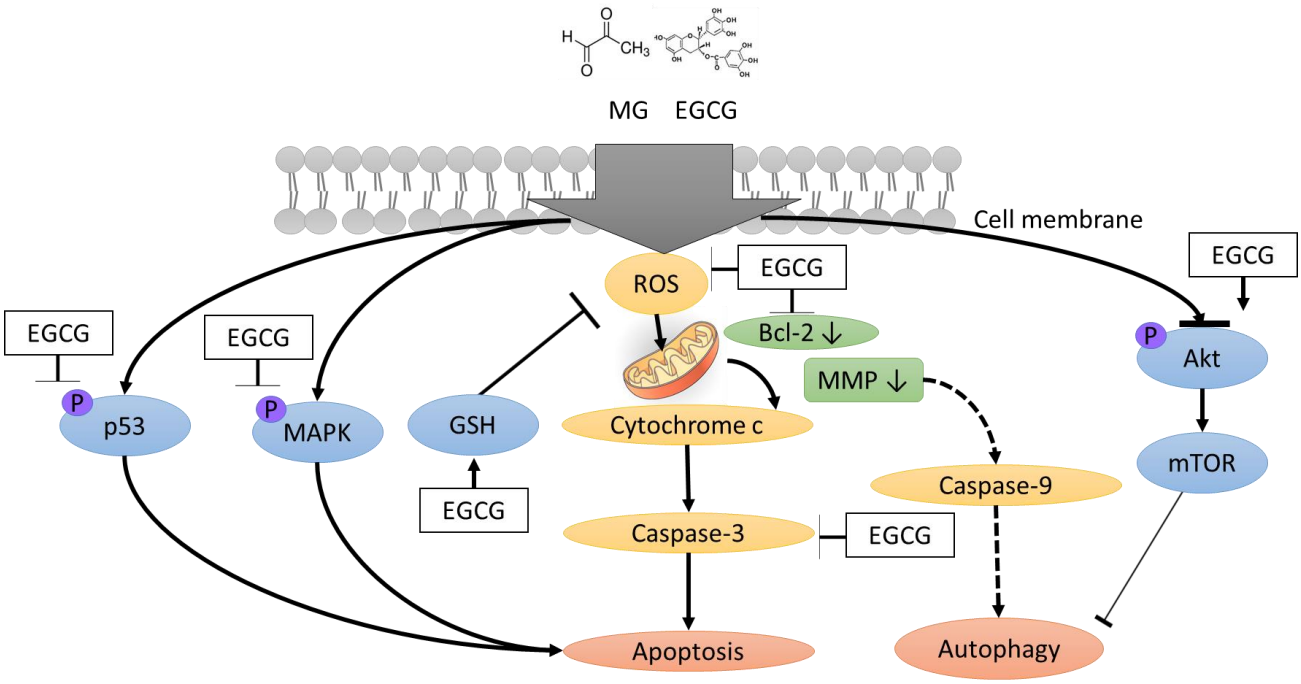

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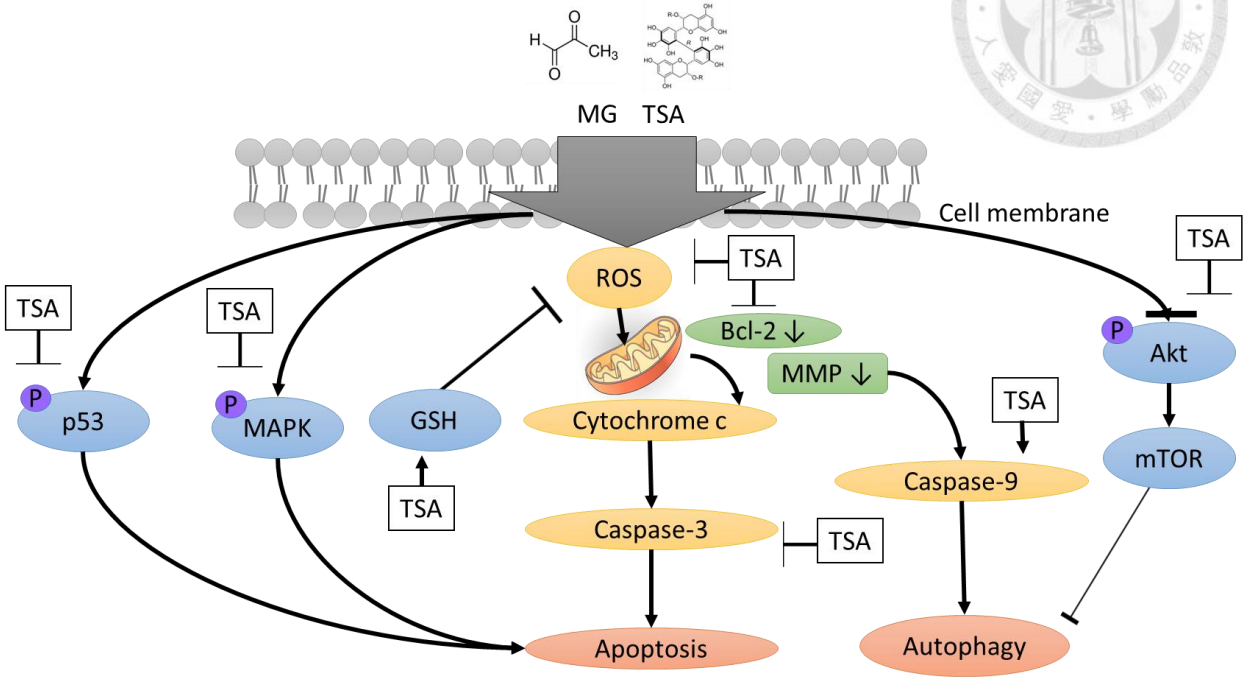

圖 13 、EGCG 和 TSA 對於 MG 誘導細胞損傷的保護機制

Figure 13 The mechanism of EGCG and TSA against MG-induced cell injury.

The graphical abstract of this study (A), mechanism of EGCG (B) and TSA (C) against MG-induced cell injury.

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The Protective Effect of Tea Polyphenol against Methylglyoxal-induced Neurotoxicity

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Keywords: Methylglyoxal (MG) 、 (-)-Epigallocatechin-3-gallate (EGCG) 、 Theasinensin A (TSA)、Apoptosis、Autophagy、Oxidative stress

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Abbreviations:

AGEs, advanced glycation end products; Akt, protein kinase B; AO, acridine orange; Apaf-1, apoptotic protease-activating factor-1; AVO, acidic vesicular organelles; Bax, bcl-2-associated x protein; Bcl-2, B cell lymphoma-2; Bid, BH3 interacting-domain death agonist; BSA, bovine serum albumin; Caspase, cysteine-dependent aspartate specific protease; CMFDA, 5-chloromethylfluorescein diacetate; DCFH , dichlorofluorescin; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DMSO, dimethyl sulfoxide; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin 3- gallate; ERK, extracellular signal-regulated protein kinases; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; JNK, c-jun n-terminal kinases; MAPK, mitogen-activated protein kinases; MG, methylglyoxal; MMP, mitochondria membrane potential; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly ADP ribose polymerase; PCD, programmed cell death; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; TSA, theasinensin A

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Abstract

Methylglyoxal (MG) is a kind of highly reactive dicarbonyl aldehyde. MG has been proved to be toxic to neuron and may be the reason of many neurodegenerative diseases. Tea is a traditional drinking and previous studies have observed that chronic tea consumption may reduce the risk of neurodegeneration. One of the most well-known tea catechin, (-)-epigallocatechin-3-gallate (EGCG), and theasinensin A (TSA), the dimer of EGCG had been found to have many bioactivities, such as anti-oxidation. However, the effect of the neuroprotective effect of tea polyphenol remains unclear. The aim of this study is to investigate the neuroprotective effects and the molecular mechanism of tea polyphenol against MG-induced toxicity in SH-SY5Y cell model. The cell viability assay demonstrated that EGCG and TSA treatment protected cells from MG-induced neurotoxicity. Besides, evidences from flow cytometry showed that EGCG and TSA inhibited MG-induced oxidative stress via reducing MG-induced reactive oxygen species (ROS) generation and induce intracellular GSH level. Through activation of caspase 3 and cleavage-PARP, EGCG would inhibit MG-induced apoptosis but TSA didn't inhibit MG-induced apoptosis. The result of acridine orange (AO) stain, detecting AO fluoresce intensity and the level of LC3, showed that TSA would induce autophagy in MG-treated neuron cell. EGCG inhibited p53 and MAPK pathway to increase cell viability via western blotting analysis; on the other hand, TSA inhibited Akt pathway to induce autophagy. In conclusion, both EGCG and TSA has the ability to protect cell from MG-

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induced cytotoxicity via different mechanisms. We expect that EGCG and TSA could be a reagent to protect neurodegenerative diseases.

Keywords： Methylglyoxal (MG)、(-)-Epigallocatechin-3-gallate (EGCG)、Theasinensin A (TSA)、Apoptosis、Autophagy、Oxidative stress

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1 Introduction

Methylglyoxal (MG) is a kind of highly reactive dicarbonyl aldehyde, which is a metabolic compound in human body and by-product of food processing. In endogenous generative pathway, MG can be majorly formed by glycolysis and less MG is formed from polyol pathway and by-product of protein or lipid metabolism (Allaman et al., 2015). MG also can be generated from exogenous pathway, such as Maillard reaction during food processing (Wang and Ho, 2012).

Epidemiological studies indicate that MG is related to diabetes, cardiovascular diseases, and neuron degenerative diseases (Sun et al., 2012). Parkinson's disease is one of the most prevalent neurodegenerative disorders around the world. The causes of Parkinson ’s disease is gradually death of dopaminergic cells by multifactorial reasons. Many study revealed that MG would increase intracellular reactive oxidative stress (ROS) level or generate metabolic products, which is very toxic to dopaminergic neuron cells (Xie et al., 2014). Thus, MG may play a crucial role in the etiology of Parkinson ’s disease (Sun et al., 2012).

The generation of ROS is very important in MG-induced cell toxicity (de Arriba et al., 2007). ROS is an oxygen-containing molecule has one or more unpaired electron group, including oxygen ion, peroxide. This kind of chemical structure would have high

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activity that can easily reaction with other chemicals, such as DNA or protein molecules. Under normal condition, ROS is a by-product of energy metabolism, such as electron transport chain. To ensure regular cell condition, cell has anti-oxidation systems to control ROS level between acceptable range. However, when the regulatory system is loss of function, extremely high concentration of ROS would induce senescence or diseases (Fernández-Mejía, 2013). Thus, how to regulate ROS level is very critical for cell viability.

Previous study found that MG not only increase intracellular ROS but also increase cell death. It is generally considered that unwilling cell death is linked to diseases. The ways of cell death may be classified into three categories: apoptosis, autophagy, and necrosis. The cell would have different appearance and mechanisms depending on different type of cell death. First, apoptosis, also called program cell death I, is a way of cell death to maintain normal body operation by eliminating damaged or unwanted cells without immune response. Identically, autophagy is also a regulated cell death, which called program cell death II. Different level of cell self-consumption would induce different effect, including cell-protective autophagy and cell-death autophagy (Boya et al., 2013). Finally, necrosis is the most undesirable cell death because necrosis would make cell bursting and initiate immune response. However, without proper regulation, cell death would create serious consequence.

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Tea is a traditional drinking which consumes all over the world. Previous studies revealed that tea has many bioactivities, such as anti-cancer, anti-diabetes or anti-oxidation (Chacko et al., 2010). According to epidemiological studies, tea consumption would lower the risk of having Parkinson's disease (Tanaka et al., 2011). Tea polyphenol is the major bioactive compound in green tea water extraction and catechins occupy the major proportion of tea polyphenol (Sang et al., 2005). Epigallocatechin-3-Gallate (EGCG) is not only the highest ratio of catechins but also the most effective catechins. Many research found that EGCG have many effects, including anti-oxidation and anti-inflammation. As the increasing of processing degree, EGCG would be decreased and polymerized catechins would increase. Among these polymerized catechins, theasinensin A (TSA) is the most abundance polymerized catechins in Oolong tea (Stodt and Engelhardt, 2013). Yet, TSA's bioactivity remains unclear. Hence, the purpose of this study is to investigate the protective effects of tea polyphenol, EGCG and TSA, on MG-induced cytotoxicity in SH-SY5Y cell.

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2 Materials and methods

2.1 Chemicals and antibodies

TSA was using a well-establish synthesis protocol (Shii et al., 2011) and using HPLC to confirm the purity of TSA was above 95%. EGCG was provided by Rutgers University. All reagents were using for lab experiment. The methylglyoxal (MG), acridine orange (AO), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3′-dihexyloxacarbocyanine iodide (DiOC6), and 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Sigma Chemical Co. Antibodies against phospho-PI3K (Tyr508), and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PARP antibody, Anti-LC3 antibody, Anti-ERK antibody, Anti-p- ERK antibody, Anti-JNK antibody, Anti-p-JNK antibody, Anti-Akt antibody, Anti-p-Akt antibody, Anti-p53 antibody, Anti-p-p53 antibody were purchased from Cell signaling (Cell Signaling Technology, Beverly, MA).

2.2 Cell culture

SH-SY5Y cell was obtained from American Type Culture Collection Rockville, MD) and cell was grown in DMEM-F12 medium added with 10% heat-inactivated fetal bovine serum (FBS) bovine serum (Gibco BRL, Grand Island, NY), penicillin

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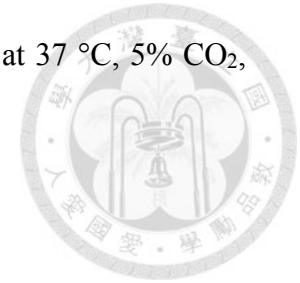
(100U/mL), and streptomycin (100 μg/mL). Cells were incubated at 37 °C, 5% CO_2, and cells were subcultivation when cells were at 80% confluence.



2.3 MTT assay

The cells were seeded at a density of 5×10^5 cells/mL into 24-well plates. EGCG and TSA were dissolved in dimethyl sulfoxide (DMSO) and treated different concentrations for an hour, and then cell was treated with MG for 3 hours. After incubation, cell was centrifuged (2000 rpm, 10 min) and removed the supernatant. MTT solution (0.02%) was added 1 mL per-well and then incubated at 37°C for 2 hours. When the purple crystal was formed, 200 μl DMSO was added to each well. The absorbance was measured at 570 nm by ELISA reader.

2.4 Western blot assay

Cells were treated with tea polyphenol for an hour and then MG treated for different time period. After incubation, cells were scraped, centrifugation and then added lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1% NP-40, and 10 mg/mL leupeptin) vortexing every 5 minutes for 12 of times on the ice. The protein extract was harvested by collecting supernatant after centrifugation. The level of protein was determined by Bio-Rad protein assay. Equal

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amount of protein would be aliquot into small eppendorf and then the protein samples would mix with 5× sample buffer and boil at 100 ˚C for 10 minutes. When the protein sample was cooling, loading protein sample into SDS-polyacrylamide gel and separated proteins. After electrophoresis, the gel would put on polyvinylidene difluoride (PVDF) membrane and put into transfer buffer (25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol) to transfer overnight. When the transfer was completed, the PVDF membrane was took out and soak into blocking solution (20 mM Tris-HCl pH 7.4, 0.2% Tween 20, 1% BSA, and 0.1% sodium azide) for an hour and immersed in primary antibodies (1:1000) overnight at 4 °C. Subsequently, PVDF membrane was washed by PBST (0.02% Tween 20 dissolved in PBS) for 3 times and was probed with secondary antibodies (1:5000) for an hour. Before PVDF membrane was dripped with enhanced chemiluminescence (ECL) (Amersham), PVDF membrane was washed by PBST for 3 times. Then, protein expression was detected by Bio Top Image Processor and the western blotting data were quantified by Image J software.

2.5 ROS production determination

SH-SY5Y cells were seeded with the concentration of 5×10^5 cells/mL into 24-well plates and grown 12 hours. Cells were treated with different concentrations of EGCG and TSA for an hour, and then added MG to incubation for 30 minutes. Cells

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were then harvested, washed with PBS, resuspended in 200 μL of PBS, and added trypsin to remove cell plate into eppendorf. Cells were centrifuged (2000 rpm, 10 min) and removed the supernatant. DCFH-DA (20 μM) was added to the PBS for 30 min at 37 °C dark. ROS production was monitored by flow cytometry. Quantitation of the ROS production was performed by Flow Jo software.

2.6 GSH production determination

SH-SY5Y cells were seeded at a density of 5×10^5 cells/mL into 24-well plates. Cells were treated with different concentrations of TSA and EGCG for an hour, and then added MG to incubation 30 minutes. Then, Cells were harvested, washed with PBS, resuspended in 200 μL of PBS, and added trypsin to remove cell plate into eppendorf. Cells were centrifuged (2000 rpm, 10 min) and removed the supernatant. CMFDA (40 μM) was added to the PBS for 15 minutes at 37 °C in the dark. GSH production was monitored by flow cytometry and the data was analyzed by Flow Jo software.

2.7 AO staining

Coverslips was immersed into 99% alcohol and fire it to sterilized. Sterilized-coverslips putted in 6 cm dish and then cells were seeded at a density of 5×10^5 cells/mL.

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After incubation for 12 hours, cells were treated with different concentration of EGCG and TSA for an hours and then treated with MG for 3 hours. Then, the supernatant was discarded and cells were washed by 1 mL PBS. AO (1 μg/mL) was added and incubated for 15 minutes at 37 °C in the dark. After incubation, the coverslips were gently picked up and observed under confocal microscope (Excitation, 488nm; emitted, 500-540 nm (DNA) and 566-606 nm (AVO)).

2.8 Acidic vesicular organelle production determination

SH-SY5Y cells were seeded at a density of 5×10^5 cells/mL into 24-well plates and grown 12 hours. Cells were treated with different concentrations of TSA and EGCG for an hours, and then added MG to incubation for 3 hours. Cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and added trypsin to remove cell plate into eppendorf. Cells were centrifuged (2000 rpm, 10 min) and removed the supernatant. AO (25 g/ml) was added to the PBS for 15 min at dark. Acidic vesicular organelle production was monitored by flow cytometry and the data was analyzed by Flow Jo software.

2.9 Detection of mitochondria membrane potential

Cells were seeded at a density of 5×10^5 cells/mL into 24-well plates and grown

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12 hours. Cells were treated with different concentrations of TSA and EGCG for an hour, and then added MG to incubation 3 hours. Cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and added trypsin to remove cell plate into eppendorf. Cells were centrifuged (2000 rpm, 10 min) and removed the supernatant. DiOC6 (40 nM) was added to the PBS for 15 min at dark. Detection of mitochondria membrane potential was monitored by flow cytometry. Quantitation of the detection of mitochondria membrane potential was performed with Flow Jo software.

2.10 Statistical analysis

All data was arranged by Microsoft Excel and used Sigmaplot 12.0 for illustrating and statistical analysis. Using one-way ANOVA with Dunnett's multiple range test as post hoc test to analyze data. p-value <0.05 had statistically significant differences.

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3 Results

3.1 Protective effect of tea polyphenol on MG-induced cell toxicity

Previous research found that MG would induce apoptosis pathway; therefore, we use MTT assay to determine cell viability after MG treatment. First of all, using different concentration of MG treated cell for 3 hours to determine concentration of half cell death. As shown in **Figure 1B**, MG treatment would lead to cell death and have dose-dependent effect. The half toxic concentration of MG was 1.5 mM which would use as inducer concentration in subsequent experiments. Then, to confirm whether tea polyphenol was toxic to neuron cells, the cell would treat tea polyphenol for 24 hours. The MTT assay showed that 2.5, 5, 10, 25, 50, 100 μM of tea polyphenol treated for 24 hours would not have cell toxicity (**Figure 2**). To observe whether tea poly phenol have neuroprotective effect, cell was pre-treated with different concentration of EGCG and TSA for an hour then treated MG for 3 hours. As illustrated in **Figure 3**, both EGCG and TSA had the ability to protect cell from MG-induced cell toxicity. The best protective concentration of tea polyphenol was 25 and 50 μM, so the subsequent experiment would focus on 25 and 50 μM of EGCG and TSA for further molecular mechanism investigation. Moreover, the cell morphology was different between every group (**Figure 3**). MG-treated group had many floated cells, EGCG-treated group

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have more adherent cell than MG-treated group, and TSA-treated group had many adherent but bubbly cell.

3.2 Protective effect of tea polyphenol on MG-induced oxidative stress

The **Figure 4** showed that after MG (1.5 mM) treated for 15, 30, 60, 180 minutes, the intracellular ROS production reach maximum level in 30 minutes. We determine intracellular ROS production after treating 25 and 50 μM of EGCG and TSA for an hour then treating 1.5 mM of MG for 30 minutes using DCFHDA dye. As shown in **Figure 5**, both EGCG and TSA could reduce MG-induced intracellular ROS production and TSA had better scavenging ability than EGCG group. Excepting ROS, anti-oxidation system is equally important and GSH is a very crucial anti-oxidative enzyme. The result of intracellular GSH level was determined by flow cytometry. As shown in **Figure 6**, MG treatment would decrease intracellular GSH level and tea polyphenol can keep GSH level as control group. Therefore, we assumed that tea polyphenol can reduce MG-induced oxidative stress.

3.3 EGCG inhibited MG-induced apoptosis

To determine whether tea polyphenol had the ability to inhibit the activation of MG-induced apoptosis, we collected the protein of different concentration of EGCG and TSA pre-treated for an hour and MG-treated for 3 hours. According to western

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blotting results (**Figure 7A**), the apoptosis marker: cleavaged- PARP was increased in MG-treated group and EGCG-treated group can inhibit PARP cleavage. However, pre-treatment of TSA did not inhibit PARP cleavage. As illustrated in **Figure 8**, EGCG could inhibit MG-induced the level of cleavaged-caspase 3. Thus, these results show that EGCG may attenuate MG-induced apoptosis.

3.4 TSA induced autophagy to protect MG-induced cell toxicity

Because above figures show that TSA have not significant different to inhibit apoptosis, we assumed that TSA may induce other pathway to avoid MG-induced cell toxicity. The AO experiment demonstrated that both 25 and 50 μM of TSA-treated group would increase acidic vesicular organelle by confocal microscope and flow cytometry (**Figure 8A, B**). To double confirm TSA would switch MG-induced apoptosis to autophagy, we detected the protein level of LC3II, autophagy marker, via western blotting (**Figure 8C**). These data all confirmed that TSA pretreatment can protect MG-induced apoptosis through activation of autophagy pathway.

3.5 The molecular mechanism of tea polyphenol against MG-induced cell toxicity

In order to further determine the mechanism of tea polyphenol on protective effect of MG-induced cell toxicity, we used western blotting to discover changes of signaling

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pathway. When p53 pathway is activated, cell would start apoptosis pathway. According to the western blotting assay, EGCG-treated group would inhibit p53 activation (**Figure 9**). Another signaling pathway, Akt signaling pathway, has the effect of inhibit autophagy. As shown in **Figure 10**, TSA-treated group can inhibit the activation of Akt pathway. According to **Figure 11**, MG would activate JNK and ERK pathway and both EGCG and TSA treated cells would inhibit JNK and ERK pathway activation. JNK and ERK pathway is a sort of MAPK pathway, which play a multiple role in cell death. Thence, the relationship of MAPK pathway, apoptosis, and autophagy will need more research. Another pathway involve in TSA-induced autophagy is the decrease of mitochondria membrane potential and activation of caspase-9. The data of mitochondria membrane potential change is using DiOC6 dye to detect mitochondria membrane potential by flow cytometry (**Figure 12A**). Both TSA-treated group and MG-treated group can significantly decrease mitochondria membrane potential and the protein level of Bcl-2 decreased corresponding (**Figure 12B**). As shown in **Figure 12B**, TSA-treatment would increase the cleavage of caspase-9. These results would explain TSA-induced autophagy pathway through decrease mitochondria membrane potential and activation of caspasae-9.

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4 Discussion

MG is a by-product of glycolysis and the toxicity of MG has become more and more concern all over the world. Some research indicated that high MG concentration may higher the risk to have Parkinson's disease (Sun et al., 2012); therefore, researchers assumed that MG might damage neuron and cause neurodegenerative diseases consequentially. The toxicity of MG is highly related to oxidative stress and cell apoptosis. From now on, many natural compounds from edible plants were found to have anti-oxidation or anti-apoptosis effects, such as tea polyphenol (Singh et al., 2011, Weerawatanakorn et al., 2015). Previous studies indicated that tea polyphenol, EGCG and TSA, may have the potential of neuro-protection.

In this study, we investigated the effect of tea polyphenol, EGCG and TSA on MG-induced neurotoxicity and their molecular mechanism using SH-SY5Y neuroblastoma cell line. The results showed that tea polyphenol had ability to protect neuron against MG-induced toxicity by MTT assay, AO staining, flow cytometry analysis and western blotting.

Different stimulation would lead to different cell morphology, so we could observe cell morphology to determine the type of cell death roughly (Ricci and Zong, 2006). The research of MG toxicity found that the mechanism of MG-induced cell death

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majorly through ROS production, cytochrome c release, caspase-3 activation, and apoptosis cascade activation (Heimfarth et al., 2013, Kimura et al., 2009). In present study, the data from cell morphology observation and MTT assay indicated that MG-treated cell would have chromatin condensation, apoptosis body formation, and cell death (**Figure 1B, C**). These MG-induced cell damage may be prevented by tea polyphenol pretreatment (**Figure 3**).

Besides, the ROS production reached the maxim level after treating MG for 30 minutes and this result was consistent with previous studies (**Figure 4**). The tea polyphenol treatment group can reduce ROS production (**Figure 5**). These results were consistent with previous studies, which found that both EGCG and TSA had anti-oxidation effects (Weerawatanakorn et al., 2015). Moreover, the level of GSH found that pretreat of tea polyphenol could maintain GSH level (**Figure 6**). We compared the ability of anti-oxidation between EGCG and TSA and TSA had better effect of anti-oxidation. This consequence may surmise that TSA had more OH-group to eliminate ROS.

The result of MTT assay and AO staining confirm that not only EGCG but also TSA had the protective effect from MG-induced cell toxicity. The finding that EGCG inhibited MG-induced apoptosis (**Figure 7**) was consistent with that of EGCG inhibited AGEs-induced cell damage (Lee and Lee, 2007) but the ability to confront MG-induced

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292 cell death was first discovery. Present study found that TSA pretreatment would induce
293 autophagy via AO staining, LC3II level assay (**Figure 8**). This finding is a novel
294 discovery for the protective effect of TSA involved in cell death.

295 Tea polyphenol inhibited MG-induced apoptosis by inhibiting p53 signaling
296 pathway, which is pro-apoptosis pathway (Fridman and Lowe, 2003). In this study,
297 pretreating tea polyphenol could nihibit p53 pathway (**Figure 9**). On the other hand,
298 TSA inhibited Akt pathway but MG could slightly activate Akt pathway to promote cell
299 survival (**Figure 10**). Based on previous reasearch, activation of Akt pathway would
300 activate mTOR; however, mTOR would inhibit autophagy (Wang et al., 2012). So, we
301 assumed that TSA might ihhibit Akt pathway to activate autophagy.

302 Some reasearch indicated that MG would promote JNK, ERK, and p38 pathway
303 (Guo et al., 2016) because MAPK signaling pathway would activate by ROS and then
304 activate down-stream pro-apoptosis factors (Zhou et al., 2015). The same effect was
305 found in present study. Intrestingly, both EGCG and TSA can down regulate JNK and
306 ERK pathway (**Figure 11**). Previous paper revealed that the up-regulation of MAPK
307 pathway may relate to the regulation of maturation stage of autophagy (Corcelle et al.,
308 2007). Whether MAPK signaling pathway is the key regulator between apoptosis and
309 autophagy needs more experiment to confirm.

310 MG would inhibit Bcl-2 level to decrease mitochondria membrane potential (Guo

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311 et al., 2016). In this research, the level of Bcl-2 and mitochondria membrane potential
312 decreased in both TSA-treated and MG-treated cell (**Figure 12**). Besides, we also found
313 that TSA-treated group would increase caspase-9 cleavage (**Figure 12B**). The
314 phenomenon was also found in a few research (Jeong et al., 2011). Unfortunately, the
315 relation of caspase-9 and autophagy remain unclear.

316 In summary, tea poly phenol can inhibit MG-induced cell death and EGCG, and
317 TSA used different pathway to protect cell. EGCG protect cell from MG-induced
318 toxicity via inhibiting apoptosis pathway; however, TSA switch MG-induced apoptosis
319 to autophagy (**Figure 13**). The relationship between every pathways is very complex,
320 so further investigation of interaction between every pathway is needed. In addition,
321 this study was demonstrated by cell line; therefore, animal study is needed to confirm
322 these effects of tea polyphenol.

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Figure Legends

Figure 1 The structure of MG and effects of MG on cytotoxicity.

MG structure (A) and the cell viability of cells was determined by MTT assay. Cell were treated with 0.25, 0.5, 0.8, 1, 1.2, 1.5, 1.8, 2 mM of MG for 3 hours (B). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with control group by one-way ANOVA and Dunnett's multiple range test.

Figure 2 Effects of EGCG and TSA on neurotoxicity.

The cell viability of cells was determined by MTT assay. Cell were treated with 2.5, 5, 10, 25, 50 μM of EGCG (A) and 2.5, 5, 10, 25, 50, 100 μM of TSA (B) for 24 hours. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with control group by one-way ANOVA and Dunnett's multiple range test.

Figure 3 Effects of EGCG and TSA on MG-induced neurotoxicity.

The cell viability of cells was determined by MTT assay. Cell were pre-treated with different concentration of EGCG (A) and TSA (B) for an hour then treated MG for 3 hours. The cell morphology after treatment was observed via inverted microscope (C). Red arrow means shrink cells and yellow arrow means bubble type cell (magnification: 400x). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with MG treatment by one-way ANOVA and Dunnett's multiple range test.

Figure 4 MG induced intracellular production of ROS in SH-SY5Y cell.

Cell were treated with 1.5 mM of MG for 0, 5, 15, 30, 60, 180 min followed by

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incubated DCFH-DA and analyzed by flow cytometry (A) (B). * p<0.05 **p<0.01 ***p<0.001 compared with control group by student's t- test.

Figure 5 EGCG and TSA inhibited MG-induced intracellular production of ROS in SH-SY5Y cell.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated DCFH-DA and analyzed by flow cytometry (A) (B). * p<0.05 **p<0.01 ***p<0.001 compared with treatment with control group by one-way ANOVA and Dunnett's multiple range test.

Figure 6 EGCG and TSA modulated MG-decreased intracellular level of GSH.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated CMFDA and analyzed by flow cytometry (A) (B). * p<0.05 **p<0.01 ***p<0.001 compared with treatment with control group by one-way ANOVA and Dunnett's multiple range test.

Figure 7 Effects of EGCG and TSA on MG-induced apoptosis.

Cleavaged-PARP (A) and caspase-3 (B) protein levels in SH-SY5Ycell was analyzed by western blotting. The cells were treated with 0, 2.5, 5,10, 25, 50 μM of EGCG or TSA for 1 hours and then treated 1.5 mM of MG for 3 hours.

Figure 8 TSA induced autophagy on MG-treated SH-SY5Y cell.

The cell morphology after pre-treated 25, 50 μM of tea polyphenol for an hour and 1.5 mM of MG treated for 3 hours (A). The cells were stained with AO as described in experimental procedures. White solid arrow means condensed chromosome and white dashed arrows means AVO. (Scale bar=10 μm) After incubation with 25, 50 μM of tea

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polyphenol for an hour and then treated 1.5 mM of MG for 3 hours, intracellular AVO value was analyzed by flow cytometry (B). The autophagy marker- LC3 II protein levels was analyzed by western blotting (C).

Figure 9 Effect of EGCG and TSA on MG-induced p53 pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of p53 pathway protein was detected by western blotting.

Figure 10 Effect of EGCG and TSA on MG down-regulated Akt pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of Akt pathway protein was detected by western blotting.

Figure 11 Effect of EGCG and TSA on MG-induced ERK and JNK pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of ERK and JNK pathway protein was detected by western blotting.

Figure 12 Effect of EGCG and TSA on MG-induced MMP alteration.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 30 min followed by incubated DiOC6 and analyzed by flow cytometry (A). For detecting the expression of Bcl-2 and cleavage-caspase-9 by western blotting, cell was treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 3 hours (B). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ compared with control group by one-way ANOVA and Dunnett' s multiple range test.

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Figure 13 The mechanism of EGCG and TSA against MG-induced cell injury.

The graphical abstract of this study (A), mechanism of EGCG (B) and TSA (C) against MG-induced cell injury.

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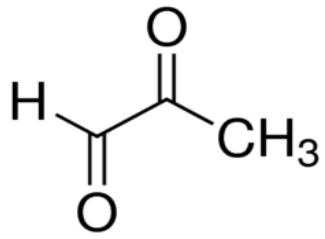
Figure Captions

(A)



(B)

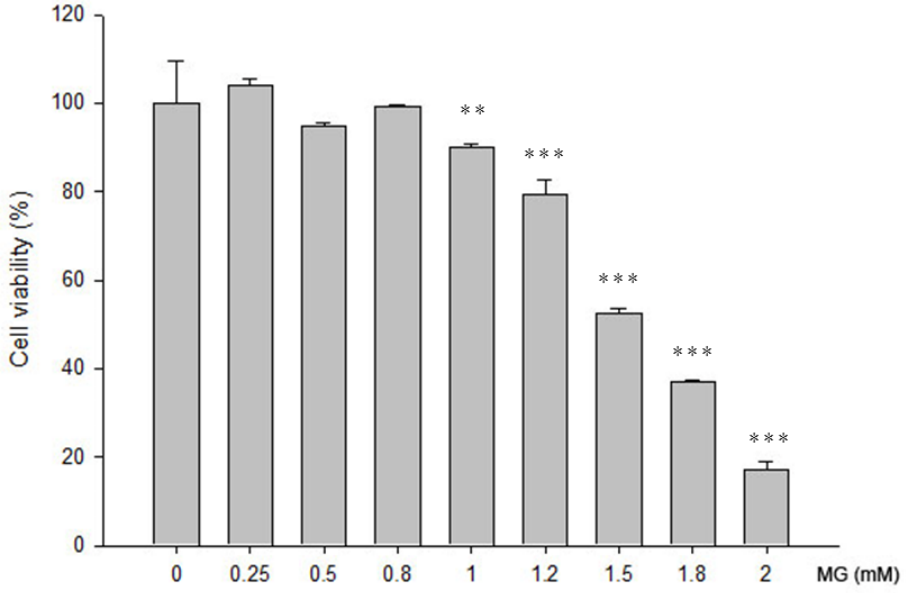
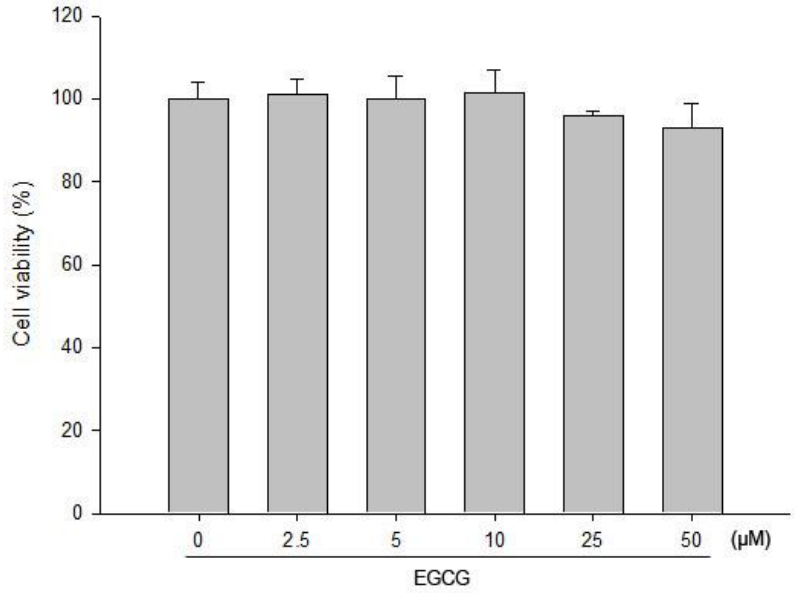


Figure 14 The structure of MG and effects of MG on cytotoxicity.

doi:10.6342/NTU201603033

(A)



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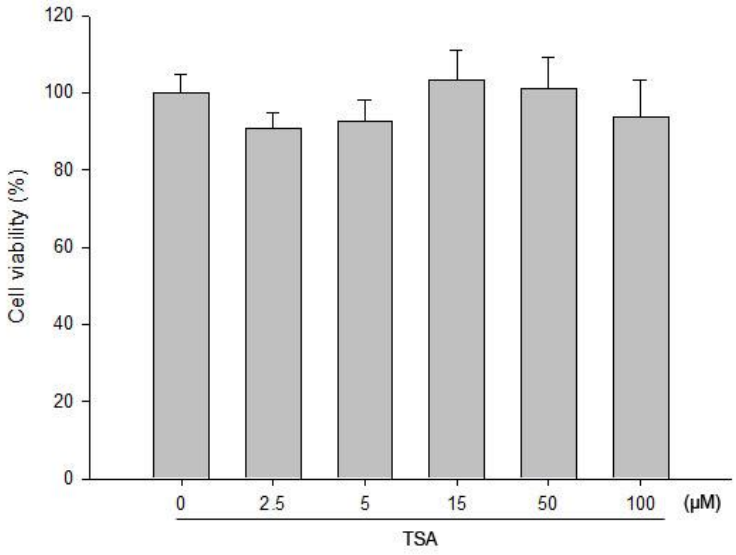

Figure 2 Effects of EGCG and TSA on neurotoxicity.

doi:10.6342/NTU201603033

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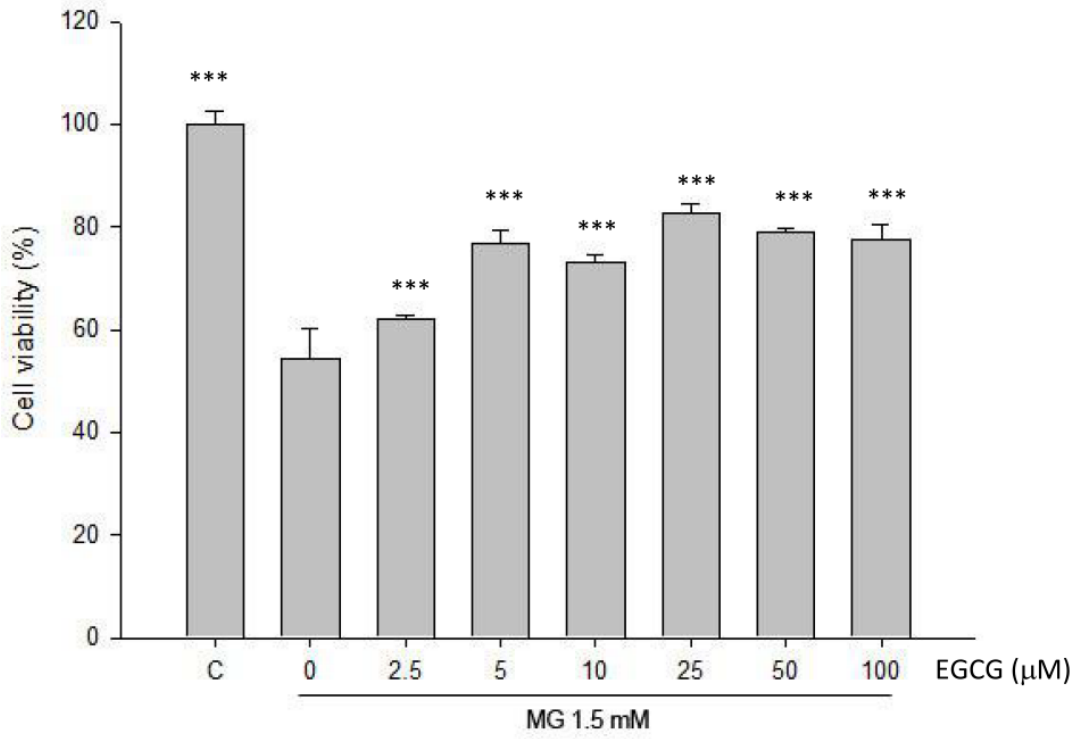

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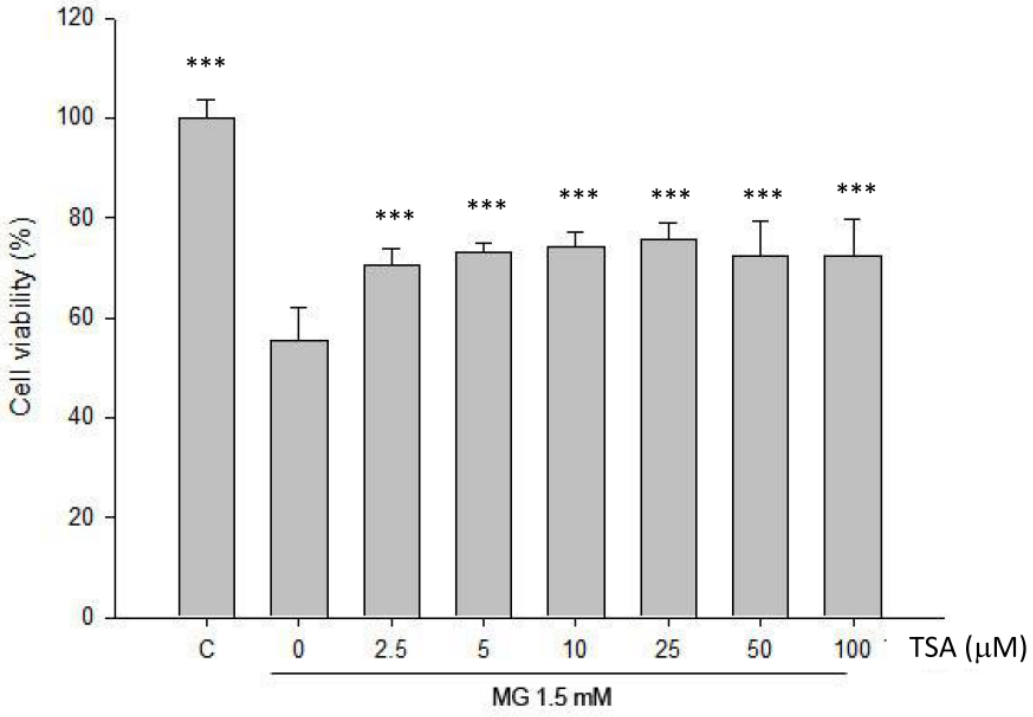


doi:10.6342/NTU201603033

(C)

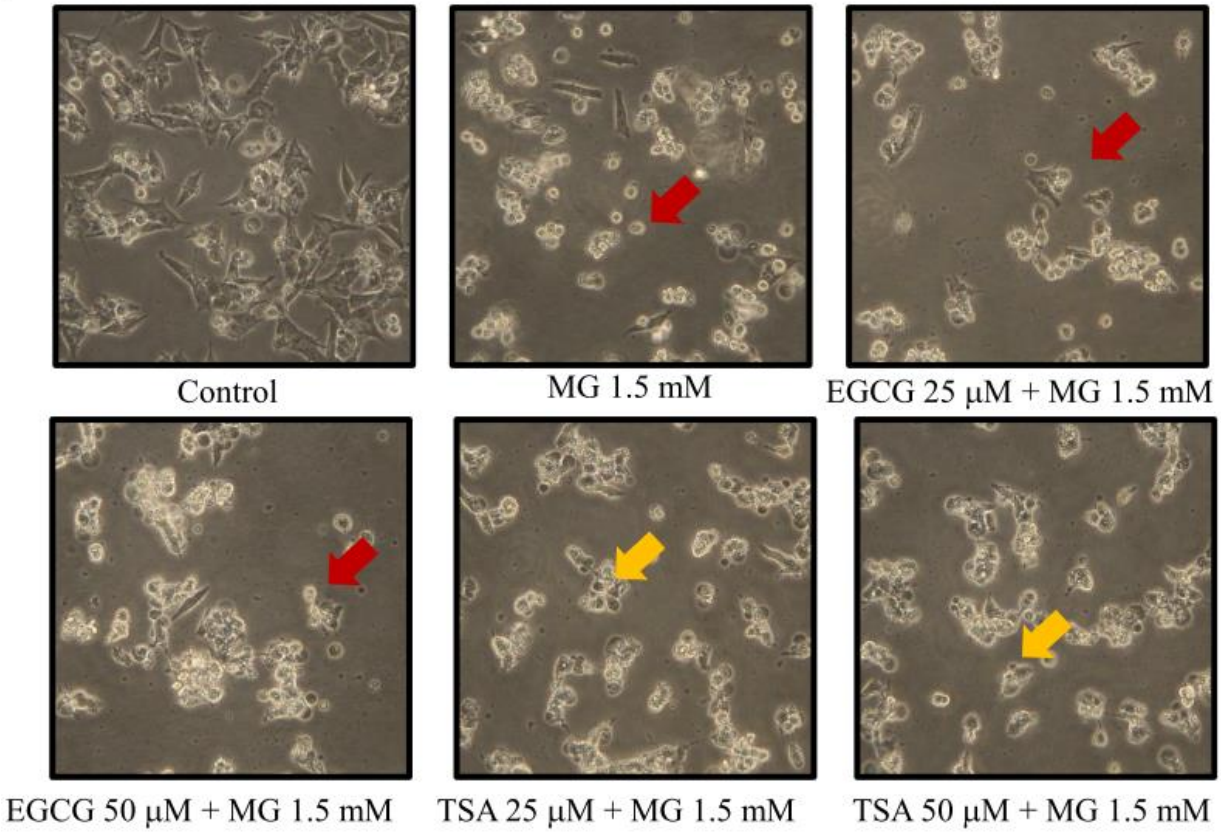
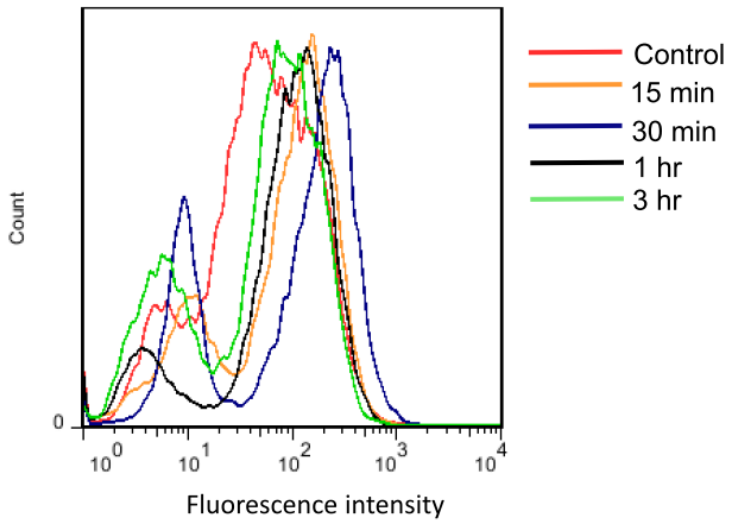


Figure 3 Effects of EGCG and TSA on MG-induced neurotoxicity.

doi:10.6342/NTU201603033

(A)



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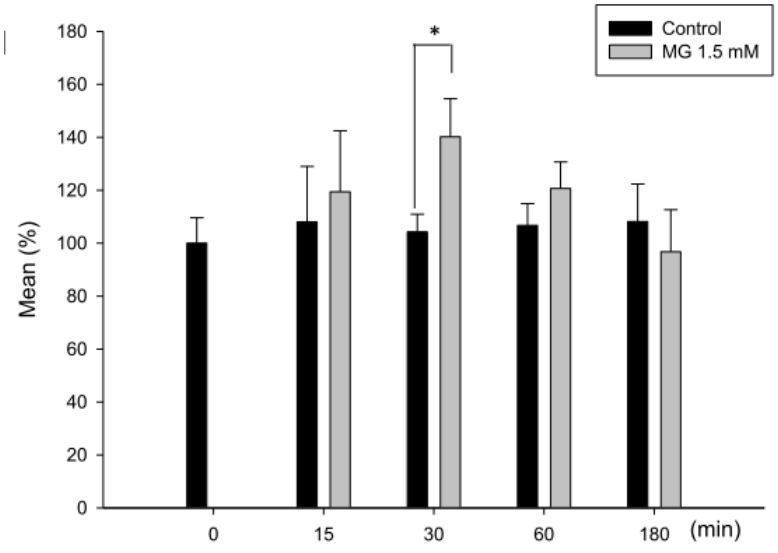
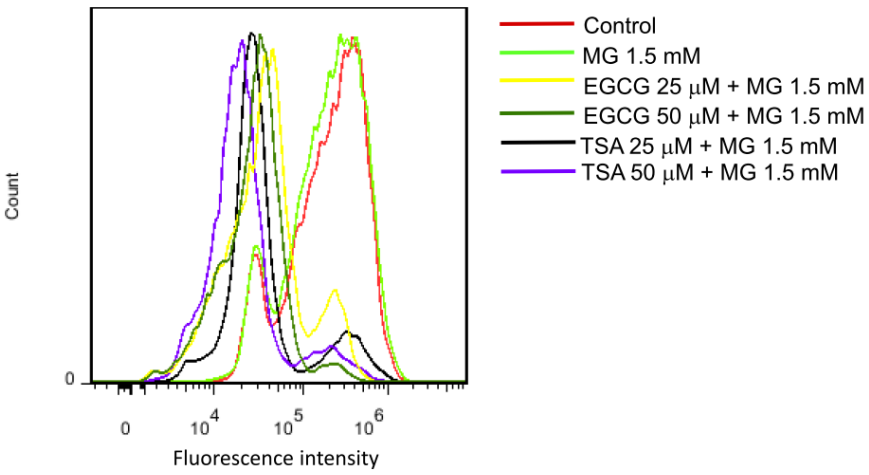


Figure 4 MG induced intracellular production of ROS in SH-SY5Y cell.

doi:10.6342/NTU201603033

(A)



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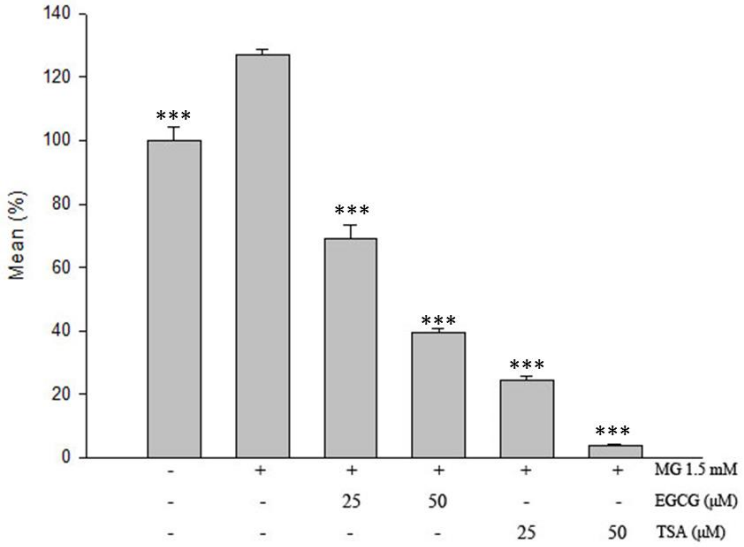
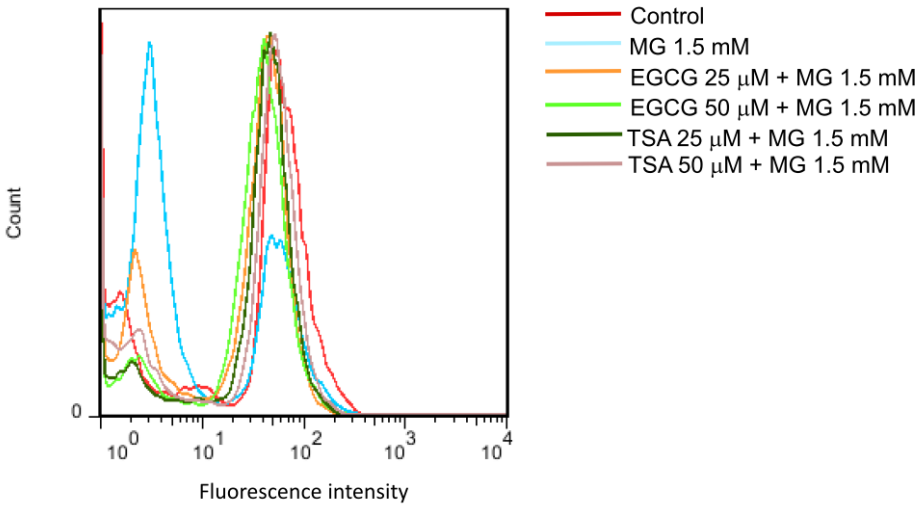


Figure 5 EGCG and TSA inhibited MG-induced intracellular production of ROS in SH-SY5Y cell.

doi:10.6342/NTU201603033

(A)



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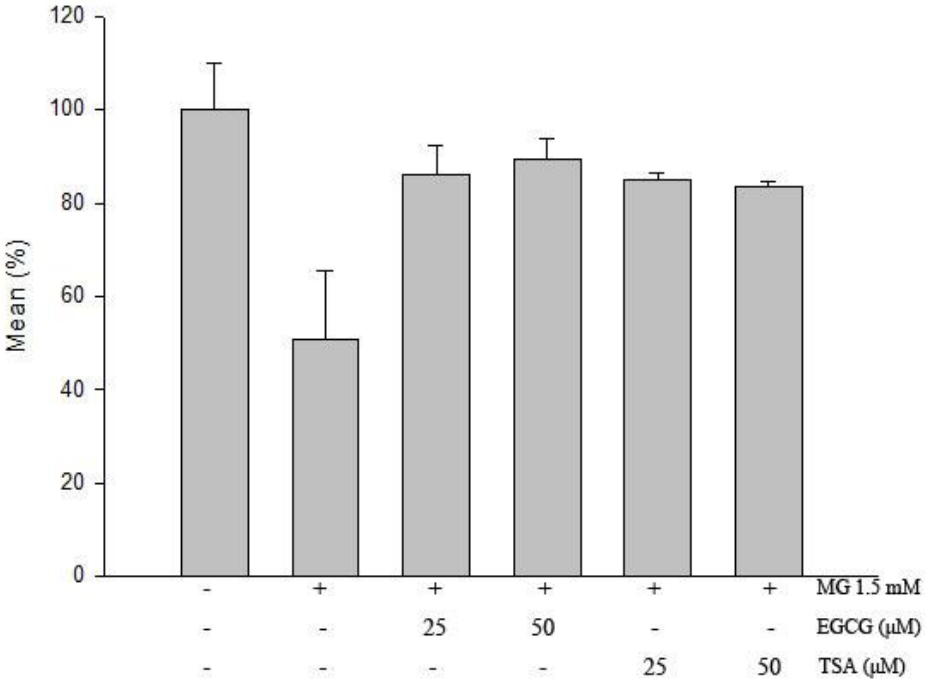
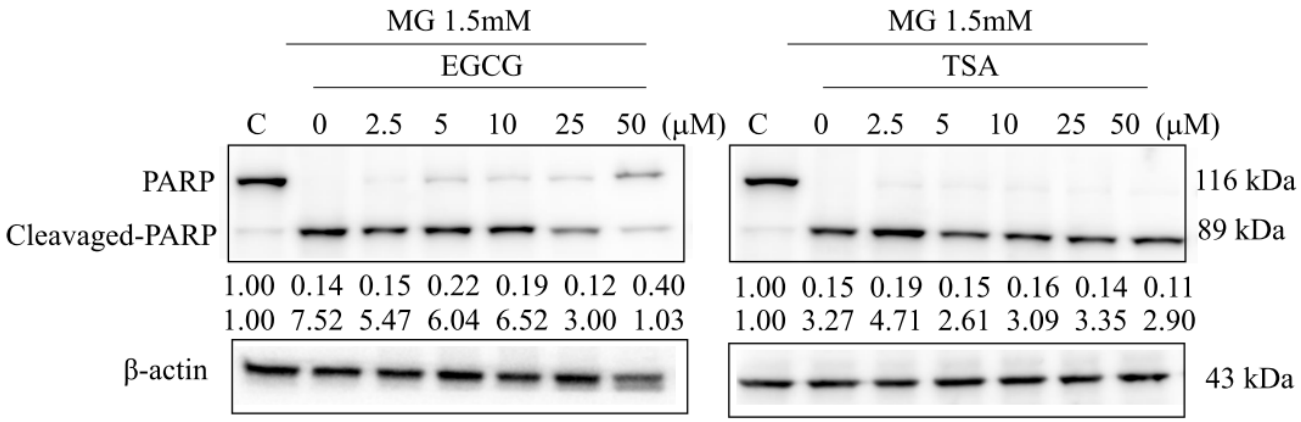


Figure 6 EGCG and TSA modulated MG-decreased intracellular level of GSH.

doi:10.6342/NTU201603033

(A)



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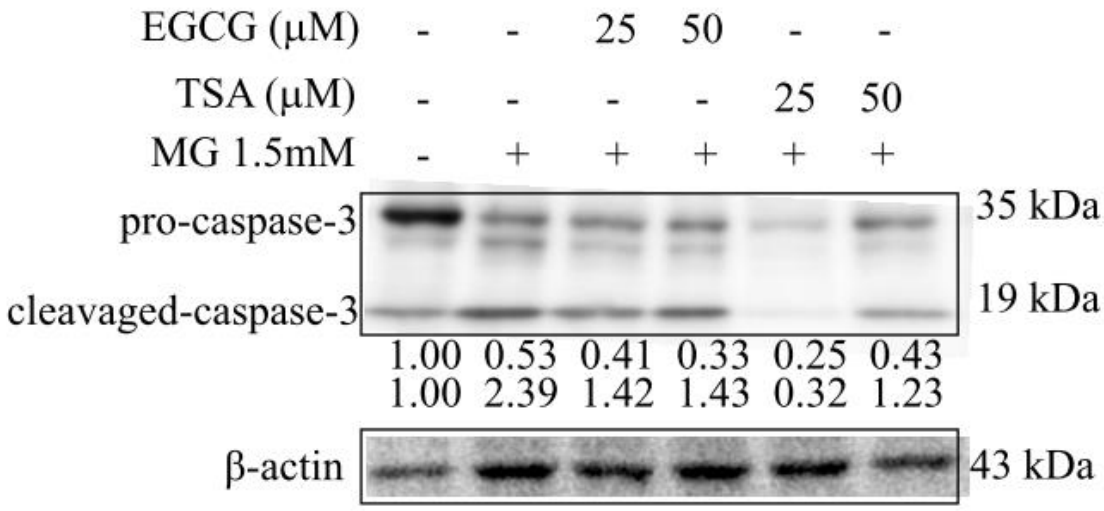
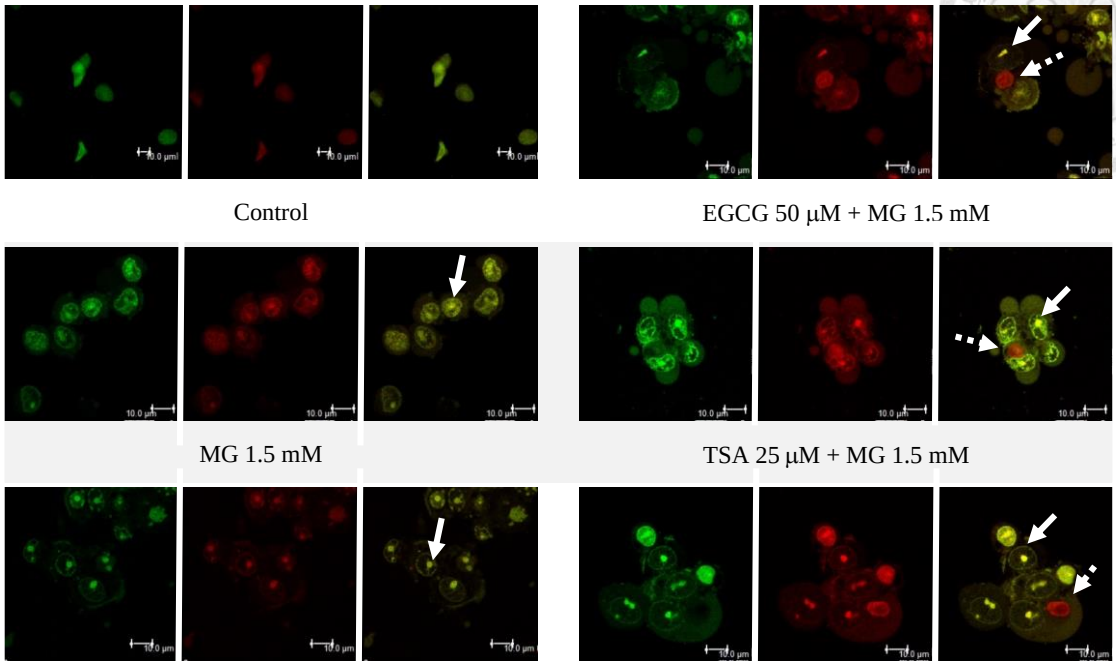


Figure 7 Effects of EGCG and TSA on MG-induced apoptosis.

doi:10.6342/NTU201603033

(A)

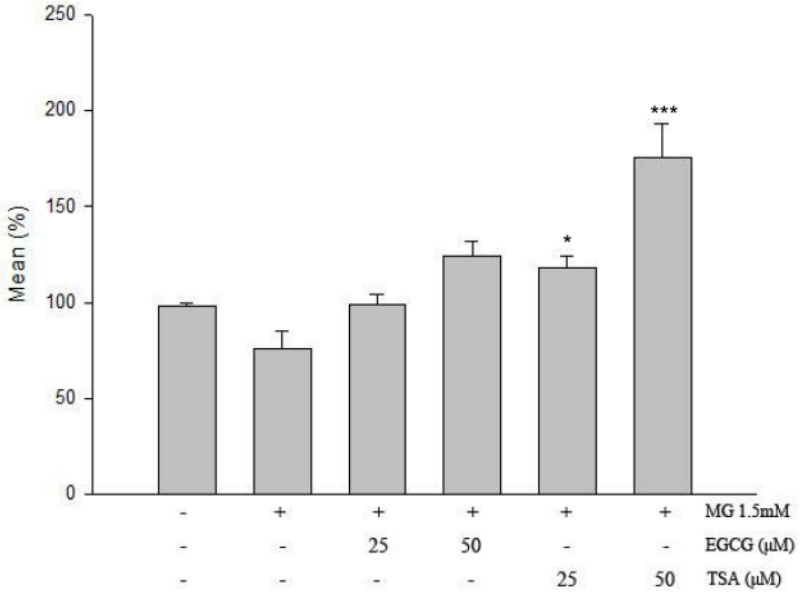


Control | EGCG 50 μM + MG 1.5 mM

MG 1.5 mM | TSA 25 μM + MG 1.5 mM

EGCG 25 μM + MG 1.5 mM | TSA 50 μM + MG 1.5 mM

(B)



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(C)

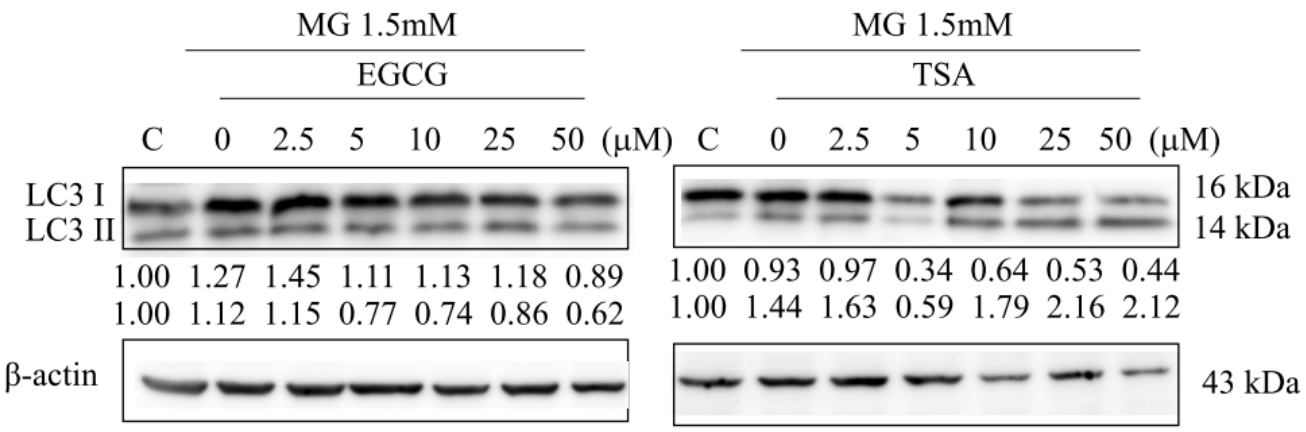


Figure 8 TSA induced autophagy on MG-treated SH-SY5Y cell.

doi:10.6342/NTU201603033

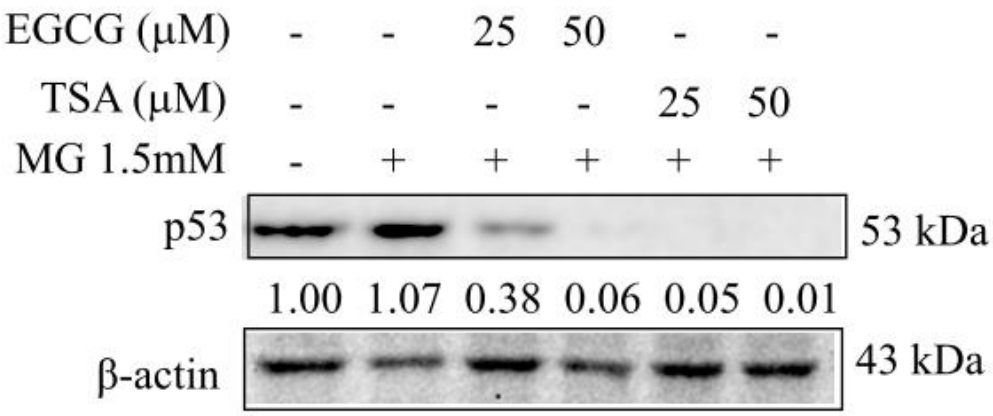


Figure 9 Effect of EGCG and TSA on MG-induced p53 pathway.

Cell were treated with 25, 50 μM of EGCG and TSA for an hour and then treated with 1.5 mM of MG for 45 min. The cells were lysed and the expression of p53 pathway protein was detected by western blotting.

doi:10.6342/NTU201603033

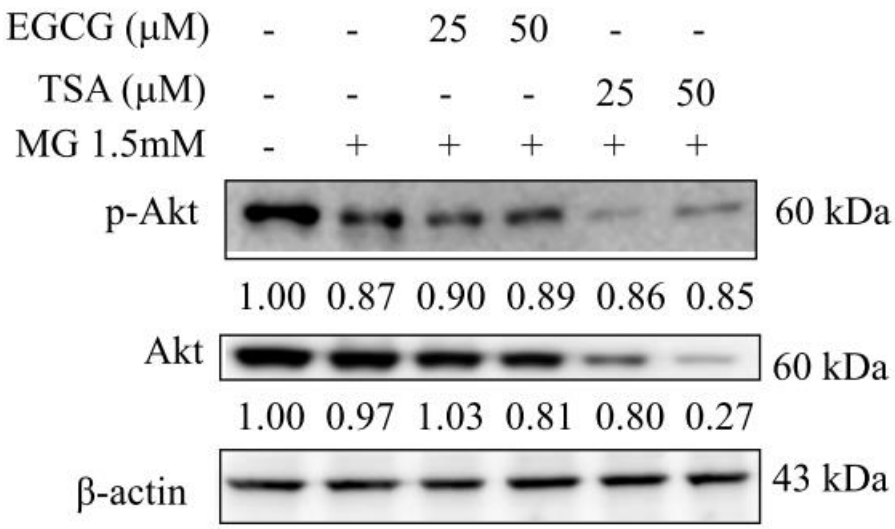


Figure 10 Effect of EGCG and TSA on MG down-regulated Akt pathway.

doi:10.6342/NTU201603033

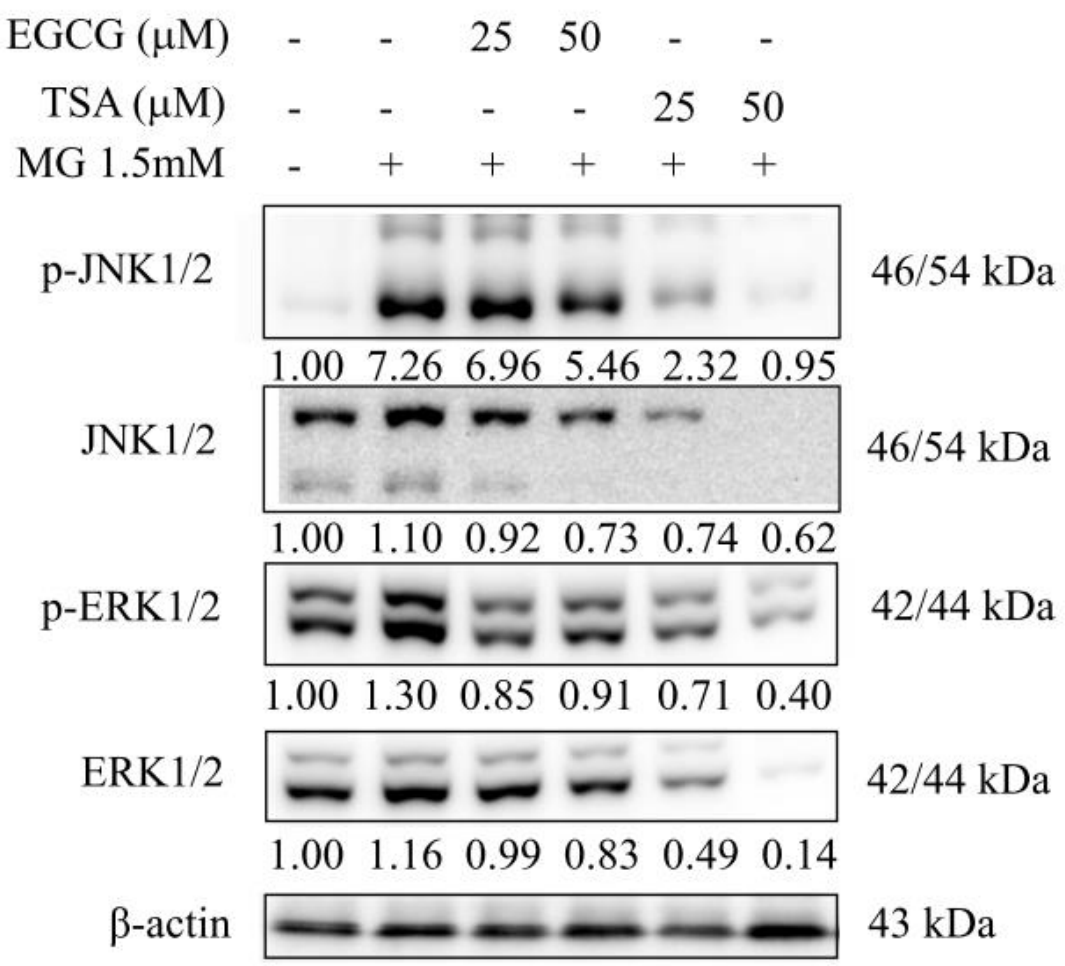
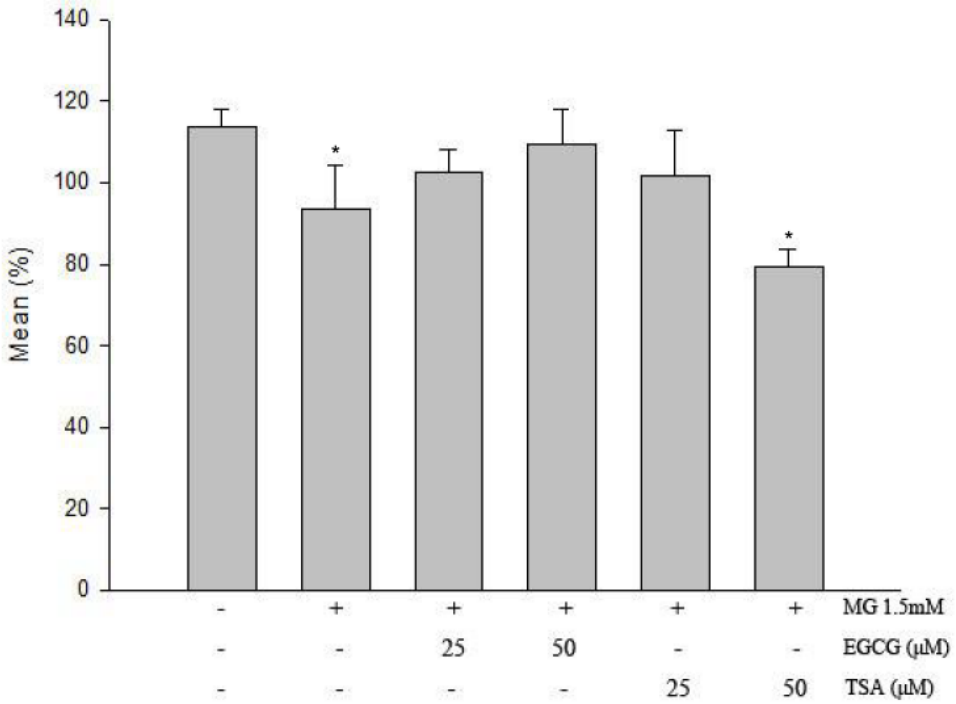


Figure 11 Effect of EGCG and TSA on MG-induced ERK and JNK pathway.

doi:10.6342/NTU201603033

(A)



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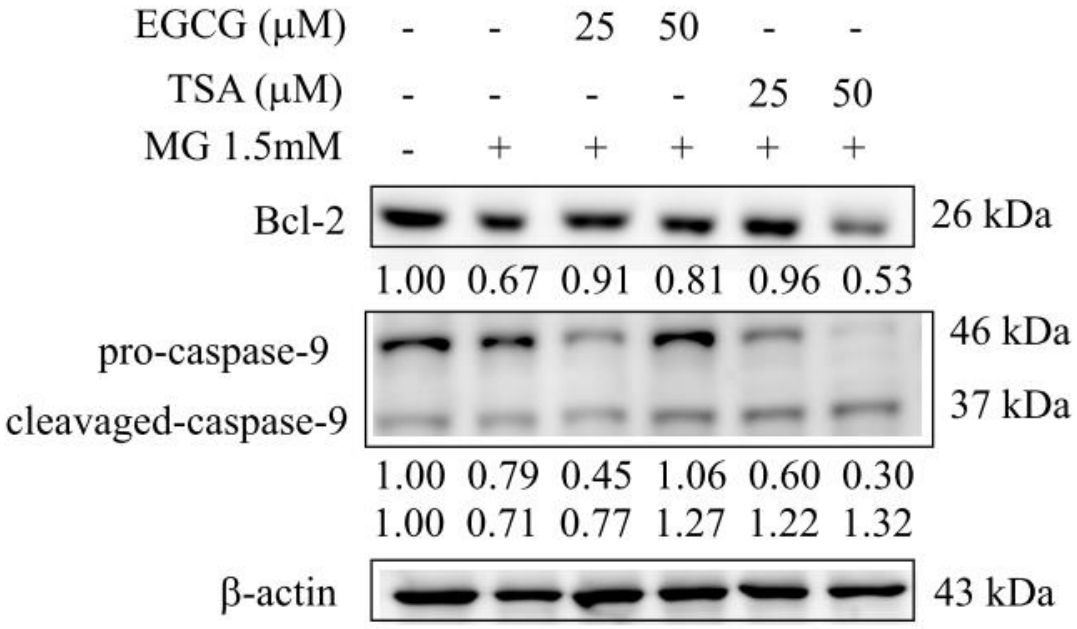
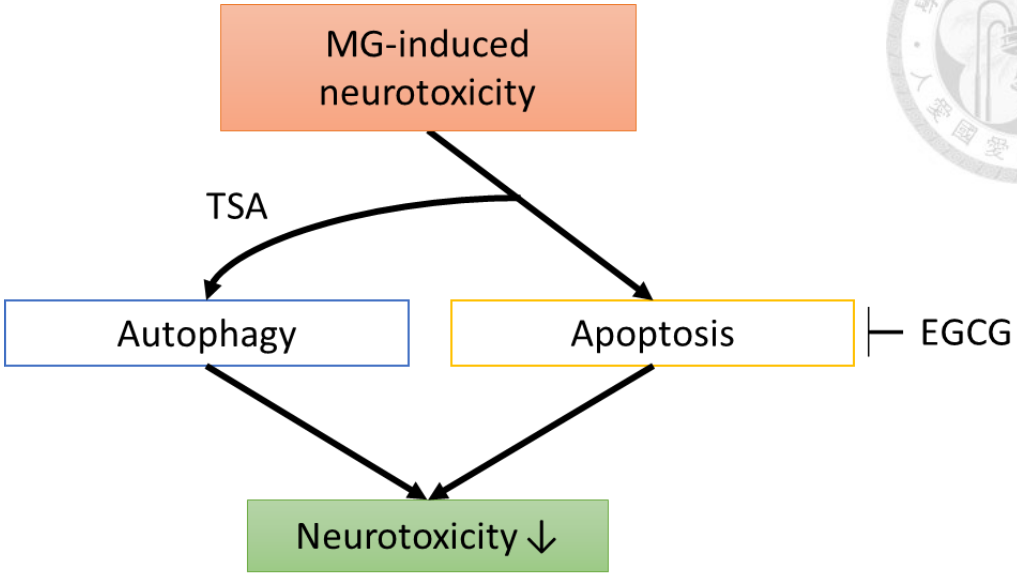


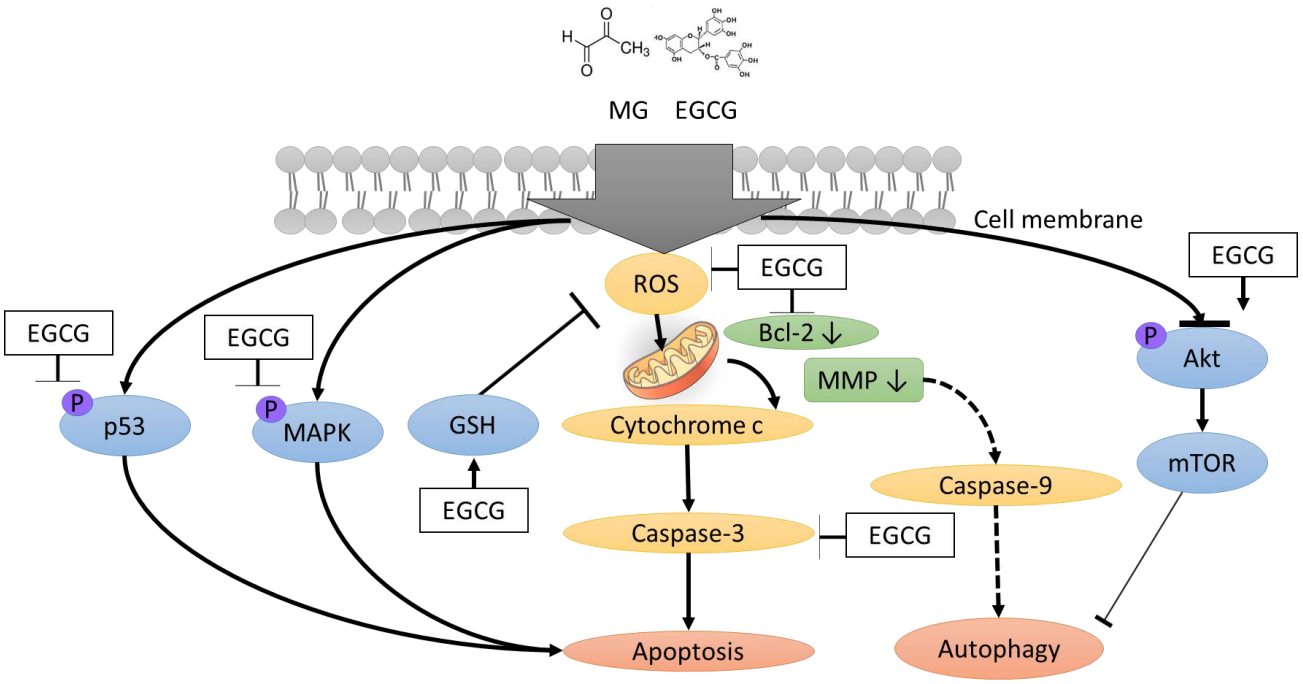
Figure 12 Effect of EGCG and TSA on MG-induced MMP alteration.

doi:10.6342/NTU201603033

(A)



(B)



doi:10.6342/NTU201603033

(C)

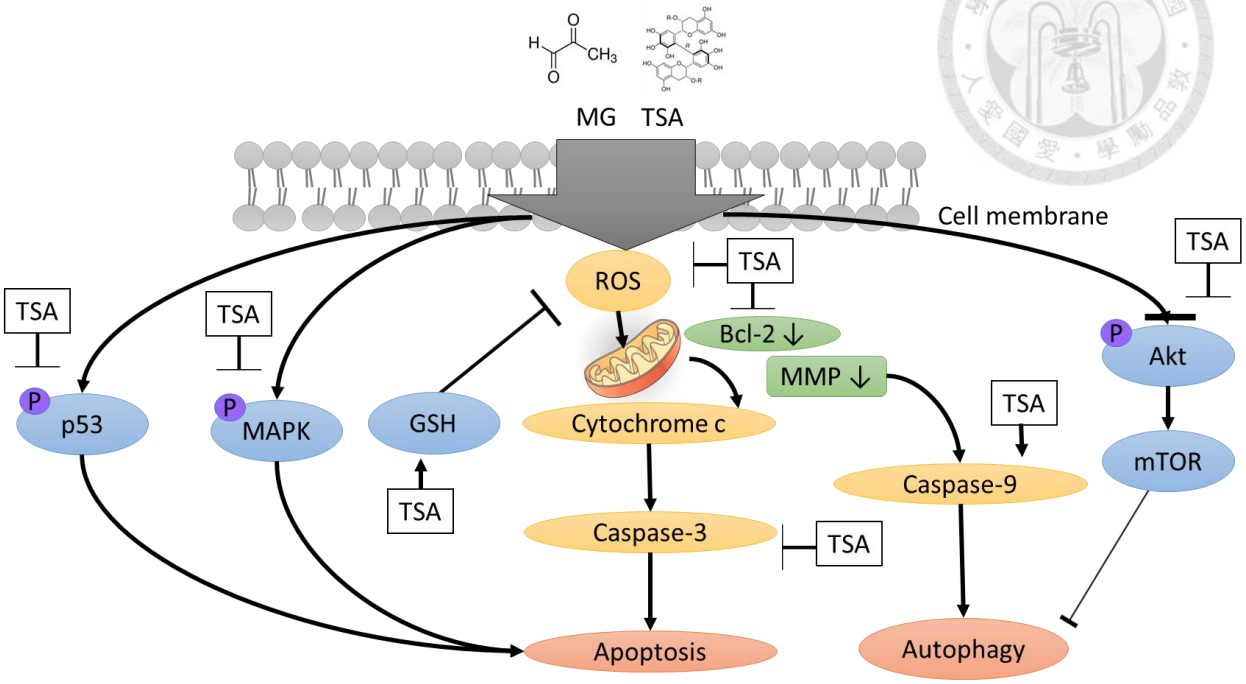


Figure 1315 The mechanism of EGCG and TSA against MG-induced cell injury.

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