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探討冬蟲夏草菌絲體於硫代乙醯胺誘導肝損傷下護肝功效

Hepatoprotection of *Ophiocordyceps sinensis* mycelium against thioacetamide-induced liver fibrosis

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傻勁，想要跟學長姐好好學習，他們不厭其煩的教導讓我至今難忘，甚至是陪我做實驗到深夜，熬夜幫我看我選的論文和報告，我認為這是我之所以日後想要走向教職的初因。這些學長姐大部分已經畢業了，包括：叡豐學長、玲玲學姊、逸帆學長、俊淵學長、孟傑學長、貝蘿學姊、致均學姊、嘉莉學姊、怡伶學姊、宜岑學姊、阿伯學長等。至於同學和學弟妹，則是跟我一起度過碩士班的好夥伴，有些也許已經工作，有一些轉跑道，但是互相幫助鼓勵當中，彼此也成長很多，包括：立盈、柏崴、珮瑄、家昇、林真、怡甄、家慈、晧倫、耀霖、壯嵩、丁毅、智淞、采潔、柏如、思筠、鈺軒等。非常感謝大家的幫助和陪伴，雖然大部分的人已經各奔東西，也不知何時再相見，但是我在這裡要由衷的祝福大家，願　神賜福你們。

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其實，整本論文最令我感到欣慰的部分就是致謝，也許我的研究之路還有相當長的距離要走，也許我博士班會換一個完全不一樣的題目，但是這些感謝的事情，我會一輩子記得，想到大家曾經對我的種種，我就有更多的動力走這個看似漫長的旅程。再次感謝所有幫助和陪伴我的人，謝謝你們。

吳奕賢

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中文摘要

肝纖維化 (liver fibrosis) 源於慢性肝損傷，這會使肝星狀細胞活化並分泌胞外基質 (Extracellular matrix, ECM)。在組織學上，肝纖維化過去被認為是被動而不可逆的過程，但 1970 年代之臨床報告指出該病症具有可逆的潛力，目前則以損傷與癒合模式看待此類病症。

本研究設計參考衛福部公告之護肝功能評估方法來探討人工培養之冬蟲夏草菌絲體 (*Ophiocordyceps sinensis* mycelium, OSM) 對於硫代乙醯胺 (thioacetamide, TAA) 誘導下肝纖維化與慢性肝損傷之保護作用。試驗之結果證實：冬蟲夏草菌絲體粉末在以 TAA 誘導大鼠之模式中可藉由改善肝臟脂質恆定而減少脂肪堆積，並提高其抗氧化能力，減緩因 TAA 造成的肝損傷。實驗結果中指出經 TAA 處理之大鼠補充 OSM，可以降低肝臟大小($p < 0.05$)及血液生化值中的 AST 和 ALT ($p < 0.05$)；TAA 所造成的低血脂現象亦顯著地回復($p < 0.05$)，且肝組織中脂質氧化程度(TBARS)減少，抗氧化能力(TEAC、SOD、CAT、GPx)顯著的上升($p < 0.05$)；在發炎相關細胞激素(TNF-α and IL-1β)與肝臟組織中所含的 collagen 含量則是顯著的降低($p< 0.05$)，並在組織病理切片的結果中發現處理組能夠有效的減緩($p <0.05$)纖維的產生並且減少($p <0.05$)發炎之區域。透過分子生物學之分析，OSM 的護肝機制主要是藉由抑制($p <0.05$)轉化生長因子(TGF-β)以及第四型類鐸受體(TLR4)相關之路徑，進而抑制($p <0.05$)核因子活化 B 細胞 κ 輕鏈增強子 (NFκB)之活化，最後該轉錄因子下游之發炎(COX2)與纖維化(Col1α、αSMA)相關之訊號也有顯著($p <0.05$)的降低。透過免疫染色分析，OSM 可以顯著減少($p <0.05$)肝臟星狀細胞活化之訊號 αSMA。本研究結果證實冬蟲夏草菌絲體之補充可以有效地減緩硫代乙醯胺誘導之肝損傷。

關鍵字：肝纖維化、硫代乙醯胺、冬蟲夏草菌絲體。

ABSTRACT

Chronic liver diseases result in persisting inflammation, progressive fibrogenesis and chronic activation of the wound healing response. This study was to exam the protective activity of compound TCM-808FB which is mycelium of *Ophiocordyceps sinensis* (OSM) via a wistar rat model. Although rats were induced by injecting thioacetamide ($CH_3C(S)NH_2$, TAA), oral OSM administration improved ($p < 0.05$) the hypolipemia and lipid accumulation in livers. Meanwhile, the smaller ($p < 0.05$) liver sizes as well as lower ($p < 0.05$) serum alanine transaminase (AST) and asparatate transaminase (ALT) values showed its potential for relieving from the fibrosis symptom. Moreover, the results of cytokine assays, liver collagen assay and pathological section supported previous hypothesis. Besides, the OSM administration also enhanced ($p < 0.05$) liver antioxidant capacities (GSH, TEAC, SOD, CAT and GPx) and decreased ($p < 0.05$) lipid peroxidation (TBARS), and alleviated liver damage against TAA. As a result, we found an evidence that the protective effect of OSM against hepatic fibrosis may be via downregulations ($p < 0.05$) of TGF-β and TLR4 pathways while OSM also decreased ($p < 0.05$) the expression of NFκB which further decreased ($p < 0.05$) the expressions of fibrotic and inflammatory genes (i.e., αSMA, Col1α, COX2). Therefore, OSM showed preventive effects on the development of TAA-induced hepatic fibrosis.

Key words: liver fibrosis, thioacetamide, *Ophiocordyceps sinensis* mycelium.

I. INTRODUCTION

In 1993, the year of summer Olympic game, *Ophiocordyceps sinensis* (Renamed from *Cordyceps sinensis* in 2007) first gained a worldwide attention when it was revealed that several Chinese laureates included it in diets during their training program. Although it lacked scientific supportings at that time, it prompted the curiosity to research. Nowaday, it is still difficult to apply in therapeutic usages or supplementation due to its rareness and expensiveness. Hence, most of studies have focused on isolating, identifing and cultivating the OSM, while finding the bioactive compound consequently. Many bioactive compounds were found, but the beneficial mechanisms of them are still unclear.

In Taiwan, the liver related disease is prevalent (MOHW, 2013) due to a westernized diet, enviromental pollution, alcoholic habitual drinking, etc. While the functional products of hepatoprotection, called a hepatoprotectant, are popular in the market, the bioactivity of OSM attracts more and more scientific attention. According to previous studies of OSM, although it performs many bioactive functions, such as immunomodulatory, anti-inflammatory, anti-apoptotic, anti-hypertensive, anti-hyperglycaemic, neuroprotective, etc., the mechanism of hepatoprotective effect in TAA-induced liver fibrosis is still unknown. Therefore, this investigation would indicate the modulation of signal cascade in livers of TAA-treated rats under OSM consumption.

II. LITERATURE REVIEW

2.1 *Ophiocordyceps sinensis*: treasure ancient medicine in scientific researches

2.1.1 Synopsis and current marketing situation

Ophiocordyceps sinensis (O. sinensis; OS*)* that is a kind of fungi that parasitizes larvae of ghost moths (*hepialus armoricanus*) or other *Lepidoptera* (Wang & Yao, 2011), it distributes above 3000 m altitude in southwest China, Tibet, north India, and so on (Li *et al*., 2011; Figure II-1). Officially, this species was first described by Mile Berkeley in 1843 as *Sphaeria sinensis*. This species had transferred into different genus twice (Saccardo, 1878; Sung *et al*., 2007) because of analytical technique progressing. The scientific name's etymology was from the Lactin *cord* "club", *ceps* "head", and *sinensis* "from china".

It is thousand years for usage of OS as herbal medicine in china. The first ancient records in Tang dynasty (618-907 AD), called "Yue-wang-yao-zhen" (月王藥診) , mentioned its protective effect of lung-related diseases. Until the Qing dynasty (1644-1912 AD), a scholar, Yi-luo Wu, experted on handling of chinese herbal materials, and in his representative writings, Ben-cao-cong-xin (本草從新), it mentioned the integrated functions of this "mild" material, including, pro-sexual

activity, asthma and chronic lung-related diseases cure, cardiac functions promotion, immune modulation, chronic renal disease prevention, anti-hypertensive, etc. Although there is no liver-related record, modern scientist proclaimed its hepatoprotective effect by many scientific evidences.

In a comparison with OS, there are diverse insect-parasitic fungi *(Ophiocordyceps sinnesis, Cordyceps militaris, Elaphocordyceps ophioglossoides, Cordyceps pruinosa;* Figure II-2) as health food and herbal medicine (Table II-1). The most famous one of them is *C. militaris*, which is similar but cheaper than OS. Although it had been proclaimed that *C. militaris* performs multi pharmacological functions in many scientific studies early, the publics still believe subjectively that legendary unimaginable functions of OS are unrevealed. Hence, it is still worthy to be a potential product for medicinal or nutraceutical studies in commercial viewpoint.

Nowadays, because the economic power of China is raising and the worldwide market demand is increasing (Cooper and Chang, 2001), the price of wild OS may be US$ 20,000 to 40,000 per kg in an international market and even higher (Sharma, 2004). However, over-harvesing in some wild habitats has been made recently (Sharma, 2004). However, OS is still extremely treasured making cultured mycelium a ray of light to those in need.

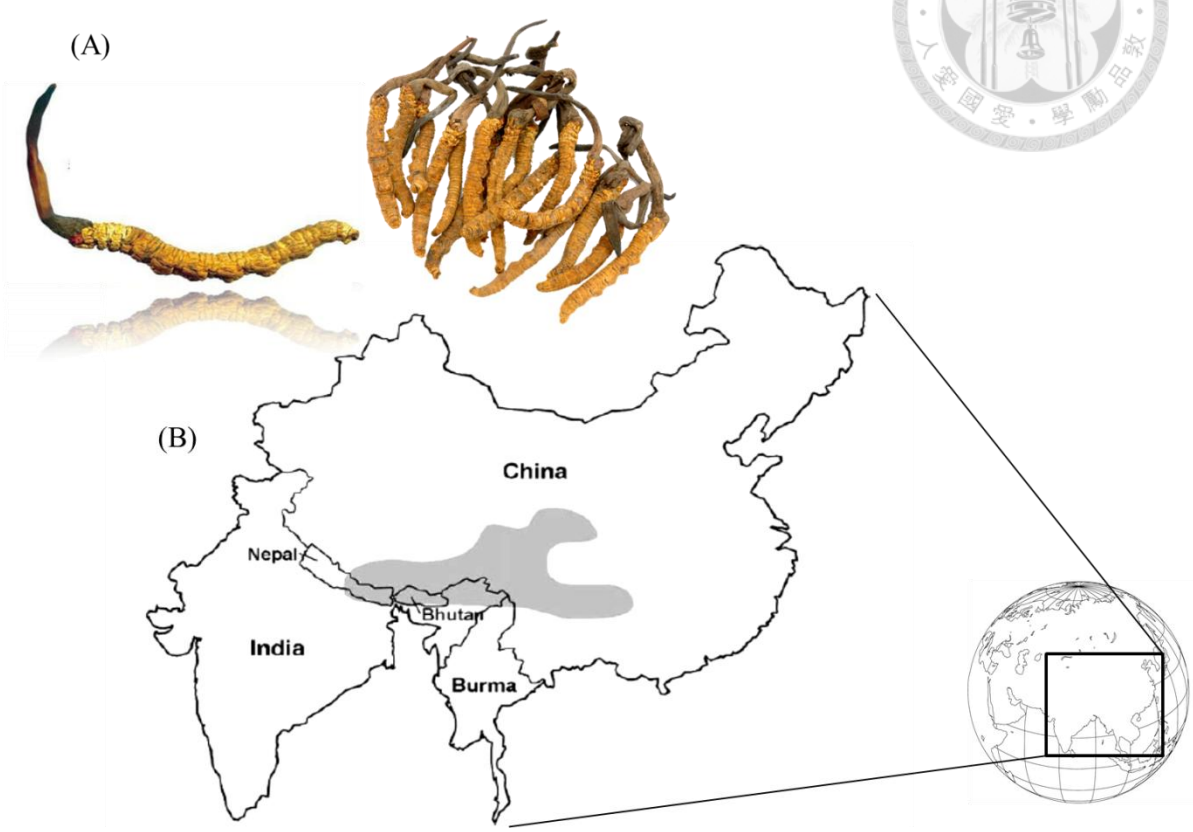


Figure II-1. Habitat range and appearance of *Ophiocordyceps sinensis*. (A) The appearance of OS fruit body that is a yellow-brown stick, and it still remain the image of larva. (B) OS distributes Asia areas where are above 3000 m altitude in southwest China, Tibet, and north India. This figure was modified from Buenz *et al*. (2005). The picture of OS is from online resource: http://www.johnsunmushroom.com/epro_show.asp?ProID=110 and http://www.bibliotecapleyades.net/ciencia/ciencia_industryhealthiermedica53.htm.

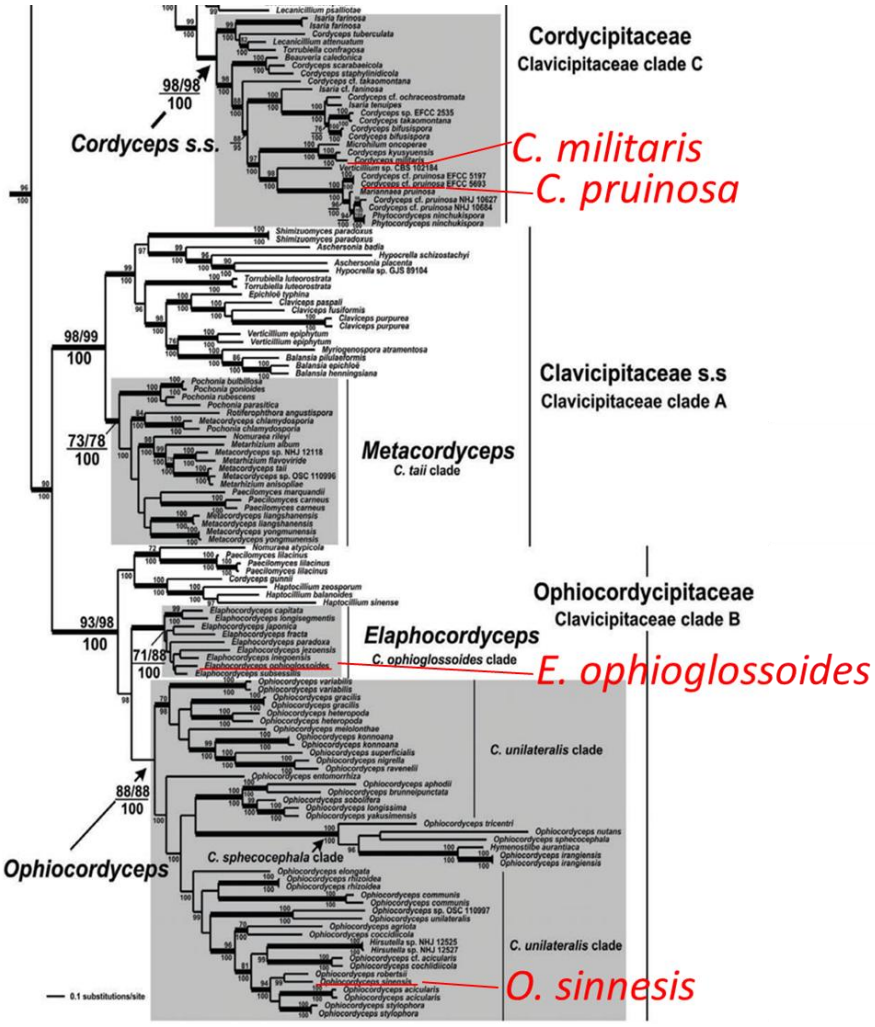


Figure II-2. Classification of *Cordyceps* and *clavicipitaceous* fungi. Bootstrap proportions (ML-BP) are obtained in maximum likelihood analyses and shown above corresponding nodes for ≥ 70 %. Internodes that are supported with both bootstrap proportions (ML-BP ≥ 70 %) and posterior probabilities (PP ≥ 0.95) are considered strongly supported and drawn in a thicker line. Portions of the tree in grey rectangular boxes indicate nomenclatural changes of *Cordyceps* (Sung *et al*., 2007).

Table II-1. Medically or nutritionally related effects of various *Cordyceps* taxa (or preparations)

Purported effect / Taxon	*O. sinnesis*	*C. militaris*	*E. ophioglossoides*	*C. pruinosa*
Immunomodulatory	(Zhang *et al*., 2005)	(Sone *et al*., 1985)		
Anti-inflammatory	(Liu *et al*., 2011)	(Yu *et al*., 2004)		(Kim *et al*., 2003)
Anti-tumor /anti-cancer	(Yang *et al*., 2005)	(Lin & Chiang, 2008)	(Ohmori *et al*., 1986)	
Anti-apoptotic	(Buenz *et al*., 2004)			
Mitogenic		(Jung *et al*., 2007)		
Pro-sexual	(Huang *et al*., 2004)			
Nephroprotective	(Lin *et al*., 1999)	(Wu *et al*., 2000)		
Hepatoprotective	(Gong *et al*., 2000) (Liu & Shen, 2003)			
Anti-Hypertensive	(Chiou *et al*., 2000)			
Anti-hyperglycaemic	(Li *et al*., 2006)	(Young *et al*., 2001)		
Anti-atherosclerotic	(Yamaguchi *et al*., 2000)			
Hematological		(Jung *et al*., 2007)	(Ikeda *et al*., 1993)	
Anti-ageing /antioxidant	(Hui *et al*., 2006)	(Yu *et al*., 2007)	(Jin *et al*., 2004)	
Neuroprotective		(Lee *et al*., 2011)		
Anti-fungal		(Wang and Yao, 2011)	(Kneifel *et al*., 1977)	
Anti-bacterial		(Ahn *et al*., 2000)	(Kneifel *et al*., 1977)	
Anti-fibrotic	(Peng *et al*., 2013)	(Nan *et al*., 2001)		
Anti-HIV		(Mueller *et al*., 1991)		
Anti-invasion	(Kuo *et al*., 2005)			

2.1.2 Collection to the submerged-fermented production

As mentioned, wild OS is over-harvested and expensive; hence, it is not enough to develop the wide-spread daily supplements or nutraceuticals for people in need. Unsurprisingly, there had already been few kind of procedure for cultivated production (Figure II-3). Liquid or submerged fermentation is usually a preferred system for efficient production in mycelial cultures because the condition of cultures could be manipulated and optimized for various fungi. Shashidhar *et al*. (2013) mentioned: "Initial attempts to develop an efficient technology for cultivation of fruiting bodies became futile, but solid state and submerged fermentations remained widely used for the production of OS mycelia biomass and components." Since different micro-environmental factors may influence the production of biomass, the end products of two fermentation systems may be various. In many cases, two cultivated systems are cut both ways, but the comparative analysis in OS mycelia production is still awaited.

In 2006, Lo *et al*. proclaimed that both extracts from wild and cultivated mycelia of OS have direct and potent bioactivities. Furthermore, cultivated mycelia may have stronger activities in results of some scientific reports (Dong and Yao, 2008). According to the aforementioned, no matter what dimensions we focus on, the cultivated mycelia of OS are the most appropriate research subject for further medical or nutraceutical application.

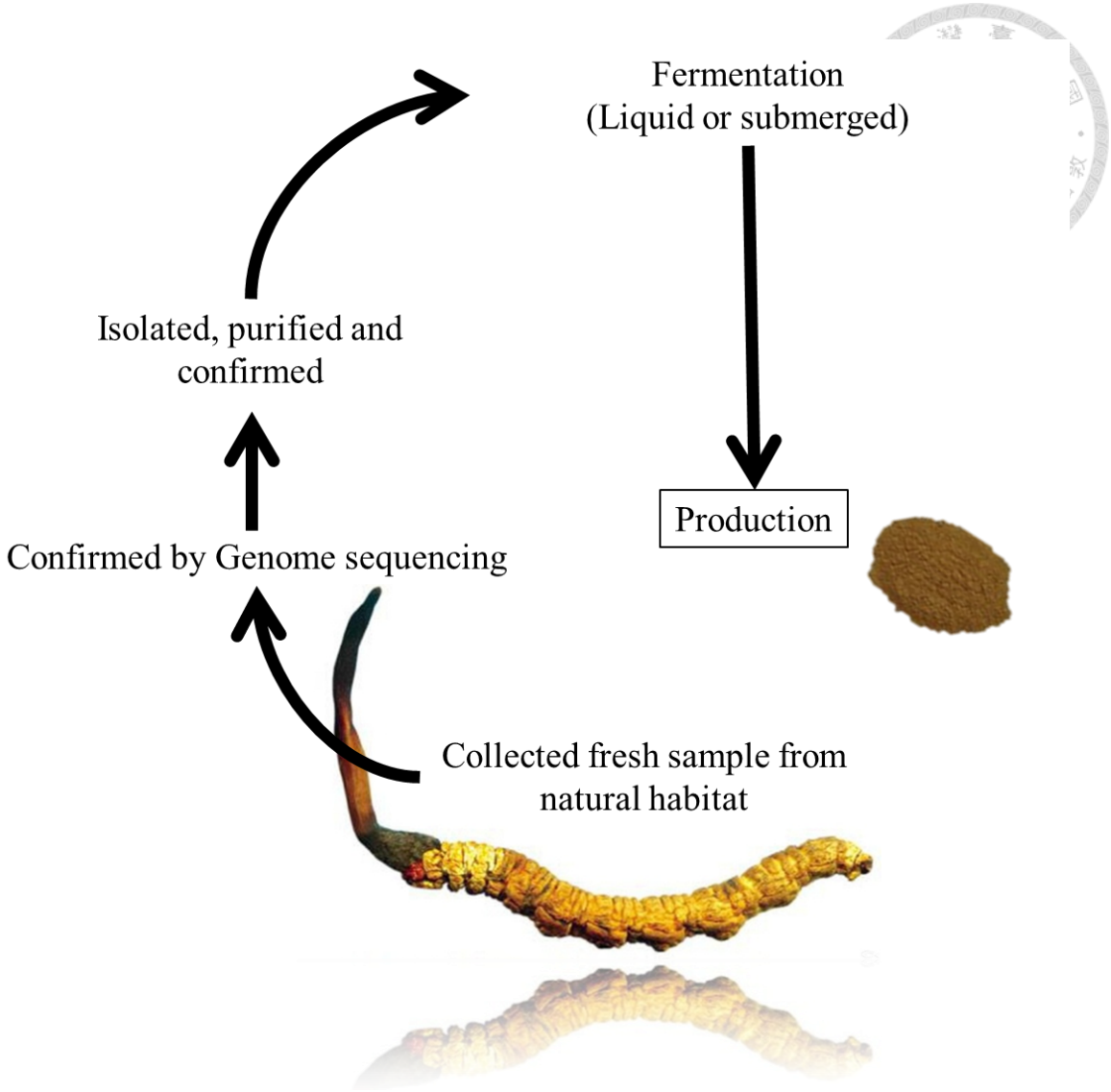


Figure II-3 Flow chart of OSM production. By improvement of biotechnology
The producer found that cultivated method of OS. First, they collected fresh sample from natural habitat, and confirmed by genome sequencing because one wild OS is consisted of variant fungi. Moreover, OS was isolated, purified, and confirmed. After all, they produced mycelium via liquid or submerged fermentation. The last, dried and grounded to become OSM product.

2.1.3 Potential medical and nutraceutical applications

As the ancient-wisdoms, the benefits of OS to human beings had been proven by scientists, and OSM also become a current subject in many studies and researches. After searching, the results are as followings: immunomodulation (Sheng *et al*., 2011), anti-inflammation (Chiou and Lin, 2012), anti-fatigue (Kumar *et al*., 2011), anti-fibrosis (Pan *et al*., 2013), anti-tumor (Kubo *et al*., 2010), antioxidation and cardiovascular (Yan *et al*., 2013), and hepatic protection (Peng *et al*., 2013).

The evidence for exercise-endurance promotion and anti-fatigue activities of OS is well known. Current study demonstrated that it is meditated by upregulation of metabolic regulators, such as AMPK, PGC-1, and PPAR in skeletal muscle (Kumar *et al*., 2011); furthermore, it was found to elevate ATP level in liver tissue (Manabe *et al*., 1996&2000). Recently, Wu *et al*. (2014) proclaimed that cordycepin, which is one of major nucleotides in OS, could active AMPK in HepG2, and decreasing intracellular lipid accumulation significantly. Qiet *et al*. (*In press*) also found Cordymin, which is a kind of peptide from OS, ameliorated the osteopenia and restored the circulating blood glucose and insulin level in diabetic rats. These results provide a strong evidence that OS can affect metabolism.

Sheng *et al*. (2011) found polysaccharides from OSM activated the immunocytes and promoted cytokines expressions. Wang *et al*. (2012) reported that peptide of OSM had a neuroprotective effect in the ischemic brain via inhibiting an inflammation, and it also increased antioxidant activities. Moreover, the anti-inflammatory effect of OSM is not only presenting in brain but also in airway tissues (Chiou and Lin, 2012). Besides, Yan *et al*. (2013) also confirmed

that OSM supplementation would maintain cardiovascular function against an ischemic-reperfusion injury. Furthermore, they demonstrated that OS regulates specific markers of oxidative stress and ameliorates cardiovascular injury.

Last but not least, the previous research showed that the polysaccharides of the OSM have been demonstrated to ameliorate liver fibrosis against CCl_4-induction in rats (Peng *et al*., 2013). Furthermore, the anti-fibrotic effects have been proved to be associated with its down-regulation on hepatic stellate cells (HSCs) activation by modulating protein expression and matrix metalloproteinase (MMP) activities (Peng *et al*., 2013). In addition, its anti-fibrotic effect was also found in renal injury model (Pan *et al*., 2013). Kubo *et al*. (2010) proclaimed that the OSM possesses an anti-metastatic activity by inhibiting the HGF-accelerated tumor invasiveness in mouse hepatic melanoma. To sum up, the fermented OSM are the potential phyto-materials for medicines and nutraceuticals indeed.

2.2 Bioactive ingredients of OS and their function

2.2.1 Nucleoside, bases and their analogues

Nucleotides are one of the major components in *Cordyceps*. There are more than 20 nucleotides and analogs discoverd in wild and cultivated OS nowadays (Supplement 1). Although it could not explain all efficacies of OS, nucleotides are widely recognized as one of the bioactive components. Futhermore, there is a significantly different quantification of nucleotides between various preparations of samples, but slight qualitative change is in HPLC-DAD analysis (Figure II-4). Five nucleotides, i.e. uridine, inosine, guanosine, adenosine and cordycepin are the majors in OS. And these nucleotides can be degraded to others or further through specific enzyme into their bases and/or related compound in wild or cultivated OS. The change pathway of nucleosides in OS was suggested as follows (Zhao *et al*., 2013):

(1). AMP → adenosine → inosine → hypoxanthine (wild OS)

(2). AMP → adenosine → adenine (cultivated OS)

(3). GMP → guanosine → suanine (wild and cultivated OS)

(4). UMP → uridine → uracil (wild and cultivated OS)

Before it goes to details, it is obvious that the investigation of relationship between nucleotide and liver function is still banal except the adenosine, Although most of studies focus on brain disease and neuronprotection, there are still some inspired results for further disscussion in this study.

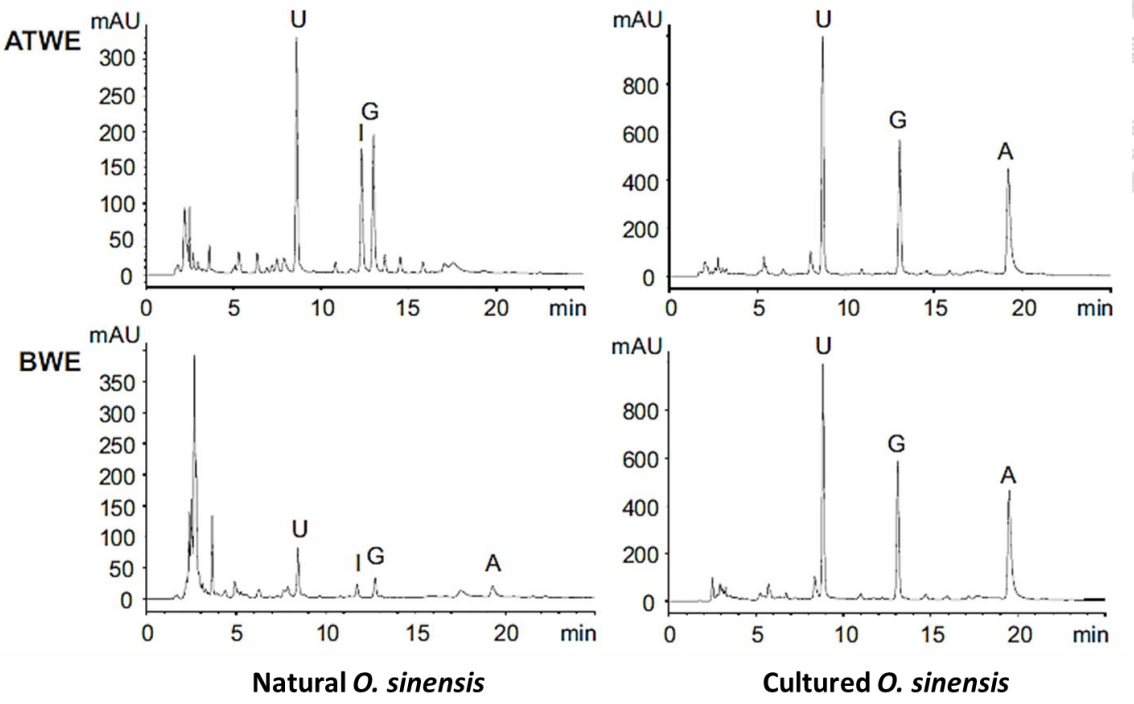


Figure II-4. Typical HPLC chromatogram of OS (left) and OSM (right) extracted by ambient temperature water extraction (ATWE) and boiling water extraction (BWE). U, uridine; I, inosine; G, guanosine; A, adenosine and C, cordycepin. (Yang & Li, 2008)

The uridine, cytidines, and their respective bases contribute to brain phosphatidylcholine and phosphatidylethanolamine sythesis via the Kennedy pathway (Figure II-5). In Kennedy pathway, the biosynthesis of PC, a crucial factor for the function of neuronal membrane, requires a cytidine nucleotide, which is involved in the rate-limiting step (Kennedy and Weiss, 1956). By some indirect evidences, an addition of uridine to cultivated medium *in vitro* or chronic dietary supplement *in vivo* may modulate such membrane-dependant processes as neurotransmitter release (Wang *et al*., 2005) and neutrite outgrowth (Pooler *et al*., 2005; Wang *et al*., 2005). As for uridine, it involves in many metabolic, i.e., glycogen synthesis, lipid and carbohydrate metabolism pathway in brain. Hence, the *in vitro* protection of uridine on astrocytes which under

immunostimulation and glucose deprivation was proved, and the protective effect may be via metabolic modulation (Choi *et al*., 2006). Meanwhile, uridine also has been demonstrated to be anti-inflammatory and protective in lung tissues (Evaldsson *et al*., 2007).

Of all nucleotide content, Guanosine, a neuronprotective element, is the highest in some investigations (Li *et al*., 2011). Oleskovicz *et al*.(2008) reported that guanosine performed neuropeotective effect on an oxygen-glucose deprivation. Also guanosine was demonstrated to present anticonvulsant and amnesic effects in rodent models (Soares *et al*., 2004; Vinadé *et al*., 2004). Moreover, guanosine is protective against mitochondrial oxidative stress *in vitro*, and the disruption is due to the pathway related to PI3K, Akt, GSK-3β and induction of antioxidative enzyme HO-1 (Dal-Cim *et al*., 2012).

Adenosine is a constitutive metabolite of all cells, and it is involved in key pathways such as nucleic acid base synthesis, amino acid and other cellular metabolic status. In addition, it not only exerted a range of generally beneficial effects in the heart and vessels (Olsson & Pearson, 1990; Shryock & Belardinelli, 1997) but also played an important role to the immuno-activity of macrophage along with reactive oxygen. Besides, adenosine was reported to have functions in liver according to Chiang *et al*. (2013). Adenosine-2A-receptor antagonist prevented and reversed fibrosis in ethanol-exacerbated liver fibrosis model. They indicated that a treatment of A2A receptor antogonist alleviated the liver injury index, hepatic stellate cells (HSC) activation, fibrosis, and dyregulated angiogensis.

Unlike the other nucleotides as mentioned above, cordycepin was initially extracted from fungi of genus *Cordyceps*. As the previous investigations, the

ability of cordycepin to reduce total cholesterol and triglycerides content through AMPK activation *in vitro* was obvious, and its *in vivo* preventive effect on hyperlipidemia model was also proved (Guo *et al*., 2010). Moreover, AMPK had been widely reported to play an important role in insulin signaling (Sriwijitkamol *et al*., 2006; Fullerton & Steinberg, 2010).

Among studies of Jeong *et al*. (2010), Ren *et al*. (2012), and Jung *et al*. (2010) cordycepin significantly attenuated the release of inflammatory mediators, i.e., NO, PGE2, TNF-α, IL-1β, phophorylated Akt and MAPKs. Ramesh *et al*. (2012) indicated that the administration of cordycepin protects the liver, kidneys, heart, and lungs of aged rats from oxidative stress by enhancing the activities of enzymatic and non-enzymatic antioxidants. Cha *et al*. (2013) also showed that a hepatoprotective effect in alcohol-induced model was attributed to cordycepin, and it could increase the activities of alcohol-metabolizing enzymes and decrease the liver injury.

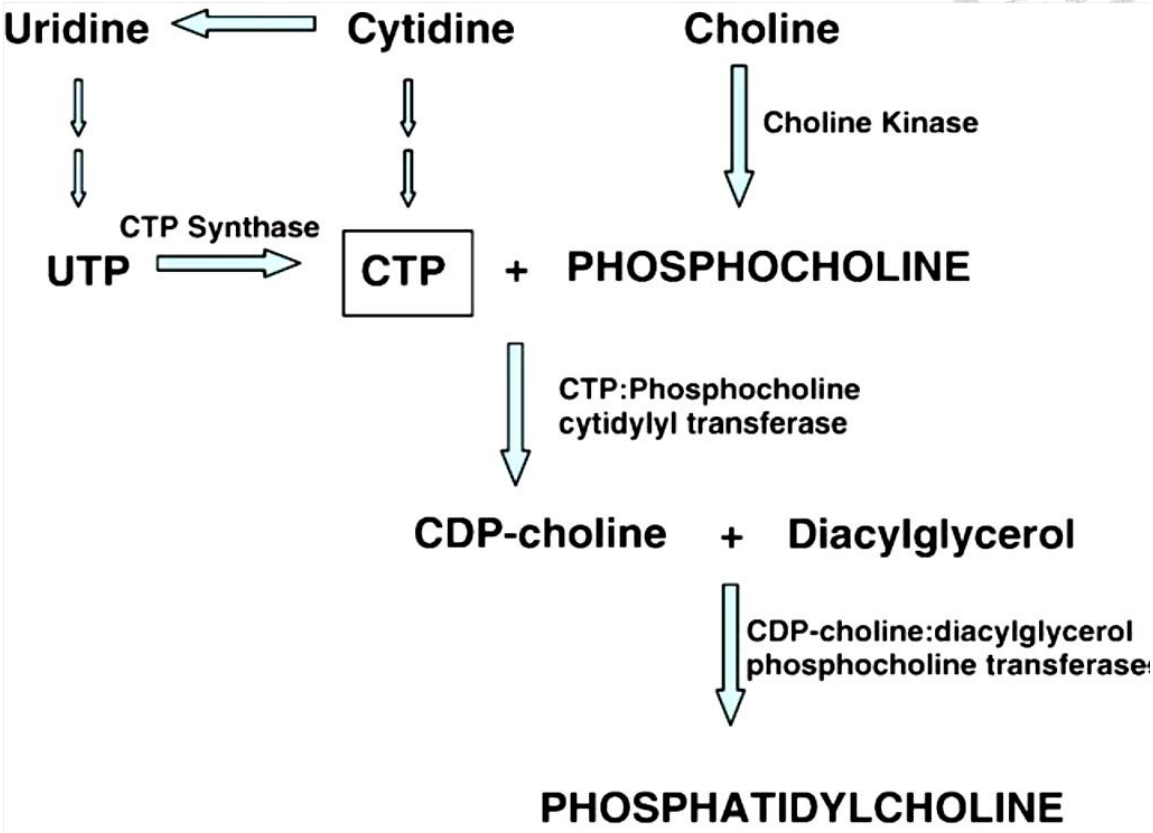


Figure II-5. Phosphatidylcholine biosynthesis via the Kennedy pathway. In rats, plasma cytidine is the major circulating pyrimidine, and however, in gerbils and humans, the primary circulating pyrimidine is uridine. Only small amounts of circulating cytidine are converted to brain CTP, since the rat blood–brain barrier (BBB) lacks a high-affinity transporter for cytidine; uridine, in contrast, readily enters the brain via a high-affinity transporter (CNT2) yielding UTP which is then converted to CTP by CTP synthase. This CTP reacts with phosphocholine to form endogenous CDP-choline, which than combines with diacylglycerol (DAG) to form PC (Cansev, 2006).

2.2.2 Proteins, peptides, and amino acids

To compare with other bioactive ingredients, proteins, peptides and other related analogues were overlooked in the past. Approximate 20 species (Supplement 1), i.e., cordyheptapeptide A/B, cicadeptin I/II, cyclosporin, cordycedipeptide A, cordysinin A-E, etc. were found, but their own bioactivity are still unclear .

Cyclosporin was proved its bioactivity in 1990s. Hozumi *et al*. (1994) proclaimed the elongation of hair-existing phase via lengthening the growing phase of the hair cycle in treated animal. Moreover, Adhirai and Selvam (1997) iondicated it would enhance retention of oxalate in hyperoxaluria, which is mediated through the altered antioxidant defensive system. Now cyclosporin is widely used in organ transplantation by its immunosupressive effect to prevent rejection in clinicals (Nakayasu *et al*., 1990).

Cordymin, which was found in early time, obtains the limelight these years because Qi *et al*. (*In press*) proclaimed that cordymin from OS retarded the diabetic osteopania in the rat model, and also restored the normal circulatting blood biochemical levels. The results suggested that cordymin may indirectly recover the function of pancreatic β-cells.

Besides, OS contains many functional amino acids (Table II-2), for example: glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), arginine (Arg) etc. Saiga *et al*. (2003) demonstrated that the composition with more acidic amino acids (Glu and Asp) or histidine could promote the antioxidative capacity and decrease the levels of lipid peroxidation because of the COO^- groups, which are electrophilic which bind to free radicals (Kang *et al*., 2012). Moreover, Glu supplementation protected hepatic function against fatty liver disease via

enhancing the glucagon in plasma, decreasing the ratio of insulin/glucagon, and increasing the output of triglyceride (Greenfild *et al*., 2009). On the other hand, it is proclaimed that alanine-additional diet results in stimulations of insulin secretion, glucose metabolism, and energy expenditure, hence it may be protective in type II diabetes (Brennan *et al*., 2002). Arg was confirmed to be multi-functional supplementary in immunomodulation (Popvic *et al*., 2007), anti-hypertension (Gokce, 2004) etc. A recent study insisted that it is beneficial by taking the multi-amino-acid formula, which comprises Glu, Ala, His, Leu, and Lys, to supress weight gain and lipid accumulation through enhancing energy expenditure in high-fat-diet treatment (Kobayashi *et al*., 2009).

Table II-2. Amino acid levels in OS and OSM

Amino acid composition (%)	natural OS		cultivated OSM	
	Corpus	Fruiting body	Mycelium	Supernatant of broth
Aspartic acid	1.70a	1.84a	1.05ab	0.31c
Threonine	0.86a	0.83a	0.65a	0.05c
Serine	0.77a	0.78a	0.49b	0.04c
Glutamic acid	2.64a	2.66a	1.12a	0.48b
Proline	1.13a	0.95a	0.72a	-
Glycine	0.82a	0.73ab	0.58ab	0.09c
Alanine	1.02a	0.95a	0.75a	0.05b
Valine	0.98a	0.80a	0.63b	0.06c
Methionine	0.26a	0.18a	0.18a	0.02b
Isoleucine	0.81a	0.53b	0.44ab	0.05c
Leucine	1.30a	0.95a	0.69ab	0.06d
Tyrosine	0.89a	0.67ab	0.31b	0.02c
Phenylalanine	0.96a	0.61b	0.50bc	0.04d
Histidine	1.07a	1.13a	0.28ab	0.02c
Lysine	1.38a	1.15a	0.80a	0.08b
Arginine	1.53a	1.60a	0.04ab	0.05b
Total	18.1a	16.4a	9.23b	1.42d

*Means with different letters in the same row are significantly different ($p < 0.05$).
**modified from Hsu *et al*. (2002)

2.2.3 Carbohydrate

Categories of carbohydrates in OS are mainly mannitol, trehalose, and polysaccharides (Supplement 1). Mannitol is a kind of phytochemicals that is unassimilable and stable in animal, and hence, it increase the osmotic pressure and micturition against edema and some renal disorders. The efficacy of mannitol to decrease intracranial pressures leads to its widespread use in neurosurgery (Wani *et al*., 2008). Xiao *et al*. (2013) found dietary mannitol increased absorption and retention of Ca^{2+} and Mg^{2+}in bone through fermentation of mannitol in large intestine. Regarding the benefits of trehalose, Rodríguez-Navarro *et al*. (2010) demonstrated that it is protective in taupathies and parkinsonism, which performed higher metabolic and oxidative stress activity in cerebral regions. Arai *et al*. (2010) also found that trehalose intake would suppress adipocyte hypertrophy under high-fat-diet induced obesity, and it could mitigate insulin resistance by decreasing insulin secretion and downregulating the mRNA of MCP-1.

Polysaccharides, which were larger amount (3-8%) than other ingredients, were regarded as the most possible bioactive compounds but they structurally diversed (Yue *et al*., 2012). Cheung *et al*. (2009) isolated and identified a kind of polysaccharide, Cordysinocan, from cultured OS. Futhermore,it activated immune respones in cultured T-lymphocytes and macrophages via cytokine induction and downstream signaling cascade. Although many investigations mentioned and emphasized those carbohydrate compounds in discussion, the isolation of each certain bioactive compund and those funtional tests are still awanting.

2.2.4 Fatty acids and sterols

Fatty acids were required nutritions for organisms, and recently some of them were proven to be critical in certain physioloigical functions (Yang *et al*., 2013; Koba and Yanagita, 2013). OS only contains few kinds of fatty acids (Table II-3), i.e., palmitic acid, linoleic acid, and oleic acid, and also sterols like ergosterol and β-sitosterol (Table II-3), showed beneficial effects to human health (Zhu *et al*., 2014; Wong *et al*., 2013)

Conjugated linoleic acid (CLA) is a well-studied representative natural conjugated fatty acid (Koba and Yanagita, *In press*). CLA showed the anticarcinogenic effect (Ip *et al*., 1991; Ip *et al*., 1994), antiobesity (Park *et al*., 1997; Pariza *et al*., 2001), antidiabetic effect (Riserus *et al*., 2002; Moloney *et al*., 2004), and antihypertensive property (Inoue *et al*., 2004). Until now, the one of most possible mechanisms in each beneficial effect was due to a modulation of peroxisome perliferator-activated receptors (PPARs) related pathway, which had been proven to regulate β-oxidation and other lipid metabolic genes (Inoue *et al*., 2006; Wang *et al*., 2006). It was shown that CLA was as an activator of PPARα (Moya-Camarena *et al*., 1999) and PPARγ (Houseknecht *et al*., 1998).

Oleic acid (OA) and human α-lactalbumin (α-LA) form a complex, named HAMLET, which was indicated to have an selective apoptotic activity (Mossberg *et al*., 2010) and be a possible cancer therapy (Ho Cs *et al*., 2012). Recently, it was mentioned that OA is an active compound in HAMLET-like complex to kill tumor cells, and the dynamic system of protein/OA molar ratio is critical in the biological properties of this complex; in other words, the solubility and aggregation state of the fatty acid significantly affect the stability of the complex and consequently its toxicity (Spolaore *et al*., 2010). The effects of

cellular processes that were shared between OA and HAMLET are membrane perturbation (Niazy, 1997; Engelbrecht *et al*., 2011), apoptosis (Yu *et al*., 2008; Maris *et al*., 2011), mitochondrial function (Köhler *et al*., 2001), cell adhesion (Trulsson *et al*., 2011), Glycolysis (Stewart and Balkely, 2000; Wilson *et al*., 2003).

Palmitic acid (PA) is also a kind of unsaturated fatty acids that were demonstrasted to activate PPARs depends on their various sizes and double bonds (Poulsen *et al*., 2012; Schup and Lazar, 2010). Bolsoni-Lopes *et al*. (2013) showed that PA significantly increased PPARα binding to its DNA consensus sequence (PPRE), concomitant with the activation of lipolysis *in vitro* and *in vivo*. However, PA is not as effective as CLA aforementioned. In addition, former investigation showed that PA increased the release of cholecystokinin (CCK) from STC-1 cells (Tanaka *et al*., 2008). Yang *et al*. (2013) indicated that dietary PA supressed appetite via releasing appetite-related hormones in male rats.

Dietary phytosterols were also proven to inhibit the growth and matastasis of human breast cancer cells in mice (Awad *et al*., 2008). Among those phytosterols, β-sitosterol (StS) and Ergosterol (ErS) are rich in OS relatively (Table II-3). Although the molecular mechanism should be further investigated, Russo *et al*. (2010) proclaimed that ErS peroxide and its derivatives attenuated the growth of human prostate cancer cells and partially triggered an apoptotic process. Zhu *et al*. (2014) found ErS peroxide may prevent the renal fibrosis against TGF-β1 induced activation of fibroblasts *in vitro*. For β-sitosterol (StS), Awad *et al*. (1996) indicated that StS inhibits cell growth as compared to cholesterol or non-sterol medium, and they inferred that the signal transduction

pathway may involve membrane phospholipids. Recently, Wong *et al*. (2013) showed that StS enhanced glutathione redox cycling via modulating mitochondrial reactive oxygen species (ROS). Furthermore, Valerio *et al*. (2013) provided strong evidences to show that StS modulated Toll-like receptor 4 (TLR4) expression and intracellular MyD88-dependent pathway activation in macrophages. TLR4 pathway is also known to be highly related to chronic liver diseases (Soares *et al*., 2010), especially hepatic fibrosis (Guo and Friedman, 2010). First and last, phytosterols may be another targeting bioactive compound for further hepatoprotective investigation.

Table II-3. The contents of ten fatty acids and four sterols in OS and OSM

	Smaples	LA	MA	PtA	PoA	PA	LoA	OA	SA	DA	LiA	ChS	ErS	CaS	StS
NOS	Sichuan	17.6	36.7	35.5	220.7	1469.5	2896.5	3978.0	800.7	35.9	52.6	88.2	134.3	+	103.6
	Qinghai	+	21.5	23.8	218.7	1760.0	2854.1	3943.6	609.2	33.1	47.9	69.3	108.0	+	105.1
	Tibeta	14.0	47.1	34.3	613.7	3842.4	6012.3	11517.6	1238.6	38.7	52.9	82.5	120.3	+	95.9
	Tibet2	+	26.8	30.2	245.9	2050.2	3581.0	5869.4	1269.2	38.9	55.4	68.8	89.7	+	78.1
CCOS	Wanfeng	+	14.7	19.1	98.3	98.3	2155.2	1471.9	1445.1	232.1	145.6	-	642.1	12.6	208.7
	Hebei	+	16.4	19.3	36.3	36.3	2561.2	895.7	2275.6	149.2	99.5	24.1	541.7	15.1	157.2
	Huadong	+	14.9	61.5	162.8	162.8	4549.2	749.3	1297.1	34.6	57.3	37.1	318.6	34.4	75.6
	Jiangxi	17.6	13.7	10.5	65.4	65.4	1780.2	909.6	799.2	48.3	59.7	+	212.0	+	164.4
	Anhui	21.9	40.3	82.9	968.3	968.3	13653.7	4182.0	3308.5	177.2	135.7	-	111.2	+	65.8
CCCM	Xiankang	31.6	23.8	89.5	77.9	77.9	2508.7	668.1	1237.2	33.3	47.5	+	273.2	+	-
	Quanxin	26.8	14.5	36.6	66.4	66.4	2132.0	386.6	621.1	35.7	51.9	-	306.6	+	-
	Guobao	+	+	133.6	75.8	75.8	1922.8	317.2	690.2	53.8	+	+	187.0	-	-
	HKUST	+	+	133.0	72.8	72.8	2564.4	442.8	803.8	60.4	95.8	+	205.2	-	+
	Aoli	18.3	36.4	31.2	233.2	233.2	1269.3	5148.2	3428.3	69.7	60.4	78.8	93.7	+	73.7
COS	C550542	+	+	-	-	-	726.8	442.1	483.9	+	48.8	-	145.2	+	51.6
	C550562	+	+	-	57.5	57.5	1020.3	332.2	295.5	+	48.2	-	397.1	+	49.8
	C550677	20.8	+	+	74.1	74.1	1052.8	604.2	762.6	36.8	60.4	-	514.5	+	-
	LCT	+	-	-	37.7	37.7	718.0	176.0	208.8	26.8	+	-	259.9	-	-

*LA, lauric; MA, myristic acid; PtA, pentadecanoic acid; PA, palmitoleic acid; LoA, linoleic acid; SA, stearic acid; DA, docosanoic acid; LiA, lignoceric acid; ChS, cholesterol; ErS, ergosterol; CaS, campesterol; StS, β-sitosterol; NOS, natural OS; CCOS, commercial cultured OS; CCCM, commercial cultured *C. militaris*; COS, self-cultured OS; LCT, isolated from the caterpillar of OS from Qinghai province.

**modified from Yang *et al*. (2009a)

2.3 Hepatotoxic thioacetamide in animal model

Detoxification/drug metabolism is one of the important physiological functions of the liver. If the injury signals were overloaded in liver tissues, the toxic effect will trigger downstream signal and influence the normal liver functions as following sections. The thioacetamide is one of well-known liver-specific toxin in hepatopathological researches.

2.3.1 Animal model for liver injury

Animal models for hepatopathological research are greatly contributive, and it successfully advances the clinical, therapeutic techniques for human beings. Tuñón *et al*. (2009) stated the importance of animal models clearly as he following: "Although a number of clinical trials testing different liver assist devices are under way, these systems alone have no significant effect on patient survival and are only regarded as an useful approach to bridge patients with AHF to liver transplantation. As a result, reproducible experimental animal models resembling the clinical conditions are still needed". The most common models are based on surgical methods, nutrient imbalance, viral infection, and chemical induction.

Surgical procedures are including total/partial hepatectomy, complete/transient devascularization. Every clinical surgery mimics the certain pathophysiological symptoms (e.g., irritative regeneration, ischemia, hypertension, cholestasis.). For hepatectomy, Mann *et al*. (1921) found that liver injury occurred when it is under stressful liver regeneration on dogs, and it has been successfully developed in various animal species (Table II-4). Furthermore, the previous studies demonstrated highly regenerative property in rodent models

while the upper limit for liver resection for rat and mice are almost up to 95% and 90% respectively (He *et al*., 2003; Makino *et al*., 2005). Clinically, this model is equivalent to massive liver damage due to liver trauma or a primary graft failure (Van de Kerkhove *et al*., 2004), and its main application was to study hepatic regeneration in the absence of toxin elimination, circulating inflammatory factors, and hepatoxin-induced liver injury (Eguchi *et al*., 1997). Next, devascularization was highly related to architectural and physiological properties of liver because it included occlusion of hepatic artery, bile duct, and/or accessory hepatic vessels (Ytrebø *et al*., 2002; Nakazawa *et al*., 2002). In this model, the inflammation and oxidative stress also occured due to toxicity of endogenous bile acid (Kosters and Karpen, 2010; Allen *et al*., 2011; O'Brien *et al*., 2013) or ischemic/reperfused signals (Kireev *et al*., 2013; Meimei *et al*., 2014). Devascularization was more useful for studying of ischemia related effects, sterile inflammation, endogenous toxic effects, and antioxidant in liver (Van Golen *et al*., 2012; Van Golen *et al*., 2013).

Besides, methionine-choline deficient diet (MCD) also induced liver injuries. Bosy-Westphal *et al*. (2003) observed that frequent hypermethioninemia and hyperhomocystinemia are proven that homocysteine-methionine pathway plays a critical role in liver metabolic function; hence, MCD had become a standard nutritional rodent model for non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) (Yamaguchi wt al., 2007; Jorgacevic *et al*., 2013; Itagaki *et al*., 2013). Because the MCD modelwas malnutrition and toxic effect due to homocysteine accumulation (Boldyrev, 2009), it increased generation of ROS by both CYP2E1 and CYP4A enzymes (Ip *et al*., 2003), and it also induced the expression of

inflammatory pathway (Mu *et al*., 2010). In other ways, choline-deficiency not only impaired very low-density lipoprotein (VLDL) secretion (Rinella *et al*., 2008) but also reduced the availability and generation of methionine (Zeisel *et al*., 1991). Comparison with other NASH or NAFLD models, it involves oxidative injury, lipid metabolism, and proinflammatory cytokines (Jorgacevic *et al*., 2013; Koek *et al*., 2011) but not insulin resistance (Rinella and Green, 2004), so it remains a useful tool for delineating the mechanism of lipid-related liver injury from other diseases of metabolic syndrome.

Despite that viral hepatitis is a main cause of liver injury in many countries, viral infected model via infective agent has been unsuccessful. The only useful models are transgenic mice overexpressing virus B protein (HBV) or infected with MHV-3 (Ando *et al*., 1993; Ding *et al*., 1997), but these models display limitations in pathophysiological procedures which compare to human clinical researches (Newsome *et al*., 2000; Rahman and Hodgson, 2000). Recently, Tuñón *et al*. (2011) described a new animal model of liver injury, especially in fulminant hepatic failure, using experimental infection of rabbits with rabbit hemorrhagic disease virus (RHDV). Furthermore, RHDV-infected rabbits have successfully represented a number of pathophysiological and biochemical features to human (Tuñón *et al*., 2003; Sánchez-Campos *et al*., 2004).

Chemicals (Table II-4) such as acetaminophen, carbon tetrachloride (CCl_4), ethanol, and thioacetamide (TAA) etc. may respectively reproduce a number of important clinical characteristics (e.g., hypoglycemia, encephalopathy, and increased serum hepatic enzymes), but not completely represent them all in single certain model (Newsome *et al*., 2000). Among all hepatotoxins, ethanol-induced model is quite different from others because it involves the

hepatic lipid accumulation additionally (Reddy and Rao, 2006). Therefore, ethanol-induced model is aimed at emulating clinical alcoholic fatty liver disease (AFLD).

Others, acetaminophen is a commonly used drug which causes dose-dependent hepatotoxic effect, and overdoses would switch the saturated normal pathway into cytochrome P-450 related pathway to form N-acetyl-p-bezoquinoneimine which induces cell damage (Sakai *et al*., 1992). However, lacking of standardization of acetaminophen administration may be the problem for its reproducibility (Newsome *et al*., 2000; Terblanche and Hickman, 1991).

Another, CCl_4 is the most common hepatotoxin of liver injured models (Slater, 1981), and it once was used as a solvent, degreaser, and cleaner both for industrial and home use until some studies proclaimed the toxicity and carcinogenicity of it. The effect of CCl_4-induced toxicity depends on species, treating dose and duration, and observing time, so the pathophysiological procedures are amalgamated including loss of Ca^{2+} storage, impairment of lipid homeostasis, release of various cytokines, apoptotic events, even fatty degeneration, fibrosis, cirrhosis, and cancer (Weber *et al*., 2003). Generally, CCl_4 induced models does not correspond to certain liver disease but generate comprehensive damages in hepatic tissues; therefore, it is often used to exam the hepatoprotective effect of nutraceuticals or some clinical drugs. Recently, the CCl_4 induced models have been gradually replaced with the TAA-induced model because of its poor reproducibility, extrahepatic toxicity, hypertoxicity to either operators or animals, and small time window before death (Tuñón *et al*. 2009).

Table II-4. Main AHF animal models in different species

Animal momdels	species	Advantage/disadvantages
Surgical		
Total/partial hepatectomy	Pig, dog, rebbit, rat, mouse	Hepatic encephalopathy; reproducible/ no reversibility; no long-term survival
Total/partial devascularization	Pig, dog, rebbit, rat	Hepatic encephalopathy; reproducible/ no reversibility; no long-term survival
Chemical		
Acetaminophen	Pig, dog, rebbit, rat, mouse	Hepatic encephalopathy; no hazard/ non-reproducible; variable interval between damage and death; species and age variability
Amanitin	Pig	Hepatic encephalopathy;specific toxic effects; large animal
Azoxymethane	Mouse	Hepatic encephalopathy; reproducible/ small size; hazard
Carbon tetrachloride	Pig, rebbit, rat, mouse	Hepatic encephalopathy/ non-reproducibility; extrahepatic toxicity; small time window before death
Concanavalin A	Rat, mouse	Hepatic encephalopathy/ small size
Galactosamine	Pig, dog, rebbit, rat, mouse	Hepatic encephalopathy; biochemical markers/ non-reproducible; hazard; variable interval between damage and death; species differences
Lipopolysaccharide	Rat, mouse	Hepatic encephalopathy; reproducible/ no reversibility; small size; hazard; small time window before death
Thioacetamide	Rabbit, rat, mouse	Hepatic encephalopathy; reproducible; large time window before death/ hazard
Viral		
Rebbit hemorrhagic disease	Rabbit	Hepatic encephalopathy; reproducible; no hazard

(Tuñón *et al*., 2009)

2.3.2 Synopsis of thioacetamide

Like most of hepatoxins, TAA was metabolized through CYP2E1 pathway, and its metabolites were proven harmful to livers. In the past, TAA had been widely used as an organic solvent in the textile, leather, and paper industries. It was as a catalyst in the vulcanization of Buna-Rubber reaction, or as a stabilizer of motor fuel, even as a fungicide. From 1981 to 1983, National Institute for Occupational Safety and Health surveyed its toxic effect on 786 workers under exposure in United States (National Toxicology Program, 2000). Nowadays, TAA has been an inducer of hepatic fibrosis (Ljubuncic *et al*., 2005; Kang *et al*., 2008).

Although TAA also generates comprehensive damage as CCl_4, the toxic effect is not quite intense and severe as aforementioned, and the time window is longer for operation. Most importantly, it has better reproducibility (Tuñón *et al*. 2009). Many results support that experimental fibrosis/cirrhosis induced model via TAA shares a number of metabolic and histological alterations with those found in the human disease (Mangipudy *et al*., 1995), i.e., apoptosis, periportal inflammatory cell infiltration (Ledda-Columbano *et al*., 1991), scar formation (Pérez *et al*., 2002), oxidative stress related damage (Bruck *et al*., 2004), and DNA strand breaks (Yogalakshimi *et al*., 2010). In conclusion, TAA models is the most appropriate experimental fibrosis models for this study that is related to common hepatoprotective effects.

2.3.3 Pathophysiological effect of thioacetamide

In 2005, Ljubuncic *et al*. (2005) reported that liver hepatic hypertrophy and lighter body weight were observed in TAA treated Sprague-Dawley rats, and it

was proclaimed that the symptom was caused by toxic metabolite of TAA, which was distinguished from high-fat diet model, non-alcoholic-fatty-liver model and other inducing factors related with obesity (Yang *et al*., 2009b). As haloalkanes is, the toxicity of TAA is metabolites not the compound itself. It has been demonstrated that flavin-containing monooxygenase (FMO) and CYP2E1 are required enzymes for the bioactivation of TAA and TAA-induced hepatotoxicity in both rat and mouse models (Sanz *et al*., 2002; Kang *et al*., 2008). Meanwhile, TAA is metabolized into further adducts through them (Figure II-6) (Porter and Neal, 1978; Porter *et al*., 1979), forming covalent-binding with other intracellular molecules were followed and an oxidative stress was induced (Bruck *et al*., 2004; Tunez *et al*., 2005). Until Chilakapati *et al*. (2005) had clarified the metabolism pathway and toxicity of TAA and its metabolites *in vivo*, it showed the mechanism to explain the characteristic of TAA intoxication. The evidences indicated that both of the first and the second step of TAA metabolic are saturable so that it lacks dose-response after reaching threshold; moreover, the TAA and TASO were eliminated in the urine under two different doses within 24h.

Reactive metabolites, which can covalently bind to cellular macromolecules, induced oxidative stress directly and indirectly (Witzmann *et al*., 1996; Okuyama *et al*., 2003; Pallottini *et al*., 2006). Due to the detoxification of TAA, the ROS production was followed by lipid peroxidation, glutathione depletion and reduction in SH-thiol groups (Pallottini *et al*., 2006). Recently it had found an endogenous multifunctional protein called thioredoxin (TRX) with redox-activity in livers, and TRX is also a stress-inducible protein that is enhanced by various types of stresses (e.g., viral infection, ultraviolet

light, X-ray irradiation, and hydrogen peroxide) (Nakamura *et al*., 1997). In TAA intoxicated mice, it showed that TRX-binding protein 2 (TBP2) could be a potent regulator of not only lipid metabolism but also innate immunity in livers (Okuyama *et al*., 2008). Although TRX and TBP2 are indicated to involve the redox pathway in injured livers, it still needs an advanced investigation to clarify the detailed mechanism.

Calcium is another key factor that responses to the TAA intoxication. The mobilization of Ca^{2+} from intracellular stores was observed (Diez-Fernandez *et al*., 1996). The TAA administration inhibited ATP-dependent Ca^{2+} sequestration by a direct influence on the endoplasmic reticulum Ca^{2+} pump *in viv*o, and elevated the cytosolic Ca^{2+} (Diez-Fernandez *et al*., 1996). Cytosolic Ca^{2+} was an important second messenger related to many cell biological procedures in many tissues even in livers (Orrenius *et al*., 2003; Gaspers and Thomas, 2005; Fracchia *et al*., 2013).

Moreover, both ROS and calcium homeostasis were determined to activate multiple mechanisms related to cell damage or proliferation (Brookes *et al*., 2004). Mitochondria are not only for ATP synthesis but also play the key role for initiating cell apoptotic signals (Kass, 2006; De David *et al*., 2011) when increased ROS formation and disruption of calcium homeostasis occurred (Bernardi *et al*., 2001). It was also reported that some direct cell damage sinduced the cell necrosis and triggered the inflammatory response (Okuyama *et al*., 2003; Laleman *et al*., 2006; Shirai *et al*., 2013). To summarize the above perspectives, the TAA intoxication results in cell death via direct and indirect pathways, which trigger the further inflammation.

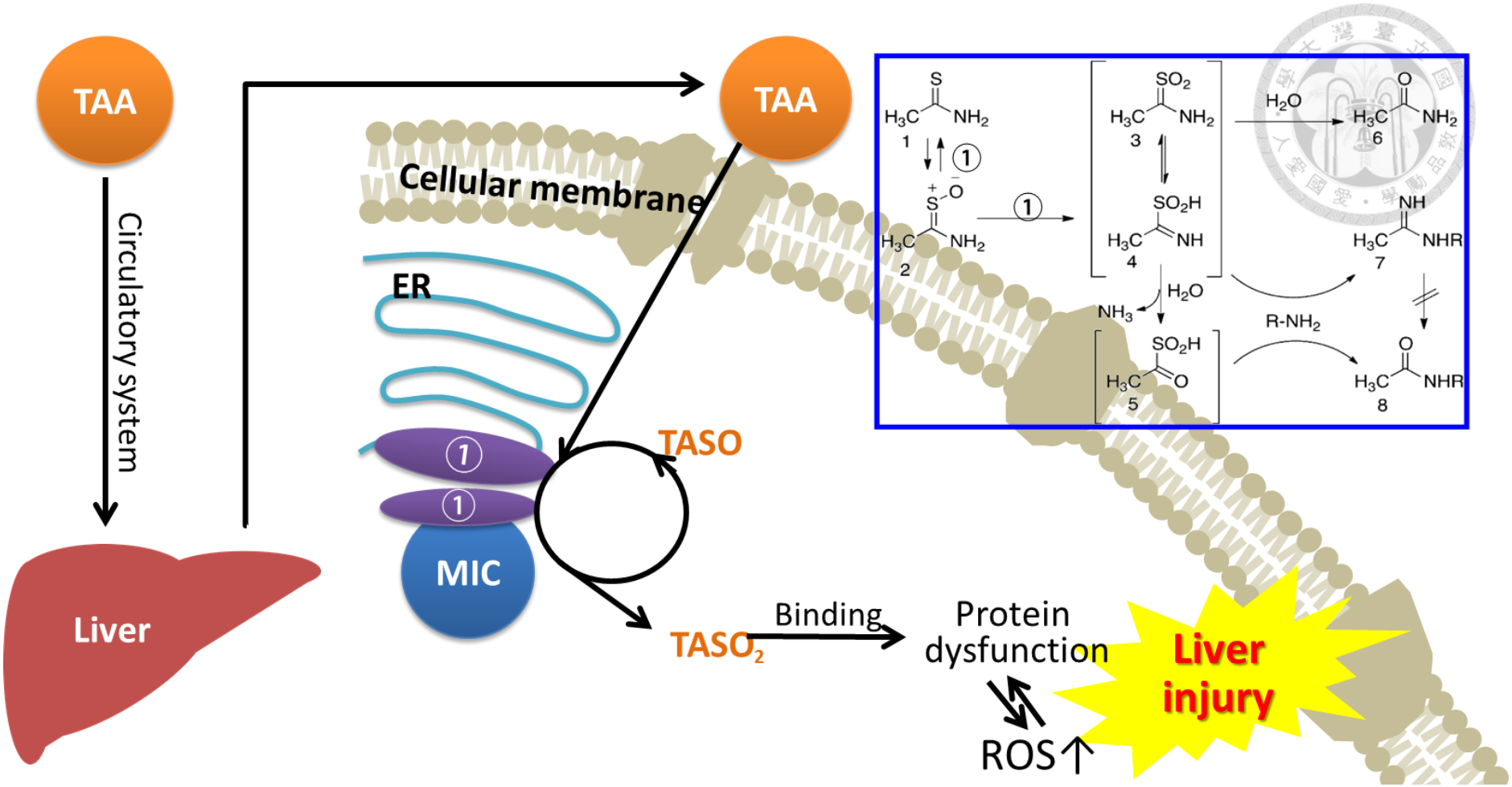


Figure II-6. Metabolic pathway and pathophysiological effect of thioacetamide. Hydrophilic TAA is diffusing into cell plasma via channel, and goes by enzymatic system (purple) within some organelles (blue). Finally, the metabolites (orange) will cause the liver injury. The sign of ER and MIC were abbreviated for Endoplasmic Reticulum and Microsome. The meanings of showing number are as following, 1: Thioacetamide, TAA. 2: Thioacetamide S-oxide, TASO. 3 and 4: Thioacetamide S, S-dioxide, TASO2 (unstable). 5: Acetyl sulfinic acid. 6: Acetamide (stabel). 7: Amidine adducts (stable). 8: Amide derivatives (stable). ①: cytochrome P450 2E1/CYP2E1 and flavin-containing monooxygenase/FMO enzymatic system on ER or MIC. (modified from Hajovsky, 2012l)

2.4 Hepatic fibrogenesis

2.4.1 Fibrogenesis and clinical research

According to a report by the International Agency for Research on Cancer in 2012, liver cancer is the second ranking of death causes among cancer categories worldwide, and it is the fifth most common cancer in male (554,000 cases, 7.5% of the total) and the ninth in women (228,000 cases, 3.4%). Besides, it is a huge problem of the eastern Asia, especially China (Figure II-7 &Figure II-8). Similarly, liver cancer was ranked first or second for cancer-related deaths within these five years in Taiwan (MOHW, 2012; Figure II-9). Furthermore, liver cancer is highly related to chronic liver diseases (CLDs), and the aetiology results in persisting inflammation, progressive fibrogenesis and chronic activation of the wound healing response (Povero *et al*. 2010). Overall, liver related diseases become a big issue in morden Taiwan society (Figure II-10).

Liver fibrosis (Figure II-11) is characterised by an excessive accumulation of extracellular matrix (ECM) proteins such as collagen, which mainly secreted by myofibroblast-like hepatic stellate cells (HSCs; also called activated HSCs). Since Hutterer and Rubin (1964) had proclaimed that terminal liver fibrosis is reversible on clinical reports, scientists had looked for a key to reduce liver fibrosis by decreasing ECM degradation and normalizing the HSCs' activation through damage-healing model until now.

According to a Bataller and Brenner (2005), the review article mentioned that the cause of liver fibrosis is due to the chronic liver damage, which may activate HSCs to become myofibroblast-like/ECM-secretion-capable cells. Although ECM deposition is proved to be acute protective response against live

damage generally (Lee and Friedman, 2010), the overloads of it, so called fibrotic scar, still leads to a conversion of the normal structure in hepatic tissues to regenerative nodules (liver cirrhosis) finally. Liver cirrhosis accompanies with liver dysfunction due to resistance of blood flows (hepatic portal hypertension), and the aforementioned progresses are irreversible (Schuppan and Afdhal, 2008). Therefore, liver therapy always focuses on fibrotic liver period. Nowadays medical technology is highly developed, but the crux that is present therapeutic drugs for liver fibrosis has many side-effects (i.e., extrahepatic toxicity, poor effectiveness to all type of liver fibrosis). Hence, many edible phytochemicals catch researcher's eye.

2.4.2 Liver injury and inflammation

Fibrosis is a wound-healing response that engages a range of cell types (Figure II-12) and mediators against liver injury. The oxidative stress and active molecule attacking are regarded as primary and direct factors among them, especially drug induced hepatic injury and fibrosis (Kaplowitz *et al*., 2002). There are two classic pathways to generate ROS (members are shown as Figure II-13) in injured tissues: an induction of cytochrome P450 2E1 (Castillo *et al*., 1992; Chitturi and Farrell, 2001), and lipid peroxidation (Paorla and Robino, 2001; Jaeschke, 2006). Oxidative stress may not only affect mitochondrial function but also enhance apoptosis via members of the TNF superfamily (e.g., TNFR1/2, FasL, DR4/5) (Shakibael *et al*., 2005), which activate redox-sensitive kinases and transcription factors (e.g., AP-1, NF-κB.) (Kaplowitz and Tsukamoto, 1996; Czaja, 2002). An otherwise effect is that covalent binding between active metabolites and intracellular molecules directly attacks proteins,

lipids or nucleic acids within targeting cells (Jaeschke *et al*., 2002; Jaeschke *et al*., 2003; Kaplowitz and Tsukamoto, 1996), and it may cause metabolic disruption, ion dysregulation, mitochondrial and cellular swelling, activation of degradative enzymes, and necrosis finally (Proskuryakov *et al*., 2003; Arends *et al*., 1994).

An apoptosis-necrosis switch was found related to intracellular ATP concentration and NO signal in hepatic cells (Leist *et al*., 1997; Nicotera and Melino, 2004). This suggests that they share common event in different types of cell death during an early injury response. Although the inflammatory pathway of necrosis has not been identified, Jaeschke (2002) proclaimed that apoptosis of parenchymal cells is no longer concequence of liver injury, but rather as an important inflammatory stimulus that activates HSCs. Hence, the inflammation is the key role to amplify of local direct damage into fibrosis via activating transcription of cytokines, chemokines, and adhesion molecules (e.g., TNF-α, IL-1β, and IL6) (Bataller and Brenner, 2005; Henderson and Iredale, 2007).

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	554	521	453	228	224	180	782	746	633
More developed regions	92	80	112	42	43	51	134	123	164
Less developed regions	462	441	341	186	182	129	648	622	469
WHO Africa region (AFRO)	25	24	17	14	13	9	39	37	26
WHO Americas region (PAHO)	40	35	35	23	23	18	63	58	53
WHO East Mediterranean region (EMRO)	20	19	12	10	9	6	29	28	18
WHO Europe region (EURO)	47	44	42	23	25	20	71	69	61
WHO South-East Asia region (SEARO)	55	52	33	25	24	15	80	77	48
WHO Western Pacific region (WPRO)	368	347	314	133	129	112	501	477	426
IARC membership (24 countries)	120	104	135	56	55	60	176	159	195
United States of America	23	17	21	8	7	7	30	24	27
China	293	282	220	101	101	71	395	383	291
India	17	17	8	10	10	5	27	27	13
European Union (EU-28)	36	32	33	16	17	14	52	48	47

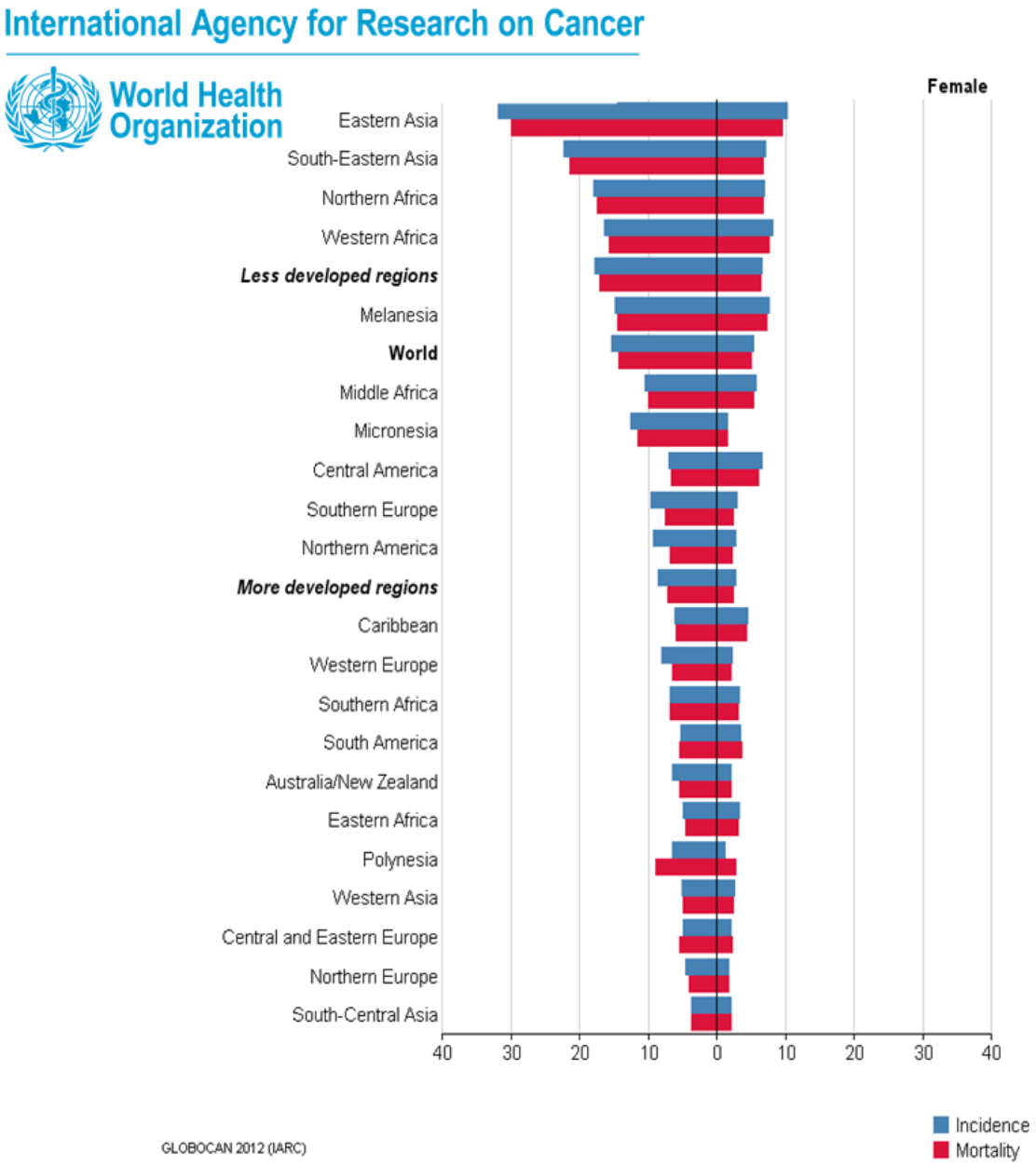


Figure II-7. The mortality and incidence of liver cancer worldwide. Liver cancer is largely a problem of the less developed regions where 83% (50% in China alone) of the estimated 782,000 new cancer cases worldwide occurred in 2012. It is the fifth most common cancer in men and the ninth in women. In men, the regions of high incidence are Eastern and South-Eastern Asia. Intermediate rates occur in Southern Europe (9.5) and Northern America (9.3) and the lowest rates are in Northern Europe (4.6) and South-Central Asia (3.7). In women, the rates are generally much lower, the highest being in Eastern Asia and Western Africa (10.2 and 8.1 respectively), the lowest in Northern Europe (1.9) and Micronesia (1.6). Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total). The prognosis for liver cancer is very poor, and as such the geographical patterns in incidence and mortality are similar. (IARC, 2012)

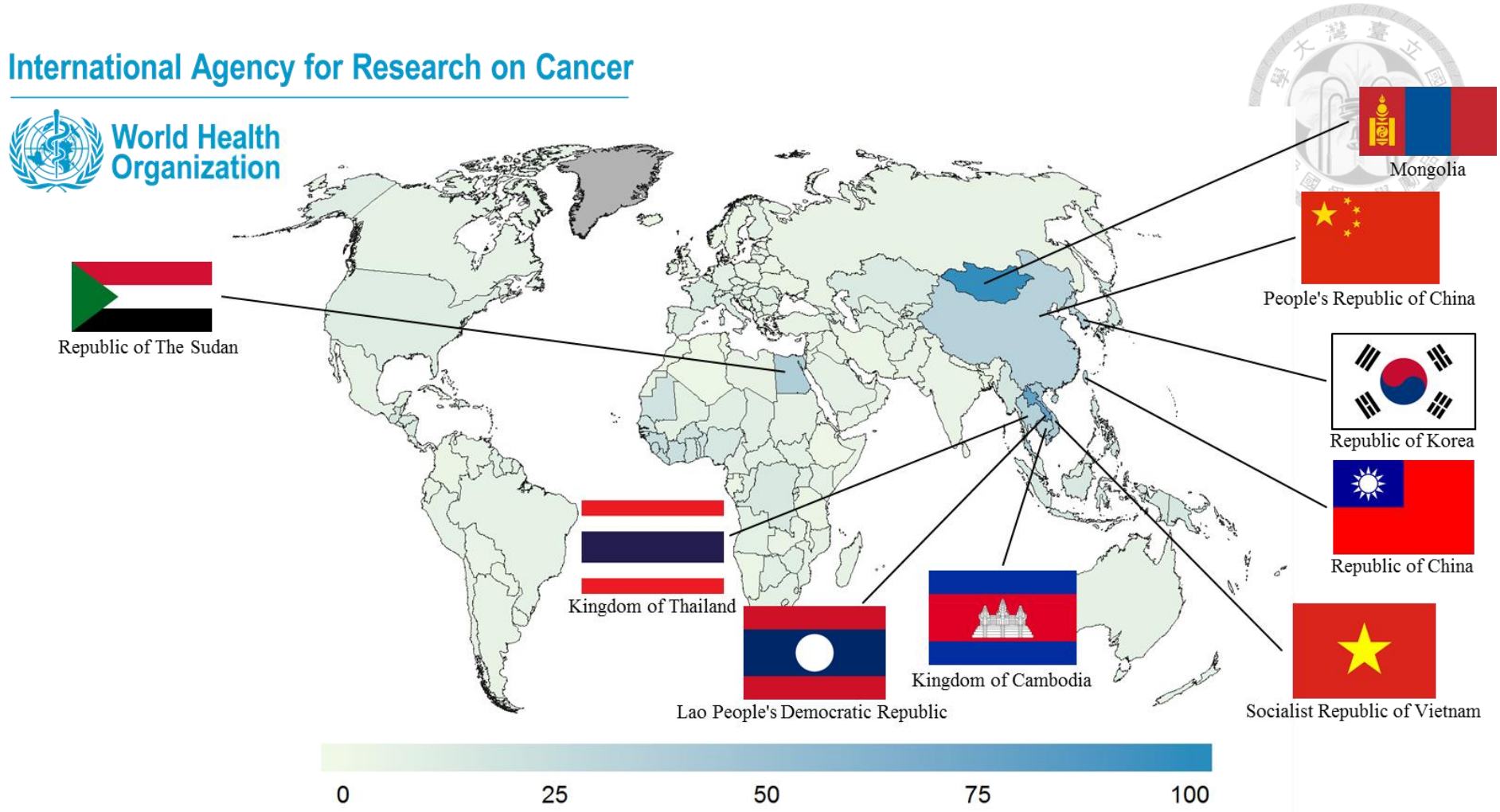


Figure II-8. The prevalence of liver cancer in different country for men. The prevalence of liver cancer in east asia is higher than other area obviously. Estimated age-standardised rates (World) per 100,000. This figure is modified from IARC (2012).

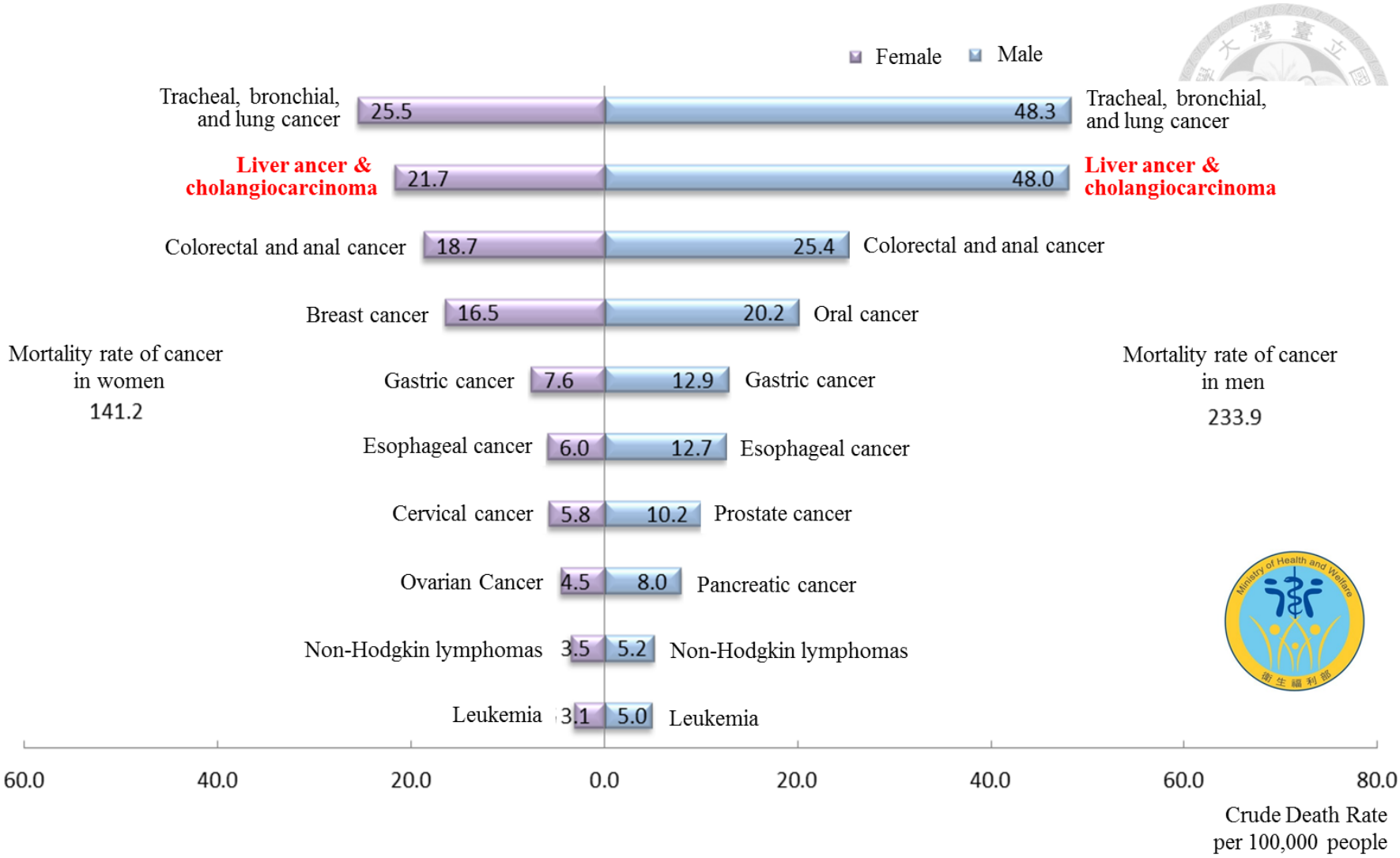


Figure II-9. Leading causes of death in cancer categories in Taiwan. (MOHW, 2012)

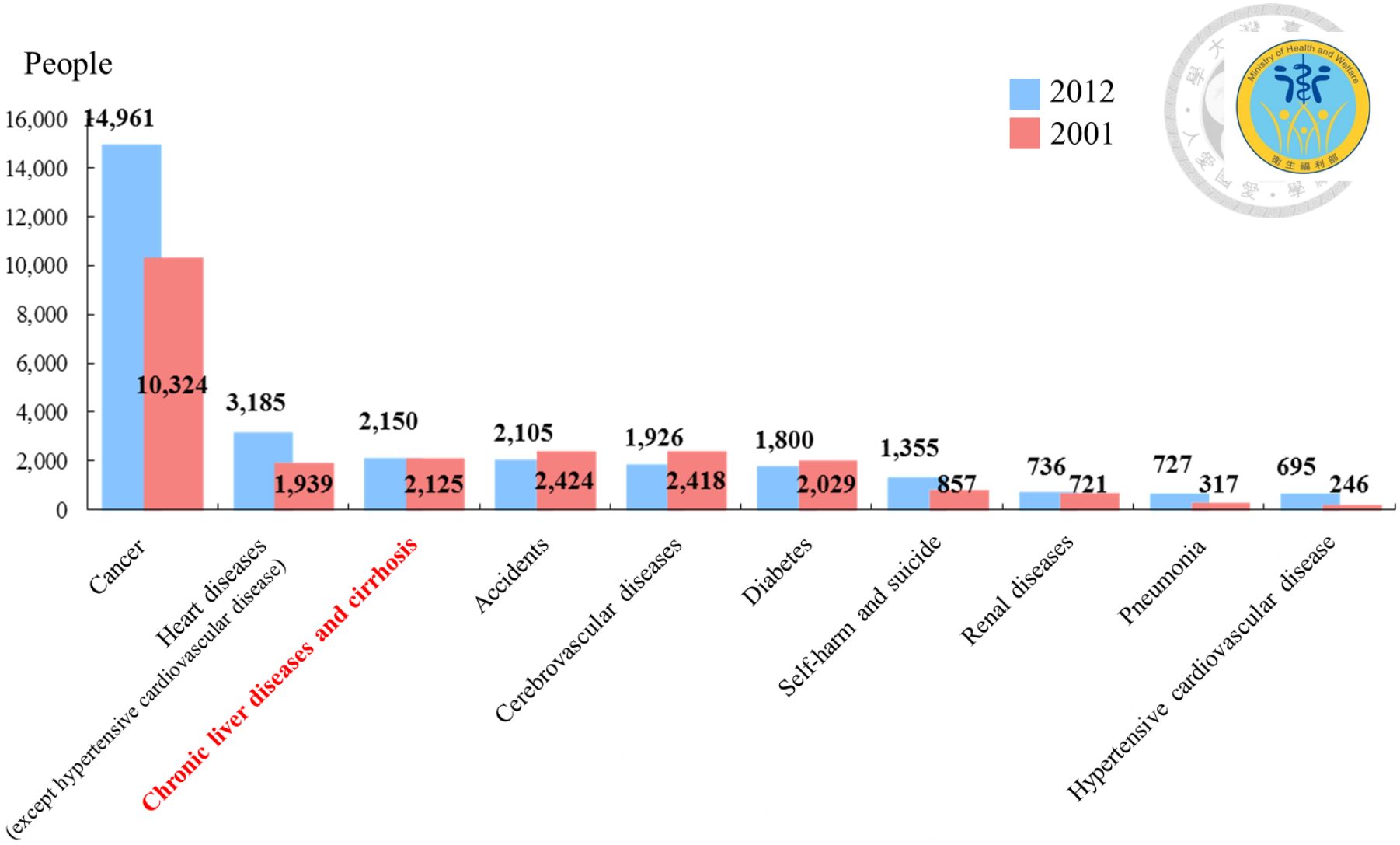


Figure II-10. The leading causes of death at 2001 and 2012 in Taiwan. (MOHW, 2012)

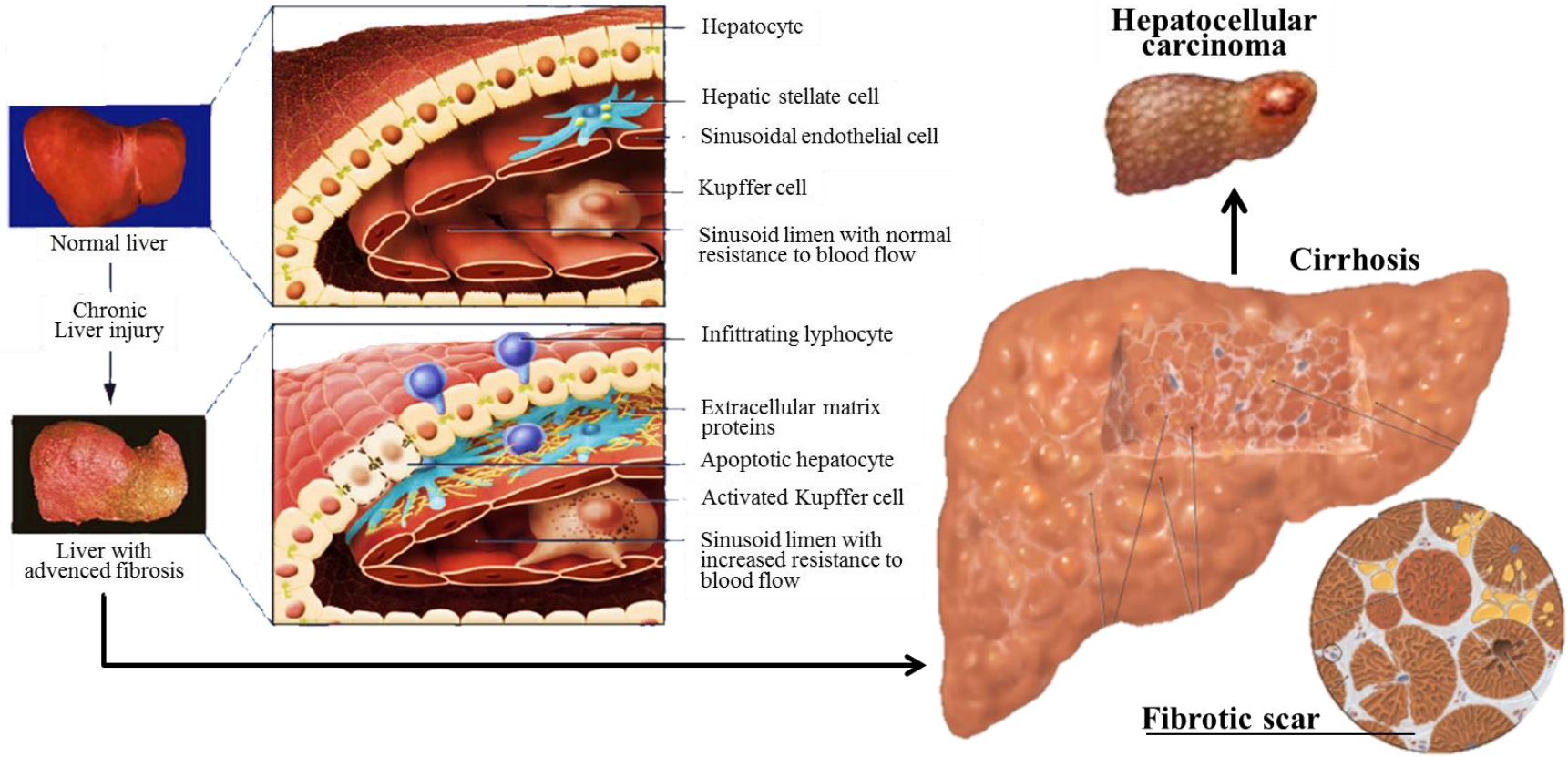


Figure II-11. The pathological process of liver injury to hepatocellular carcinoma. Liver fibrosis is characterised by an excessive accumulation of extracellular matrix proteins such under injury, and the ECM was mainly secreted by myofibroblast-like hepatic stellate cells (i.e., activated Kupffer cell, activated HSCs). The accumulated fibers will form the fibrotic scar, which will disrupt the blood flow and cause the destruction of liver. Finally, sever injury and fibrogenesis may turn into Cirrhosis (hepatic nodules and integrated fibrotic scar), and maybe hepatocellular carcinoma. Fortunately, the fibrogenesis is reverserable.

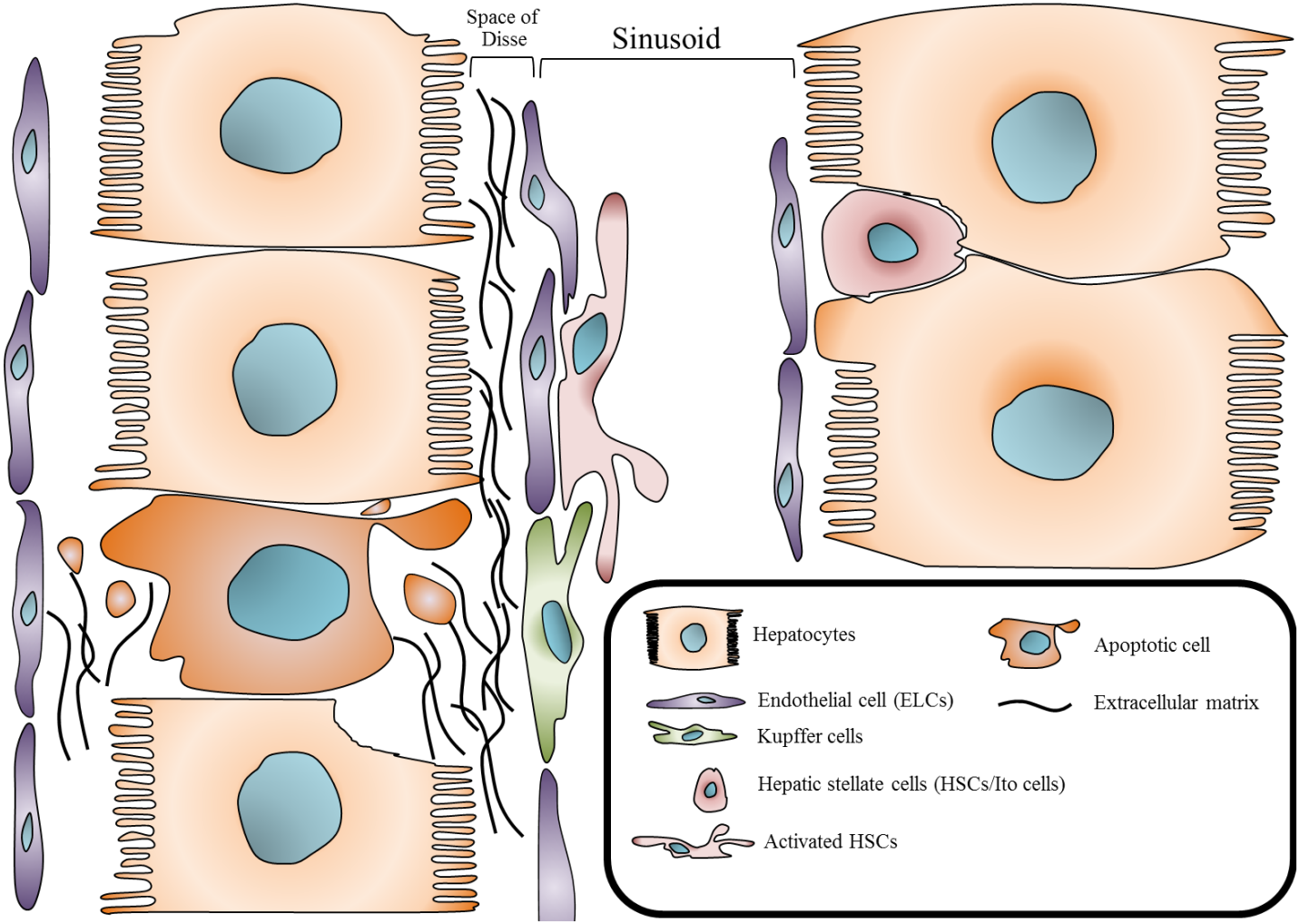


Figure II-12. Major responsive hepatic cells to fibrosis. Liver mass were mainly consisted by four kinds of hepatic cell, including: hepatocyte, endothelial cell, Kupfer cell and stellate cell. It is about 80% of live mass that is contributed to hepatocytes, which hold the critical role of hepatic metabolism, detoxification, urea formation, and sythesis of bile acid. Endothelial cells are the architectural supporter, because it will form the barrier bwtween sinusoid and space of Disse. In the sinusoid, Kupffer cells (Ito cell, fat-storing cell, perisinusoidal cell), the hepatic macrophage, will clean the fragments of dead cells and microrganisms, and it also can secrete defending cytokines. The last but least, stellate cells (10% of liver mass) modulate the balance of extracellular matrix in liver, vitamin A storage, and hepatocyte growth factor in the space of Disse.

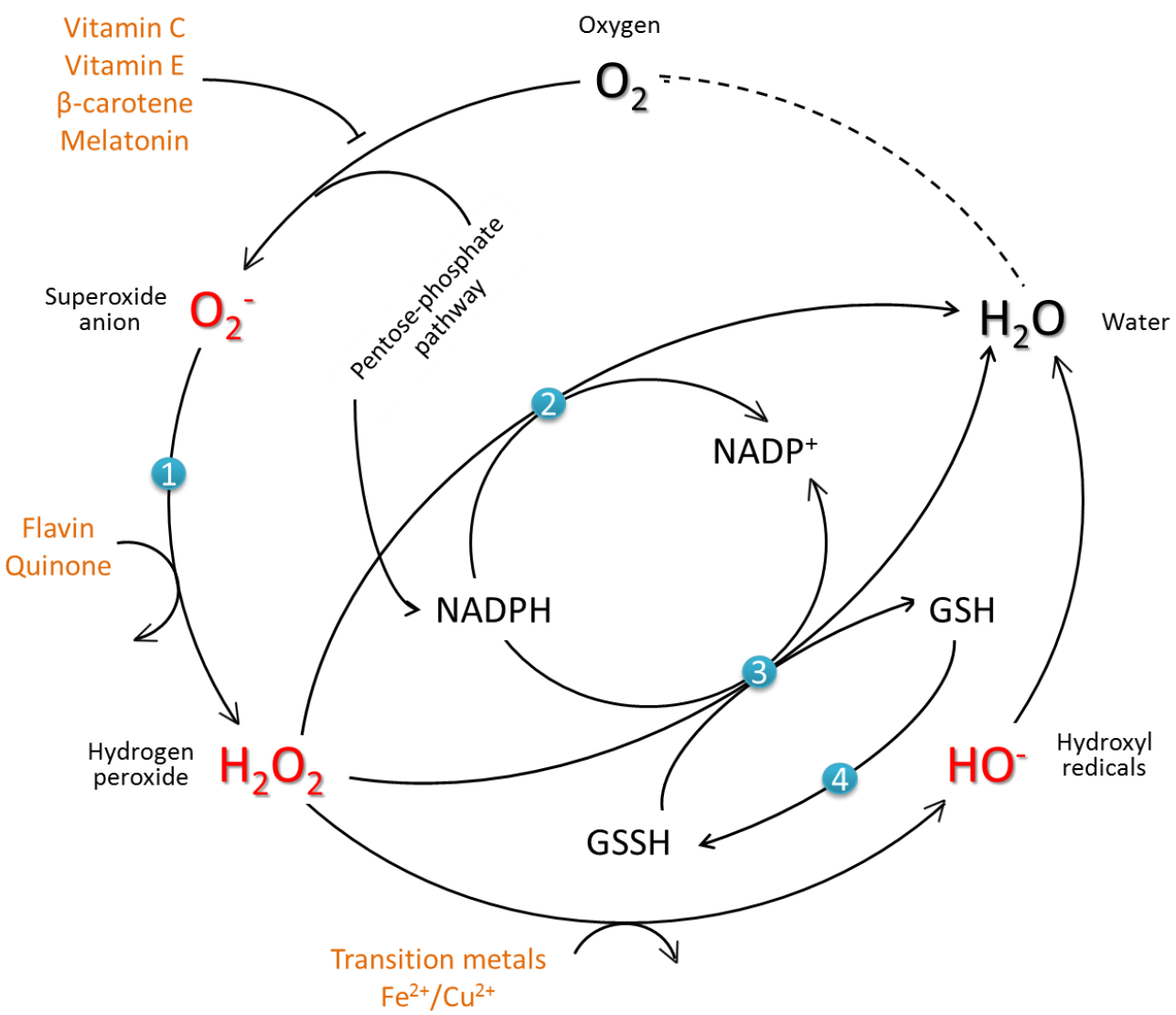


Figure II-13. Memebers of ROS and their generated pathway in antioxidativ system. The antioxidative system is divided into two categories, antioxidant (orange) and antioxidative enzymes (blue). All the players in this pathway turn the harmful molecules such as ROS (red) into oxygen and water. The numbers indicate different antioxidative enzymes as following, ①: superoxide dismutase (SOD); ②: Catalase (CAT); ③: Glutathione reductase (GR); ④: Glutathione peroxidase (GPx).

2.4.3 Hepatic stellate cell activation and its molecular mechanism

Initial events of HSC activation are occurring on a background of progressive changes (e.g., paracrine and autocrine signals) in the surrounding ECM within the sub-endothelial space of Disse. By literatures reviewing, we found three steps of this progressive changes including: 1) changing the membrane receptors, altering matrix signals, and migrating through adhesion disassembly (Yang *et al*., 2003; Melton *et al*., 2007). 2) Activating cellular MMPs to release growth factors (Schuppan *et al*., 1998; 2001). 3) Enhancing density of ECM (Wells, 2008). However, the reason that triggers HSCs activation is still unclear.

Although the opinions of regulatory pathway for HSCs activation are widely divided (Figure II-14), the Toll-like receptors (TLRs) related signals were involved, because those compelling pathways of injury were recently uncovered for innate immune signaling in liver. Paik *et al*. (2003) found that bacterial lipopolysaccharide (LPS) responsive receptors, TLR4, were unsurprisingly identified on Kupffer cells (specialized macrophages located in the liver), but also on HSCs unexpectedly. Moreover, although TLR4 signaling is essential for pathogen induced inflammation in macrophages (Hua *et al*., 2007), many scientists indicated that TLR4 signaling in HSCs was not only triggered by LPS but also possibly endogenous ligands such as called damage-associated molecular patterns (DAMPs), which includes low molecular-weight hyaluronic acid (Taylor *et al*., 2004; Jiang *et al*., 2005), free fatty acids (Lee *et al*., 2001), fibrinogen (Smiley *et al*., 2001), fibronectin (Okamura *et al*., 2001), heat shock proteins (HSPs) 60/70 (Chen *et al*., 2004; Ohashi *et al*., 2000), and high mobility group box (HMGB) 1 (Park *et al*., 2004); furthermore, damage signals and intact

ECM degradation also activated TLR4 too *in vivo* (Brunn *et al*., 2005). Therefore, Friedman (2008) recommended that the TLR4 signal in HSCs may be more important than that in Kupffer cells in eliciting a fibrogenic response.

Activated human HSCs express TLR4 and its essential co-receptors (CD14 and MD2), and they have intact TLR4 signaling and regulate the downstream signal to induce proinflammatory features, such as upregulation of chemokines (CCL2, CCL3, and CCL4), adhesion molecules (Vascular cell adhesion molecule 1, VCAM-1; intercellular cell adhesion molecule 1, ICAM-1; E-selectin), and profibrogenic features including the increasing of TGF-β signaling (Seki *et al*., 2007; Paik *et al*., 2003; Schwabe *et al*., 2006). Under TLR4 signals, an otherwise effect is that anti-apoptosis signal was provoked, and it promoted HSCs' survivals (Guo *et al*., 2009).

2.4.4 TLR4 signaling in the liver injury and hepatic fibrogenesis

TLRs mediate a tightly integrate signal transduction cascade conveying downstream signals that control transcription (Figure II-15). As De Creus *et al*. (2005) and Zarember *et al* (2002) proclaimed, the mRNA of TLR4 and its signaling molecules were very low in healthy livers in comparison to other organs, and it contributed to the high tolerance of the liver to LPS from intestinal microbiota. The LPS is elevated in experimental models of hepatic fibrosis and in patients with cirrhosis (Seki *et al*., 2007; Chan *et al*., 1997), and it may be due to changes in intestinal motility and microbiota, mucosal integrity (Wiest and Garcia-Tsao, 2005). Generously, hepatic fibrosis contributes to a failure of the intestinal mucosal barrier, and causes increase LPS levels in liver.

In liver, TLR4 is not only expressed in HSCs and Kupffer cells but both the

hepatocytes and non-parencymal cells (NPCs), and they all display a cell-specific activation pattern to TLR ligands (Chan *et al*., 1997). Hepatocytes make up 70-85% of the liver cytoplasmic mass, so it is important to understand this kind of TLR4-responsive cells. Hepatocytes play an irreplaceable role in liver metabolism and detoxification, and they also remove the intercellular LPS via absorbing from systemic circulation and secreting into the bile (Scott *et al*., 2009; Scott and Billiar, 2008). Moreover, the LPS uptake is through CD14-TLR4-MD2-dependent mechanism, and it is mediated by MAPK activation (Scott *et al*., 2009; Scott and Billiar, 2008), stimulating hepatocytes to produce IL-6 and TNF-α via the TLR4-NFκB-dependent signaling (Galloway *et al*., 2008). Obviously, TLR4-induced hepatocytes build a specific microenvironment for other cells to undergo fibrogenesis.

As Guo and Friedman (2010) mentioned, the downstream factors regulated by TLR4 signaling could divide into three parts. First, the TLR4 signal is as a causative factor of innate immune response, because it increases the expression of pro-inflammatory cytokines (TNF-α, IL-1, IL-6), chemotactic cytokines (monocyte chemotactic protein 1, MCP-1; macrophage migration inhibitory factor, MIF), pro-inflammatory proteins (ibducible nitric oxide synthase, iNOS), ROS, adhesion molecules (ICAM-1, VCAM-1), and other effectors (cyclooxygenase 2, COX-2). Second, the TLR4 signal regulates cell-cycle progression and the apoptotic threshold via related proteins (e.g., cyclin D, Bcl-2, and Bcl-xL). Third, the TLR4 signal activates the HSCs that may interact to classic TGF-β signal. The overall evidents showed that TLR4 signaling may be the linkage of inflammation and HSC activation in hepatic fibrogenesis progressing.

2.4.5 The role of TGF-β signaling in liver fibrosis

TGF-β related pathway is the classic target in variant-organ fibrosis all along such as renal cells (Gentle *et al*., 2013), pancreatic cells (Brandl *et al*., 2013), colonic cells (Grau *et al*., 2006), osteoclasts (Gingery *et al*., 2008), and astrocytes (Hsieh *et al*., 2010) *in vitro*. In liver, although scientists had found the relationship among HSCs activation, proliferation, and existence of TGF-β proteins for much long time (Saile *et al*., 2001), the up-regulator of them is still unclear.

Peng *et al*. (2013) reported that the anti-fibrotic effect was presented by suppressing the TGF-β1/Smad signaling in OS-polysaccharide-fed rats, moreover, it also inhibite the activation of HSCs, recovering the balance of MMPs and tissue inhibitor of metalloproteinases (TIMPs), which cotrol the ECM degradation so that it may also regulate the TLR4 signals. At the same year, Salama *et al*. (2013) found a kind of phytochemicals could protect liver in TAA-induced rats, and it suppress the expression of NFκB protein and decrease the apoptotic protein. In fact, Bitzer *et al*. (2000) had revealed the relevance between TGF-β signal and NFκB protein in fibroblasts *in vitro*.As aforementioned, TLR4 signaling pathway may interact with TGF-β signaling in HSCs (Guo and Friedman, 2010). NFκB may be the cross point resumptively (Figure II-15).

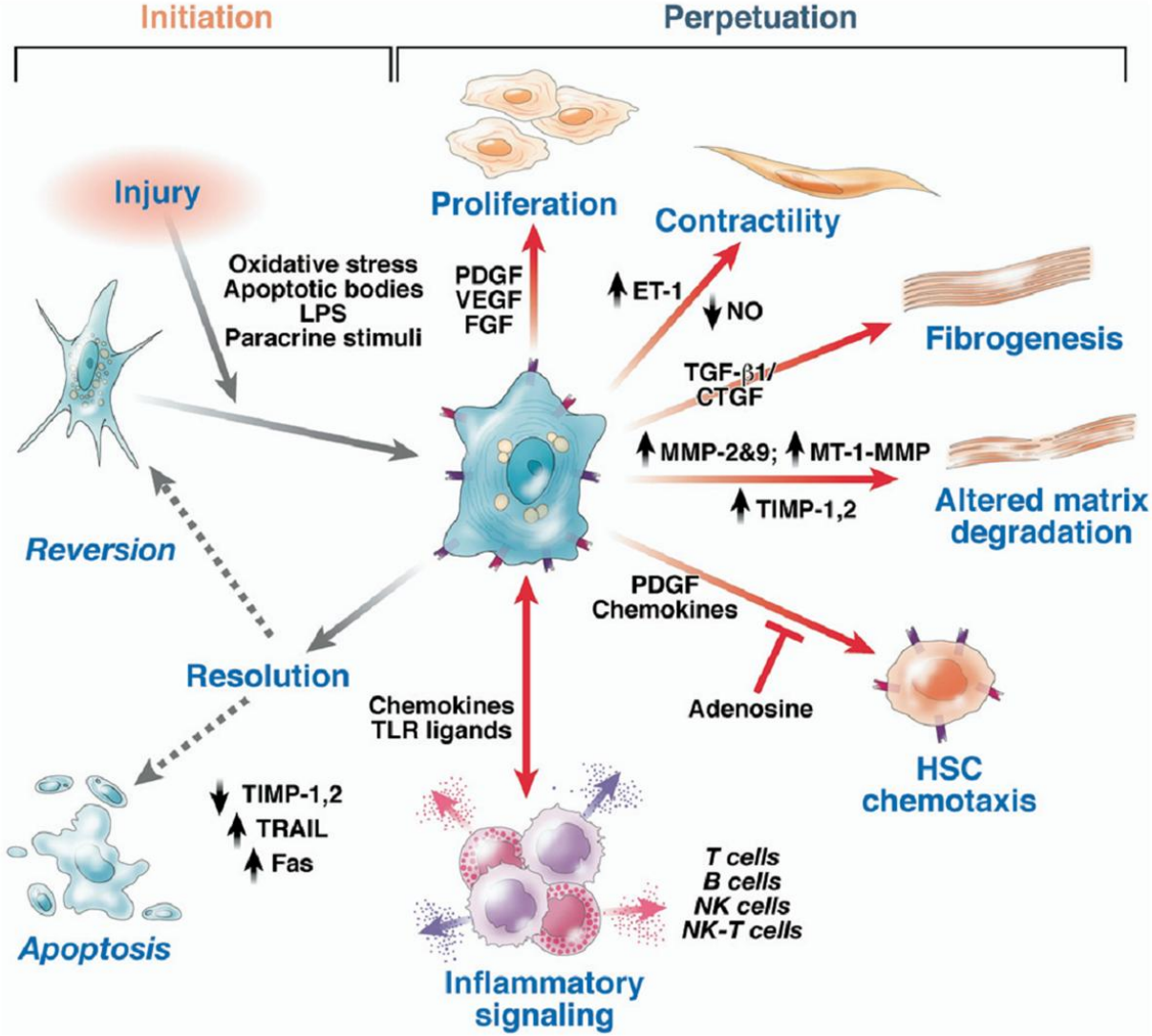


Figure II-14. Pathways of hepatic stellate cell activation. Features of stellate cell activation can be distinguished between those that stimulate initiation and those that contribute to perpetuation. Initiation is provoked by soluble stimuli that include oxidant stress signals (reactive oxygen intermediates), apoptotic bodies, lipopolysaccharide (LPS), andparacrine stimuli fromneighboring cell types including hepaticmacrophages (Kupffer cells), sinusoidal endothelium, and hepatocytes. Perpetuation follows, characterized by a number of specific phenotypic changes including proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis, and inflammatory signaling.FGF, fibroblast growth factor;ET-1, endothelin-1;NK, natural killer; NO, nitric oxide;MT, membrane type. (Friedman, 2008)

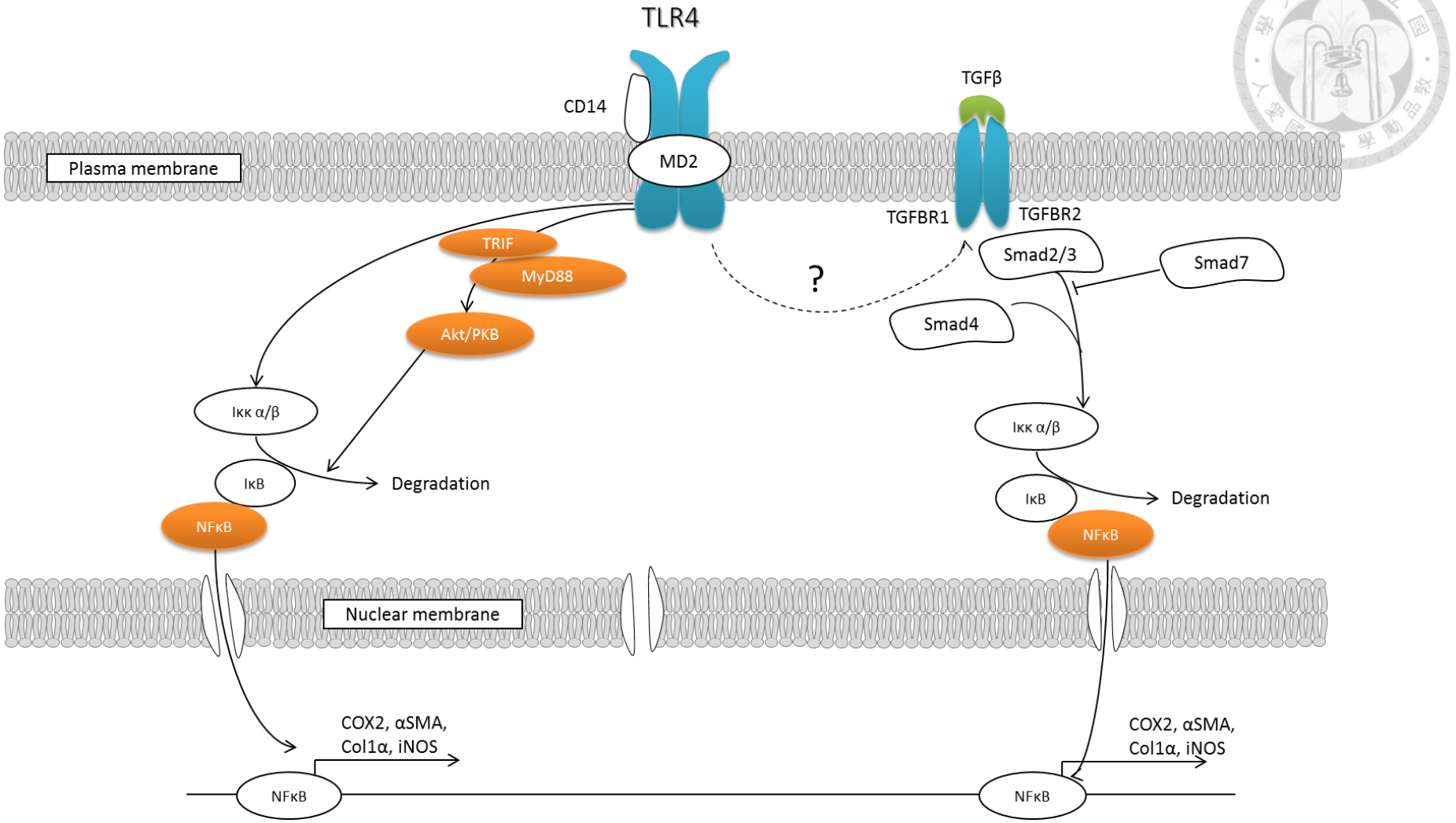


Figure II-15. TLR4 and TGF-β related pathway in liver fibrosis.

III. MATERIALS AND METHODS

3.1 Chemicals

TAA were purchased from Sigma-Aldrich (St. Louis, MO, USA). As a chemical inducer for hepatic fibrosis, TAA was dissolved in sterile phosphate-buffered saline (PBS). The *Ophiocordyceps sinensis* mycelium (OSM) powder (TCM-808FB®), which contains 10% polysaccharide and 0.25% adenosine, was kindly provided by the TCM Biotech International Corp. (Taipei, Taiwan). According to the composition analysis of TCM-808FB, the dry matter mass is 72.82%.

3.2 Animal and treatments

A total of 48 male Wistar rats (6 weeks old) weighting between 250-300 g were used in this study, and they were obtained from BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). In animal room, every two rats were housed in one cage under the condition of 22+2^{o}C, approximately 60-80% relative humidity, and 12-h light-dark cycle. Otherwise, the diet and water were *ad libitum* for them.

All rats were acclimatized for one week prior to the experimental treating (Figure III-1). Rats were randomly divided into 4 groups: 1) Control: saline (i.p.) and pure water (gavage); 2) TAA: TAA (100 mg/kg BW, i.p.) and pure water (gavage); 3) OSML: TAA (100 mg/kg BW, i.p.) and OSM (280.8 mg TCM-808FB /kg BW, gavage); 4) OSMH: TAA (100 mg/kg BW, i.p.) and OSM (1404 mg TCM-808FB /kg BW, gavage). TAA and OSM treatments were both given three times per week during a persistent 8-week period. At the end of the treatment period, rats were fasted, and sacrificed. Immediately, blood and tissue samples were harvested for further analyses. Animal use and protocol were reviewed and approved

by the National Taiwan University Institution Animal Care and Use Committee (IACUU No.: 100-015).

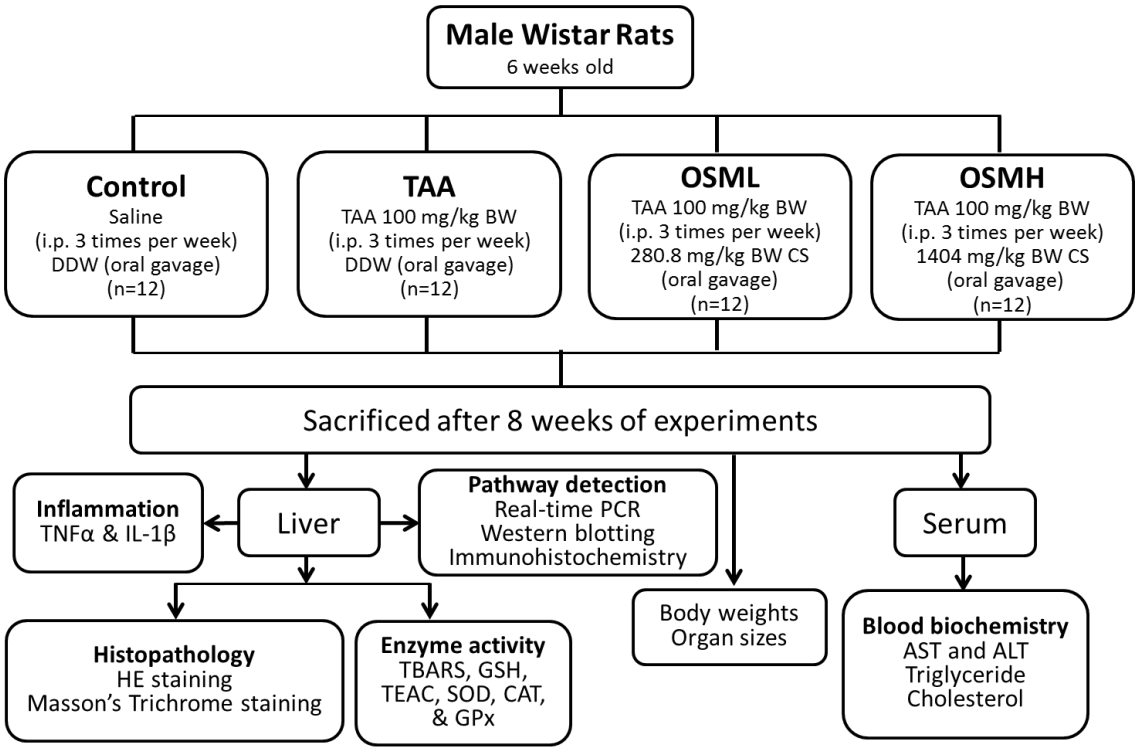


Figure III-1. Experimental design of this study

3.3 Sample collection

During the experimental period, food and water intakes, and body weight were recorded weekly. At the end of experiment, rats were fasted overnight. Then they were euthanized by ethoxyethane (Sigma-Aldrich, St. Louis, MO, USA), and harvested blood samples by abdominal aorta puncture. The liver, heart, and kidney from each rat was removed and weighted individually. Liver was cut off two or three one cubic millimeter soaking with 10% formalin (Sigma-Aldrich, St. Louis,

MO, USA) for histopathological section, and the others were placed into liquid N_2 for further analysis. Finally, all the samples but the aforementioned samples for section were stored at -80 ^{o}C for subsequent analyses.

3.4 Serum biochemical value analyses

Blood samples were collected from the abdominal aorta and placed for 1 hour at room temperature to allow clotting. Then the serum were collected by a centrifugation at 3,000 x g for 15 min (Model 3740; KUBOTA, Tokyo, Japan) and then stored at -80 ^{o}C for subsequent analyses. The serum alanine aminotransferase (ALT; GPT), aspartate aminotransferase (AST; GOT), triglyceride (TG), and cholesterol (TC) levels were assayed by using an ARKRAY SPOTCHEM SP-4410 automatic dry chemistry analyzer (ARKRAY, Kyoto, Japan) with respective kits.

3.5 Preparation of liver homogenate

A 0.5 g amount of liver was homogenized on ice in 4.5 mL of phosphate buffer saline (PBS, pH 7.0, containing 0.25 M sucrose) and centrifuged at 12,000 x g for 30 min (Model 3740; KUBOTA, Tokyo, Japan). The supernatant was collected for further analyses. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (catalog #500-0006; Bio-Rad Laboratories, Inc., Hercules, CA, USA) using bovine serine albumin as a standard.

3.6 Liver lipid analyses

Liver triglyceride and cholesterol concentrations were measured according to the procedures (Yang *et al.*, 2010). Briefly, liver lipid was extracted by chloroform and methanol (2:1, v/v). The extract was dried under N_2 and resuspended in

isopropanol via an ultrasonic cleaner (Model: DC150H, Taiwan Delta New Instrument, TW). Triglyceride (TAG), cholesterol (TC), and bile acid concentrations were measured by using commercial kits (Randox Laboratories Ltd., Lodon, United Kingdoms).

The former two commercial kits were both developed and based on the methods from Trinder (1969). The triglycerides of samples were hydrolysed into glycerols and fatty acids, and the glycerols may be phosphorylated to glycerol-3-phosphate via glycerol kinase, consequently, forming the dihydroxyacetone, phosphate, and H_2O_2 by glycerol-3-phosphate oxidase. Last, the end products transformed into light-pink-targets, quinoneimine, by peroxidase. Reading the O.D. values under 500nm wavelength and calculating by following formula:

The triglycerides of sample $\left({}^{mg}/_{dL}\right)$

$$= \frac{O.D.value_{500nm}\ of\ sample}{O.D.value_{500nm}\ of\ standard} \times the\ concentration\ of\ standard$$

The method of cholesterol analysis was quite similar. Cholesterol esters of sample were hydrolysed into cholesterol and fatty acids via cholesterol esterase, oxidizing into cholestene-3-one and H_2O_2 by cholesterol oxidase. Similarily, the end products and additive 4-aminoantipyrine reacted and formed the quinoneimine. After all, reading the O.D. values under 500nm wavelength and calculating by following formula:

The cholesterol of sample $\left({}^{mg}/_{dL}\right)$

$$= \frac{O.D.value_{500nm}\ of\ sample}{O.D.value_{500nm}\ of\ standard} \times the\ concentration\ of\ standard$$

3.7 Antioxidative capability analyses

3.7.1 Thiobarbituric acid reactive substances (TBARS)

MDA, by-product of lipid peroxidation, which binds to thiobarbituric acid (TBA, J. T. Baker; Malinckrodt Baker, Inc., Philipburg, NJ, USA) undergoes color reaction to pink. Both MDA and GSH content were performed according to procedures by Yang *et al*. (2009a). Liver homogenate or PBS for blank was added 1 mL into pre-mix solution that contained 1.5 mL TBA and 8.5 mL Trichloroacetic acid-hydeoxyl chloride reagent (TCA-HCL; Sigma-Aldrich, St. Louis, MO, USA). The samples were heated in 95 °C water-bath for 30 min, cooling on ice for 10 min, centrifuging in 4℃ 9170 x g for 5 min (Model 3740; KUBOTA, Tokyo, Japan), last, the supernatant will measure under 535 nm wavelength.

$$\text{TBARS of liver tissue}\left(nmole\ MDA/_{mg\ protein}\right) = \text{sample}\ O.D._{535nm} \times \frac{705.15}{protein\ Con.of\ liver\ tissue}$$

3.7.2 Glutathione (GSH)

Basically, the cysteine among GSH binds to 5.5'-dithiobis (2-nitrobenzonic acid, DTNB; Sigma-Aldrich, St. Louis, MO, USA) and undergoes color reaction into yellow (thio-2-nitrobenzonic acid). Before the analysis, 0.5 mL 10% tissue sample was added into 0.5mL 10% Trichloroacetic acid (TCA; Sigma-Aldrich, St. Louis, MO, USA), mixing, reacting for 5min at room temperature and centrifugated (2290 x g, 10 min, 4 °C; Model 3740; KUBOTA, Tokyo, Japan). Then the 50 μL supernatant was added into the tube with 1mL Tris base (Apolo,

TW) - EDTA (ethylenediaminetetraactic acid, J. T. Baker; Malinckrodt Baker, Inc., Philipburg, NJ, USA) buffer (20mM) and read for value A1 under 412 nm wavelength. The 20 μL DTNB was added into the former interacting buffer, and incubating for 5 min. Last, the optical density value of each well was measured by the Synergy H1 hybrid reader (Bio-Tech Instruments, Inc., Wooski, VT, USA) as value A2. Importantly, the values were calibrated with the blank (value B), whose sample was replaced by water in final reacting buffer, due to the background color of DTNB.

$$\text{GSH content of liver}\left({}^{nmole}/_{mg\ protein}\right) = \frac{(A_2 - A_1 - B) \times 15.7 \times 20 \times 10^3}{protein\ Con.of\ liver\ tissue}$$

3.7.3 Trolox equivalent antioxidant capacity (TEAC)

The TEAC value was analyzed according to a method described by Hung *et al*. (2006). A free radical, $ABTS^+$, can be generated by mixing ABTS (100 μM; Sigma-Aldrich, St. Louis, MO, USA) with H_2O_2 (50 μM; Sigma-Aldrich, St. Louis, MO, USA) and peroxidase (4.4 U/mL; Sigma-Aldrich, St. Louis, MO, USA). The TEAC value was expressed as a scavenging capacity against $ABTS^+$. Briefly, a 0.25mL mixture of ABTS, H_2O_2, peroxidase, and 1.5 mL of dd H_2O were mixed well, consequently, it was placed under a dark room. After 30 min, 0.25 mL of diluted liver homogenate (1%, v/v) was then added. The absorbance was measured at 734 nm wavelength after the interaction of sample solution for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. A standard curve was plotted for Trolox on scavenging $ABTS^+$ capacity and was used for calculation for TEAC of samples. The higher TEAC value of a sample results in the stronger antioxidant activity.

3.7.4 Superoxide dismutase (SOD)

The SOD was measured by the inhibitory effect of SOD on pyrogallol autoxidation (Mueller *et al.*, 2009). Under the condition of pH 7, pyrogallol undergone auto-oxidation and formed the anion free radicals, purporalin, which turned into yellow. Therefore, the tissue origin SOD could retard the pyrogallol auto-oxidation and reduce the formation of purporgalin.

Briefly, 100 μL of 10% liver homogenate was mixed well with 650 μL of PBS (pH 7.0; Bio-kit, Inc., Maoli, Taiwan). After centrifugation at 6000 x g for 10 min under 4 ^{o}C (Model 3740; KUBOTA, Tokyo, Japan), 10 μL of supernatant was mixed with 3 mL of Tri-HCl buffer (50mM, pH 8.2; APOLO, Taipei, Taiwan) and 15 μL of pyrogallol (0.2 mM; Riedel-de Haën; Sigma-Aldrich, St. Louis, MO, USA). The absorbance change caused by the formation of the yellow pyrogallol oxidation product, purpurogallin, was recorded at 420 nm wavelength in 3 min. One unit of SOD activity was defined as the amount of enzyme that inhibited the autoxidation of pyrogallol by 50%. The hepatic SOD activity was expressed by unit/mg protein.

$$\text{Activity of SOD}\left({}^{unit}/_{mg\ protein}\right) = \frac{(standard\ O.D._{\cdot 60s} - standard\ O.D._{\cdot initail}) - (sample\ O.D._{\cdot 60s} - smaple\ O.D._{\cdot initial})}{(standard\ O.D._{\cdot 60s} - standard\ O.D._{\cdot initail}) \times 50\% \times protein\ Con.of\ liver\ tissue}$$

3.7.5 Catalase (CAT)

The CAT activity was performed according to the procedure as described by Hong and Lee (2009) with a slight modification. Briefly, 450 μL of liver homogenate was mixed well with 50 μL of triton X-100 (10%v/v; AMRESCO,

Solon, OH, USA). After centrifugation at 6000 x g for 10 min under 4 °C, a mixture of 10 μL of supernatant and 990 μL of PBS (pH 7.0; Bio-kit, Inc., Maoli, Taiwan) was reacted with 0.5 mL of H_2O_2 (30 mM; Sigma-Aldrich, St. Louis, MO, USA). The difference in absorbance between initial and 3 min incubation was measured at 240 nm wavelength. The hepatic CAT activity was calculated by taking the extinction coefficient of H_2O_2 to be 0.0395 $mM^{-1}cm^{-1}$. One unit of CAT was expressed as the amount of enzyme that decomposes 1 μmole H_2O_2 per min at 25 °C. The hepatic CAT activity was expressed by unit/mg protein.

$$\text{Activity of CAT}\left({}^{unit}/_{mg\ protein}\right) = \frac{\left\{\left[\left(sample\ O.D._{\cdot initial} - smaple\ O.D._{\cdot 60s}\right)/_{0.0395\ (mM^{-1}cm^{-1})\times 1\ (cm)}\right]\times 0.75\ (mL)\right\}/1(min)}{0.75\ (mL)\times protein\ Con.of\ liver\ tissue\ (mg/mL)}$$

3.7.6 Glutathione peroxidase (GPx)

The GPx activity was measured according to the procedure as described by Hong and Lee (2009) with a slight modification. Briefly, 2 μL of liver homogenate was mixed with 988 μL of reaction solution containing 100 mM PBS (pH 7.0; Bio-kit, Inc., Maoli, Taiwan), 10 mM EDTA (J. T. Baker; Malinckrodt Baker, Inc., Philipburg, NJ, USA), 10 mM NaN_3 (Sigma-Aldrich, St. Louis, MO, USA), 2 mM NAPDH (Sigma-Aldrich, St. Louis, MO, USA), 10 mM GSH (L- Glutathione reduced; Sigma-Aldrich, St. Louis, MO, USA), and 1 U/mL GSH reductase (from Bakers yeast; Sigma-Aldrich, St. Louis, MO, USA). After 3 min, 100 μL of H_2O_2 (2.5 mM; Sigma-Aldrich, St. Louis, MO, USA) was then added. The difference of absorbance between 0 and 3 min was measured at 340 nm wavelength.

$$\text{Activity of GPx}\left(nmole\ NADPH/_{min \times mg\ protein}\right)$$

$$= \frac{(sample\ O.D._{\cdot 60s} - smaple\ O.D._{initial}) - (standard\ O.D._{\cdot 60s} - standard\ O.D._{initial})}{622 \times 10^6 \times 2 \times protein\ Con.of\ liver\ tissue}$$

3.8 Inflammatory cytokine analyses

Hepatic tumor necrosis factor-α (TNF-α) (Quantikine Rat TNF-α immunoassay, CAT: RTA00) and interleukin-1β (IL-1β) (Quantikine Rat IL-1β/IL-1F2 immunoassay, CAT: RLB00) content were measured by sandwich enzyme-linked immunosorbent assay (ELISA) and the procedures were followed according to the commercial manufacturer's instructions (R&D system, Inc., Minneapolis, MN, USA).

First, 10% liver homogenous, control buffer, washing buffer, standard solution, and substrate solution were prepared before the experiments. 50μL of assay diluent (TNF-α: RD-1-41; IL-1β: RD1-21) were added into 96-well plate, then 50μL standard and samples were added into plate and incubated for 2 hours at room temperature. Then the plate was washed by washing buffer and then conjugate was added 100 μL into plate which next incubated for 2 hours at room temperature, and repeated the washing processing. Finally, 100μL substrate solution was added into plate then incubated for 30 min at room temperature in the dark room, and then 100μL of stop solution was added into each well. After 30 min reaction, immediately, the optical density value of each well was measured in an ELISA reader. Hepatic TNF-α and IL-1β levels were both expressed by pg/mg protein.

3.9 Histopathological sections and staining

For a histopathological study, liver tissues were placed in formalin

(Sigma-Aldrich, St. Louis, MO, USA) up to 24 hours, and all samples were fixed in a new neutral-buffered formalin solution again. Consequently, they were dehydrated in graded alcohol (ECHO chemical, Taipei, Taiwan), cleared in xylene (Merck Millipore, Darmstadf, Germany), and embedded in paraffin wax (Leica Microsystems, Singapore) by embedding plate (Electron Microscopy Sciences, Hatfield, USA) and plastic ware (Simport, Beloeil, Canada) under the Hybridization Oven (RH-800; YIH DER, Taipei, Taiwan) and Digital Dry Bath h Incubator (Genepure Technology, Taipei, Taiwan). These blockers were later sectioned using a HM315R microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Moreover, the paraffin-embedded sections were put in water bath (WB100, Taiwan) for spreading out, and dried by electronic heating plate (HP1200 , Taiwan). All sections were dehydrated in graded alcohol, embedded in paraffin section, and stained with hematoxylin (Merck Millipore, Darmstadf, Germany) and eosin (Merck Millipore, Darmstadf, Germany). The Masson's trichrome method is a specific staining for collagen. The commercial kit was purchased from Sigma-Aldrich Co. (Product No. HT15). All the fixed, dehydrated, embedded and hematoxylin staining processing is same to the preceding paragraph, but the sections were stained with Biebrich-scarlet-acid fuchsin solution, hosphomolybdic- phosphotungstic acid solution, aniline blue solution and acetic acid solution.

The values of fibrosis and inflammation were given by trained observers in double blind assay. The former one was following the METAVIR fibrosis staging system (Figure III-2) that was usually for clinical diagnosis. The samples were categorized into 5 levels depend on the severity. Finally, the different scores were given to each group for analysis. Whereas the later one followed the general international standards, histology activity index score (HAI score; Table II-1). The

activity of inflammatory level was valuing according to area condition such as portal inflammation, intralobular degeneration and periportal-bridging necrosis.

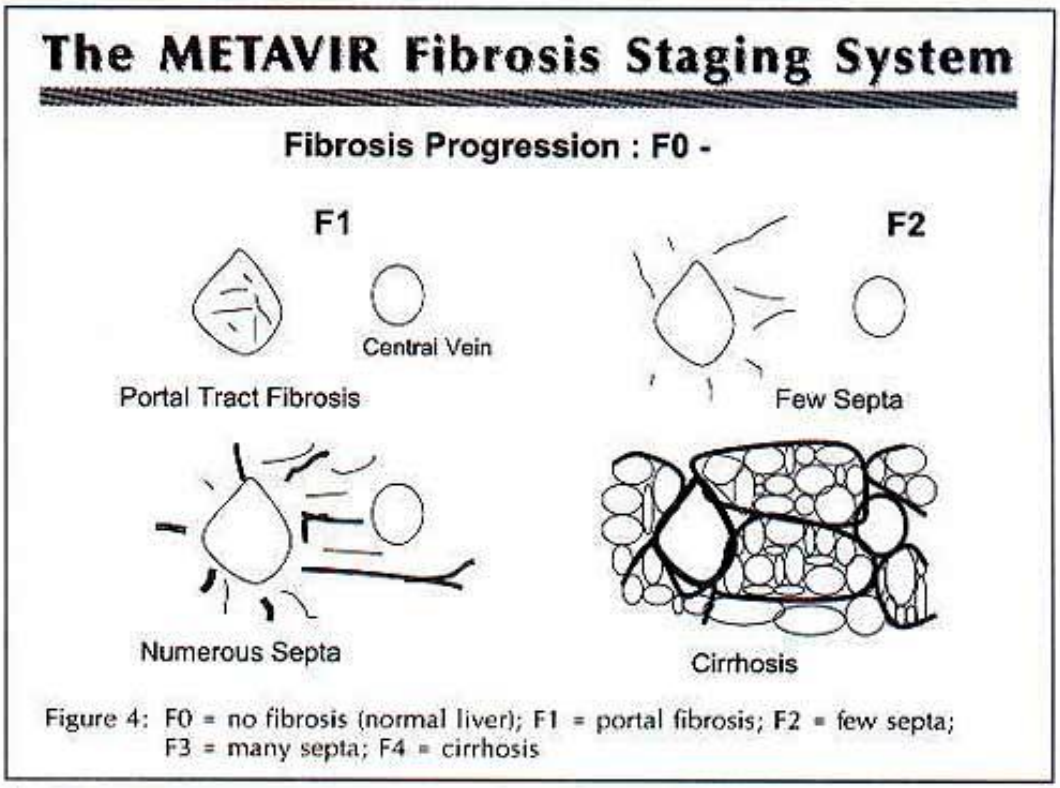
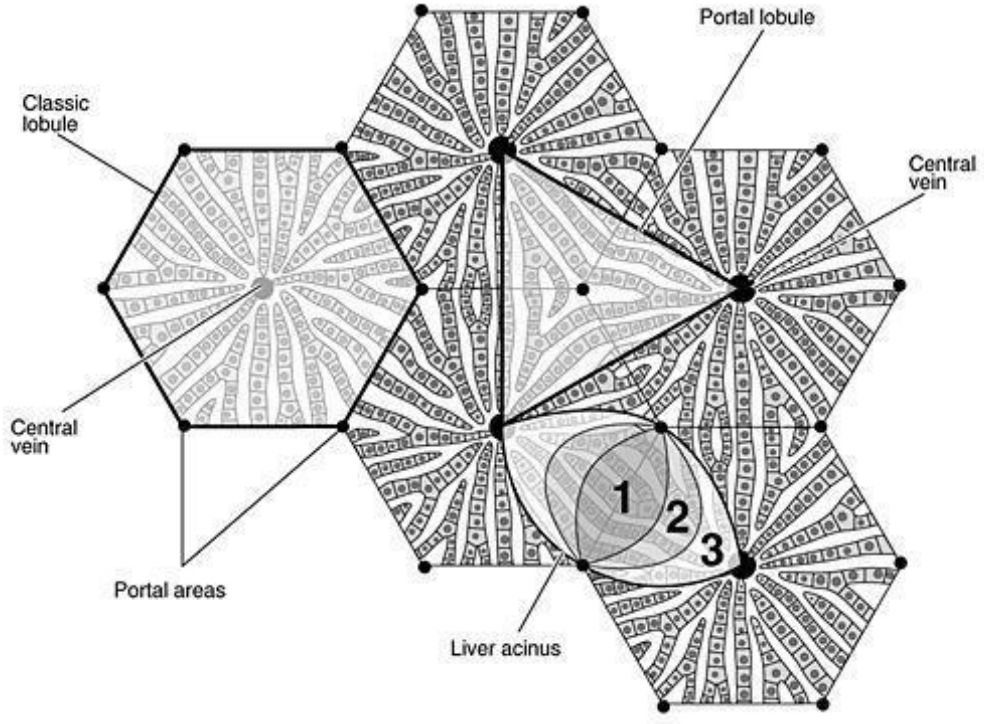


Figure III-2. The criteria of METAVIR fibrosis staging system (Brunt, 2000)

Table III-1. International histology activity index (HAI) score

EVENT	OBSERVED DETAIL	SCORE
Portal inflammation	No inflammation	0
	Slight inflammation (less than 1/3)	1
	Middle inflammation (about 1/3 to 2/3)	3
	Sever inflammation (more than 2/3)	4
Intralobular inflammation	No inflammation	0
	Slight inflammation (less than 1/3)	1
	Middle inflammation (about 1/3 to 2/3)	3
	Sever inflammation (more than 2/3)	4
Periportal degeneration	No periportal degeneration	0
	Slight focal necrosis (less than 10%)	1
	Middle focal necrosis (less than 50%)	3
	Sever focal necrosis (more than 50%)	4
	Middle focal and bridging necrosis	5
	Sever focal and bridging necrosis	6
	Multi-lobe necrosis	10

(Brunt, 2000)



3.10 Collagen assay

The collagen assay was performed according to the procedure as described by Reddy and Enwemeka (1996) with a slight modification. The concentration of hydroxypeoline (Sigma-Aldrich, St. Louis, MO, USA) is representative value for collagen because every 7.46 g collagen contains 1 g hydroxyproline.

Before analysis, there were three essential buffers required, and all the chemicals in this analysis were purchased from Sigma-Aldrich, Inc. First, the acetate-citrate buffer (pH=6.5) which contains 120 g sodium acetate trihydrate, 46 g citric acid, 12 mL acetic acid, and 34 g sodium hydroxide in 1 L deionized water. Second, Chloramine-T (0.056 M) contains 1.27 g chloramine-T, 20 mL 50% N-propanol in 1 L deionized water. The last, Ehrlich's reagent required 15 g dimethylaminobenzaldehyde in 100 mL solvent that was consisted of N-propanol and perchloric acid (2:1 in volume).

Briefly, the serial dilution of hydroxyproline stock was added into 96-well plate as standard, and the sample were added into plate too, but they were required to be autoclaved at 120 ^{o}C for 20 min before use. First, hydroxyproline was oxidized into pyrrole by chloramine-T, and reacted with Erlich's reagent for color reaction.

$$\text{Con. of tissue collagen}\left({}^{g}/_{g\ liver}\right) = \text{Con. of hydroxyproline} \times 7.46$$

3.11 Real-time polymerase chain reaction (Realtime-PCR)

After sacrificing the experimental mice, 0.1 g liver tissue in 0.5 mL RNA later (Qiagen, Valencia, CA, USA) at room temperature overnight, and then stored at -80 ^{o}C until analysis. A commercial kit (E.Z.N.Z^{TM} Tissue RNA kit, Omega Bio-Tek, Norcross, GA, USA) was applied to extract the mRNA from liver tissues, and the cDNA was synthesized from mRNA by the reverse transcriptional reaction according to the manufacturer's protocol offered by ImProm-II^{TM} Reverse Transcriptase (Promega, Medison, WI, USA). According to the principle of quantitative real time PCR, all the primers (Table III-2) were designed for the specific gene sequences in order to amplify the specific cDNA fragments. Two μL cDNA (50 ng/μL), 5 μL SYBR Green (Fast SYBR® Green Master Mix, Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 μL RNase free water, and 1 μL primer were mixed together. After a centrifugation (KUBOTA8700, KUBOTA, Tokyo, Japan; Rotor RS-3000/6 053-5930), the remnant was spun down by 1350 x g for 3 min. the fluorescence signals of each target gene were detected by using the StepOne Real time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAPDH was used as an internal control, and the values of all genes in TAA, OSML, and OSMH rats were expressed relatively to the average value of Control rats, which was set to 1.0.

Table III-2. Primer sequences and PCR product size for quantitative real time PCR

Gene	Size	Accession number	Primer sequence (5'-3')
Gapdh	264	NM_017008.4	F: GACCCCTTCATTGACCTCAAC R: GGAGATGATGACCCTTTTGGC
Tgfb1	86	NM_021578.2	F: CCTGAAAGGGCTCAACACC R: CAGTTCTTCTCTGTGGAGCTGA
Tgfbr1	159	NM_012775.2	F: TCCAAACCACAGAGTAGGCAC R: TGGATTCCGCCAATGGAACA
Tgfbr2	78	NM_031132.3	F: CCGTGTGGAGGAAGAACGAC R: TGAAGCCGTGGTAGGTGAAC
Tlr4	165	NM_019178.1	F: CTACCTCGAGTGGGAGGACA R: TGGGTTTTAGGCGCAGAGTT
Myd88	118	NM_198130.1	F: CTCGCAGTTTGTTGGATGCC R: TCGATGCGGTCCTTCAGTTC
Trif/Rnf138	95	NM_053588.2	F: CCTATGTCGCGGAAGTGTGA R: TGCAGCTACCAGAAACCCTC
Akt	178	NM_033230.2	F: GAAGTCTGAGCCCACCTTTCA R: CCACTGGCTGAGTAGGAGAAC
Nfkb1	145	NM_001276711.1	F: TGGGGCCTGCAAAGGTTATC R: TTTGCAAAGCCAACCACCAT
Cox2	94	NM_017232.3	F: TGTATGCTACCATCTGGCT R: GTTTGGAACAGTCGCTCGTCATC
Col1a1	102	NM_053304.1	F: GACTGTCCCAACCCCCAAAA R: CTTGGGTCCCTCGACTCCTA
Acta2/αSMA	200	NM_031004.2	F: TTCGTTACTACTGCTGAGCGTGAGA R: AAAGATGGCTGGAAGAGGGTC

3.12 Immunohistochemical analysis (IHC)

The commercial kit of immunohistochemistry assay was purchase from VECTOR Laboratories (Mouse IgG, PK-6102; Rabbit IgG, PK-6101). The procedure was according to the instructions manual.

Before the immunochemical reaction, all sections should be deparaffinized and hydrated through xylene, graded alcohol, and tap water. Consequently, antigen retrieval was necessary step for antigen presenting, all the sections were sunk into sodium-citrate buffer which contain 2.581 g citric acid (Sigma-Aldrich, St. Louis, MO, USA) and 500 μL Tween-20 (Chumeia chemical Inc.) in 1 L deionized water; the sections were heated by microwave for 10 min three times. After cooling, blocking procedure were taken via biotin/avidin blocking kit (SP-2001; VECTOR Laboratories, Inc., Burlingame, CA, USA) and H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA)/methanol (Sigma-Aldrich, St. Louis, MO, USA) buffer 30 min each step, which would eliminate the endogenous noise from peroxidase, biotin, and avidin. Finally, blocking buffer which contained the animal serum was added upon the tissue area for 20 min. PBS was added to wash the sections 5 min four times between the different buffers.

Sections were incubated with primary antibody (Table III-3) that diluted to optimal concentration, and they were washed and added the diluted biotinylated secondary antibody solution for optimal reacting time. Next, all samples were incubated with VECTASTAIN® Elite ABC Reagent for 30 min each buffer. Last, slides were hybridized with DAB peroxidase substrate (Cat. No. SK-4100, VECTOR Laboratory, CA, USA) until desired stain intensity develops. After all, we rinsed sections in tap water, and it gone to counterstain, clean, and mount. When the sections were completely dry, the Axioskop microscope (ZEISS, Oberkochen,

Germany) was used to observe the tissue section. The microphotography was taken through commercial program, AxioVision Rel. 4.8, from ZEISS.

Table III-3. Product sheets of primary antibody for IHC

Primary Antibody	Cat. number	Company
NFkappaB/p65	PA5-16545	Thermo Fisher Scientific, Inc., Waltham, MA, USA
Anti-Actin, Smooth muscle, clone ASM-1	CBL171	Merck Millipore, Darmstadf, Germany

IV. RESULTS

4.1 Effects of OS mycelium on growth performance of rats

The growth performance is an important factor of liver injury, and the body weight is the representative value for metabolic efficiency. At the end of 8-weeks experiment, the body weight of control group was heavier ($p < 0.05$) than TAA-treated group (Figure IV-1), but there were not ($p > 0.05$) difference on body weight among TAA-treated groups (TAA, OSML, and OSMH). During the experimental period, the food intakes were not different ($p > 0.05$) (Table IV-1), but water intakes of TAA-treated groups (43.24~45.51 mL/rat/day) were less ($p < 0.05$) than that of Control group (54.29 mL/rat/day) (Table IV-1). Regarding to organ sizes, there was no ($p > 0.05$) different on heart size among groups, but liver and kidney sizes were increased ($p < 0.05$) in TAA-treated groups compared to those of Control group. The liver size of TAA group was 3093.84 mg/100g BW, but control group is only 2459.16 mg/100g BW (Table IV-1). It may be due to the hepatic hypertrophy and inflammation induced by TAA. As this observation, it was speculated that OS mycelium treatment could alleviate the TAA-induced damage for liver and kidney.

4.2 Effects of OS mycelium on serum and liver lipids

According to the data from National Laboratory Animal Center, Taiwan, the rat's TG and TC levels in this study coincide to their normal physiological ranges. At the end of experiment, while lower serum lipids (TG and TC) occurred in TAA-treated groups, OS mycelium supplementation increased ($p > 0.05$) serum lipids, especially TC which normalized to the level of Control groups (Figure IV-2;

C and D). In liver lipids, it showed opposite results to serum lipid results. The liver lipids of TAA-treated groups are higher ($p > 0.05$) than those of Control group, but OS mycelium supplementation reduced ($p < 0.05$) them (Table IV-1). These results imply that OS mycelium can reduce lipid accumulation in livers.

4.3 Effects of OS mycelium on antioxidative capacity of livers

The liver antioxidant capacities are shown on Table IV-2. It was reported that a raising intrahepatic oxidative stress easily results in the liver injuries (Kaplowitz, 2002). The MDA, a by-product of polyunsaturated fatty acid peroxidation, increased ($p < 0.05$) in TAA-treated groups (Table IV-2), but decreased ($p < 0.05$) when supplemented with OS mycelium (Table IV-2). Although there was no ($p > 0.05$) differences on liver GSH contents among groups, rats injected by TAA without OS mycelium had lower ($p < 0.05$) liver TEAC value than Control rats but OS mycelium supplementation normalized ($p < 0.05$) liver TEAC values to the Control group (Table IV-2). Similarly, the activities of liver antioxidative enzymes including SOD, CAT, and GPx were decreased ($p < 0.05$) by TAA treatment, but OS mycelium supplementation increased ($p < 0.05$) them (Table IV-2).

4.4 Effects of OS mycelium on liver damage

Serum AST (GOT) and ALT (GPT) values are often used as biochemical markers for liver injury. As expected, while TAA group had higher ($p < 0.05$) values than Control group, but OS mycelium supplementation decreased AST and ALT values ($p < 0.05$) (Figure IV-2; A and B). For another, inflammatory cytokine such as TNF-α and IL-1β were determined in this study. Based on the results, the TAA treatments indeed raised ($p < 0.05$) hepatic TNF-α and IL-1β values (Figure IV-3),

and OS mycelium supplementation decreased ($p < 0.05$) them (Table IV-3). Similarly, the hepatic collagen content was increased ($p < 0.05$) by TAA compared to that of Control group but decreased ($p < 0.05$) by OS mycelium (Table IV-3). Regarding to histopathological analyses, the visible signs of fibrosis and intrahepatotic inflammation were directly observable (Figure IV-3 &Figure IV-4). By microscopic images and double-blind experimental design the results show that OS mycelium supplementation alleviated ($p < 0.05$) the processing of liver damages (Figure IV-3 & Figure IV-4). In the microscopic image of trichrome staining, visible fibrotic scar and collagen is strong evident for fibrosis in TAA group, and those observations of TAA-treated groups were reduced ($p < 0.05$) by OS mycelium supplementation (Figure IV-3) which is corresponding to the results of hepatic collagen assay (Table IV-3). For H&E staining, the values determined with the size of monocyte- aggregated area (black arrow), the amount of cell under necrosis (black triangle), the morphology of portal area and intralobular area. The results were also similar as the results I Masson's trichrome staining (Figure IV-4). In Figure IV-5, arrow heads indicated αSMA positive areas. Apparently, TAA group had larger those areas than Control group. OSM supplementation significant reduced them wich is corresponding to the observation of Masson's trichrome (fibrotic scars in Figure IV-3).

4.5 Protective mechanism by OSM supplementation

According to the mRMA expressions of NFκB induction pathways, the TGF-β Figure IV-6) and TLR4 pathway (Figure IV-7), TAA treatment significantly induced ($p < 0.05$) TGF-β, TGFBR1, and TGFBR2, as well as TRIF and Akt, respectly, while TLR4 and MyD88 gene expressions were not influenced ($p > 0.05$).

Regarding to the fibrosis related gene expressions (Figure IV-8), αSMA, Col1α, NFκB, and COX2 were upregulated ($p < 0.05$) by TAA intoxification. Those changed patterns of gene expressions were downregulated ($p < 0.05$) by OSM supplementation, especially a high dosage of OSM. In the immunohistochemical analysis of NFκB protein (Figure IV-9), there were several NFκB positive cells (dash circles) in liver tissues of TAA treated groups. However, an inflammation was induced only by the entrance of NFκB subunit (p65) into nuclei (arrow head). In a comparison of NFκB p65 entrances, liver sections in TAA treated mice without OSM supplementation showed more positive nuclei than those with OSM supplementation.

V. DISCUSSION

Ljubuncic *et al*. (2005) reported that liver hepatic hypertrophy and lighter body weight are observed on TAA treated Sprague-Dawley rats where the results were also observed in our experiment (Figure IV-1 & Table IV-1). Meanwhile, it has also proclaimed that these symptoms are caused by toxic metabolite of TAA, which differs from high-fat diet animal model, non-alcohol fatty liver animal model and other inducing factors related with obesity (Yang *et al.*, 2009b). A recent report from our team also showed the lower body weight and larger liver size of mice treated with TAA but co-treatment of silymarin reversed these observations (Chen *et al.*, 2011). Based on our data, it is speculated that OS mycelium is potentially hepatoprotective. Otherwise, TAA often causes kidney damage with chronic liver damage both in human clinical and animal researches (Water *et al.*, 2005). The reason is that liver fibrogenesis forms the intrahepatic nodules which press the portal vein and cause portal hypertension during the pathological processing. The efficiency for filter and sodium re-absorprion was decreased in glomerulus that decreases the effective blood volume (Natarajan *et al.*, 2006; Hocher *et al.*, 2011), so it is reasonable to explain the increased kidney size and decreased water intake presented in this study (Table IV-1).

Synder *et al*. (2012) proclaimed the relation between serum albumin and muscle mass changes. According to our results, serum albumin levels were consistent among all treating groups (Table IV-2), and it may indicate that decreasing of body weights in this study did not comprise of muscle mass changes. Precisely, under same daily food intake, the body weights of TAA treating rats were decreased due to liver-related metabolic dysfunction, the lipid trended to accumulate in liver (Table IV-2) rather than circulation system (Figure IV-2). Although OSM supplementation could attenuate those levels significantly, the lipid homeostasis was not rebuilt completely.

In many pharmacological cases, there were not dose-dependent. According to the results of this study (Table V-1), it seems to be dose-independent. Although results of OSMH may not effective as those of OSML, the hepatoprotective effects of it still presented compared to TAA group. Incidentally, OS was classified to “mild” herbal materials by Chinese ancient records, it means there may not be acute toxicity.

Table V-1. The hepatoprotective effects in different treatments

	TAA-induced	Hepatoprotective effects	
		OSML	OSMH
Body weight	Decreased	-	-
Liver size	Increased	●	-
Albumin	No change	-	-
Liver TG, TC	Increased	●	●
Serum TG, TC	Decreased	●	●
Serum AST	Increased	●	●
Serum ALT	Increased	●●	●
TBARS	Increased	●	●
GSH	No change	-	-
TEAC	Decreased	●	●
SOD, CAT, GPx	Decreased	●	●
IL-1β & TNFα	Increased	●	●
HAI score	Increased	●	-
Fibrotic score	Increased	●●	●

Literatures reported that the metabolites of TAA can form a covalent binding to intracellular molecules such as membrane protein which causes nuclear stress, ER stress, mitochondria stress and oxidative stress (Pumford *et al.*, 1997; James *et al.*, 2003; Kaplowitz, 2004). Oxidative stress is defined as the exposures of the cell to an

excessive level of oxidants, free radicals, superoxide or hydroxyl radicals, which commonly refer to as reactive oxygen species (ROS). The major site of production of ROS is the mitochondria so the high oxidative stress affects many cellular functions and leads to severe adverse effects of target cells including lipid peroxidation and protein thiol oxidation (Dai *et al*., 2014). The consequences of these effects are as following: 1) The covalent binding leads to membrane destruction of hepatic cell, and the target cells will go under necrosis (Singh and Handa, 1995) (Figure IV-4B), and then AST and ALT will release into outer space and diffused into blood with extracellular liquid (Figure IV-2A & B); 2) Because of irregular protein thiol oxidation, Golgi structure and ER stress, the production and delivery efficiency of approtein B, and low-density lipoprotein (VLDL) are decreased (Sabesin *et al.*, 1977), and causes lower serum lipids but higher liver lipid accumulation (Table IV-1 & Figure IV-2); 3) The lipid peroxidation by-products, i.e. MDA increase, while the quantity and activity of antioxidants and relative enzymes, respectively, decrease under the oxidative stress state (Sanz *et al.*, 1995; Dai *et al.*, 2005) (Table IV-2); 4) The mitochondria is also an important site of the cell death (Kaplowitz and Tsukamoto, 1996; Czaja, 2002). While TAA induces the ROS productions, the apoptotic messengers are activated indirectly to lead to hepatic parenchymal cells going under mitochondria-dependent apoptosis pathway (intrinsic pathway).

The study on hepatoprotection of OSM against carbon tetrachloride indicated decreases of serum AST and ALT values; meanwhile, increased anti-oxidative capabilities by OSM supplementation were also observed (Ko *et al.*, 2010). Several previous studies had proclaimed *Cordyceps sinensis* can inhibit the formation of MDA, delay the peroxidation of VLDL and reduce the accumulation of esterfied cholesterol in microphage (Shin *et al.*, 2001; Tsai *et al.*, 2001; Wang *et al.*, 2004).

Although bioactive compounds are not the major concerns in this study, the OSM powder (TCM-808FB), which contains 10% polysaccharide and 0.25% adenosine (offered from TCM Biotech International Corp., Taipei, Taiwan). Adenosine was proved to inhibite the macrophage-origin cytokine production and reversed the immuno reaction (Hasko *et al*., 1996), which was similar to the hepatoprotective effect in results. Moreover, the previous research that hepatoprotection of polysaccharides extracted from *Lucium barbarum* against alcohol in rats was attributed to decreased serum lipid levels, oxidative stress, and liver damage (Cheng and Kong, 2011). Our previous studies also indicated that amelioration of noni juice on serum lipids, oxidative statuis, and inflammatory reaponeses in high-cholesterol/fat dietary hamsters or alcohol-treated mice is related to the phytochemicals in noni juice (Lin *et al*., 2012&2013; Chang *et al*., 2013). In addition, Cordycepin modulated the NFκB activation and the expression of apoptotic proteins (Jeong *et al*., 2010; Ren *et al*., 2012). Ko *et al*., (2010) indicated that OSM decreases of serum AST and ALT values in carbon tetrachloride treated rats; meanwhile, increased anti-oxidative capabilities were also observed. Several previous studies had proclaimed *Cordyceps sinensis* can inhibit the formation of MDA, delay the peroxidation of VLDL and reduce the accumulation of esterfied cholesterol in microphage (Shin *et al.*, 2001; Tsai *et al.*, 2001; Wang *et al.*, 2004).

The oxidative stress and cell death signals in direct damage are the trigger of many signal transductions such as fibrogenesis, immune response, inflammatory response etc. (Losser & Payen, 1996; Jaeschke *et al*., 2002). The ROS which stimulated by injury and inflammation will directly activate hepatic stellate cells (Robino *et al.*, 2000), and then upregulate the expressions of fibrosis related genes (Parola and Robino, 2001; Bataller and Brenner, 2005; Novo *et al.*, 2006). The active HSCs are known as initiator of liver fibrosis because they are main secretors of ECM such as collagen, and in this study the

scoring of Metavir was the same as the expectation (Figure IV-3). It has been semonstrated that the mitochondria damage and lipid peroxidation stimulate Kupffer cells or adjacent sinusoidal endothelial cells to secret cytokines, including TNF-α and IL-1β, the main cytokines in hepatotoxin related inflammation (Michie *et al.*, 1988; Holst *et al.*, 1996; Laskin and Laskin, 2001), which lead to sever inflammation (Shakibaei *et al.*, 2005) and apoptosis (Chou *et al.*, 1999; Meier and Silke, 2003) (Table IV-3), and the scoring of inflammation by HAI is also support the this estimation (Figure IV-4).

Crucial concern in this thesis is screening the molecular pathway for OSM-preventing effect of the liver fibrosis. As aforementioned, TGF-β relative pathway is classic fibrotic signaling cascade in various tissues, and it is proved to be necessary factor in HSCs activation (Saile *et al*., 2001; Liu *et al*., 2003). Although the activation mechanism of TGF-β may be tissue specific, but it is acceptable that pro- and activated-TGF-β molecule are released from ECM via immune cells, such as T-cell, Kuffer cell, etc. (Annes *et al*., 2003; Taylor, 2009). The OSM supplement could be effective to avoid the inflammation (Table IV-3; Figure IV-4), even though the type of immune cells are not identified respectively, so that it distrupts the downstream signal of TGF-β (Figure IV-6) and attenuates the activation of HSCs (Figure IV-3, Figure IV-5, &Figure IV-7). Yang *et al*. (2008) proclaimed that HSCs were activated by ROS and calcium flux which is induced by TGF-β signal cascade; meanwhile, OSM supplement also hold the antioxidative system obviously (Table IV-2) that could scavenge the ROS efficiently (Figure II-13), possibly, the modulation may be attributed to the phytosterol content (Wong *et al*., 2013).

NFκB, a key player in inflammatory pathway, was found an apoptotic modulator in liver (Sonenshein, 1997). Although, the general function had been revealed, the specific

function in liver fibrosis is uncertained due to the complicated cell composition in liver. In fibroblast, NFκB is an upstream suppressor of apoptotic signal from extracellular TGF-β through enhancing expression of Smad7 protein (Bitzer *et al*., 2000). In the activated HSCs, the dirving force of fibrogenesis, apoptotic signal from TGF-β and TNF-α also involves the downstream NFκB (Saile *et al*., 2001). In our data, the expression pattern of NFκB was changed in OSM supplement *in vivo* (Figure IV-8 & Figure IV-9), and the transcription pattern of downstream molecules (i.e., COX2, TNF-α, IL-1β) are also similar (Table IV-3 & Figure IV-8), but it need in vitro data to further clarify the critical role of NFκB in different hepatic cells. Incidentally, the NFκB-related immuno-modulated ability of cordycepin may share the above bioactivity (Jeong *et al*., 2010; Ren *et al*., 2012).

Overall, inflammatory signal is highly influence progressing of liver fibrosis through TGF-β pathway. Recently, many researches indicate that TLR4 signaling participates in chronic liver diseases including hepatic fibrosis (Soare *et al*., 2010; Guo *et al*., 2010). In addition, TLR4 signaling is upstream factor that affect the activation of NFκB, and Seki *et al*. (2007) proved it will mediate the regulation of TGF-β pseudoreceptor in HSCs, so it is reasonable to involve this targeting beneficial effect. In this thesis, although the receptor is not responsive to TAA-intoxification in mRNA level, the downstream signals (i.e., TRIF, Akt) are decreased slightly (Figure IV-7). In spite of lacking of confirmation in protein level, it is difficult to ignore the connection between these two vital pathways.

VI. Conclusion

According to the results from this study, the hepatoprotective effects of OSM against the TAA treatment are due to reducing hepatic lipid accumulation, increasing antioxidative capabilities, and decreasing hepatic cytokine secretion and collagen accumulation. All beneficial effect may involve the NFκB induction pathway including TGF-β and TLR4 related pathway. In addition, not only observation of physiological values but also investigation of antioxidant and anti-inflammatory/anti-fibrotic mechanisms on OS mycelium against TAA induced liver fibrosis were indicated (Figure VI-1). Furthermore, we sincerely hope that the research frame in this study could offer future workers an original thinking and strategy of hepatoprotective researches.

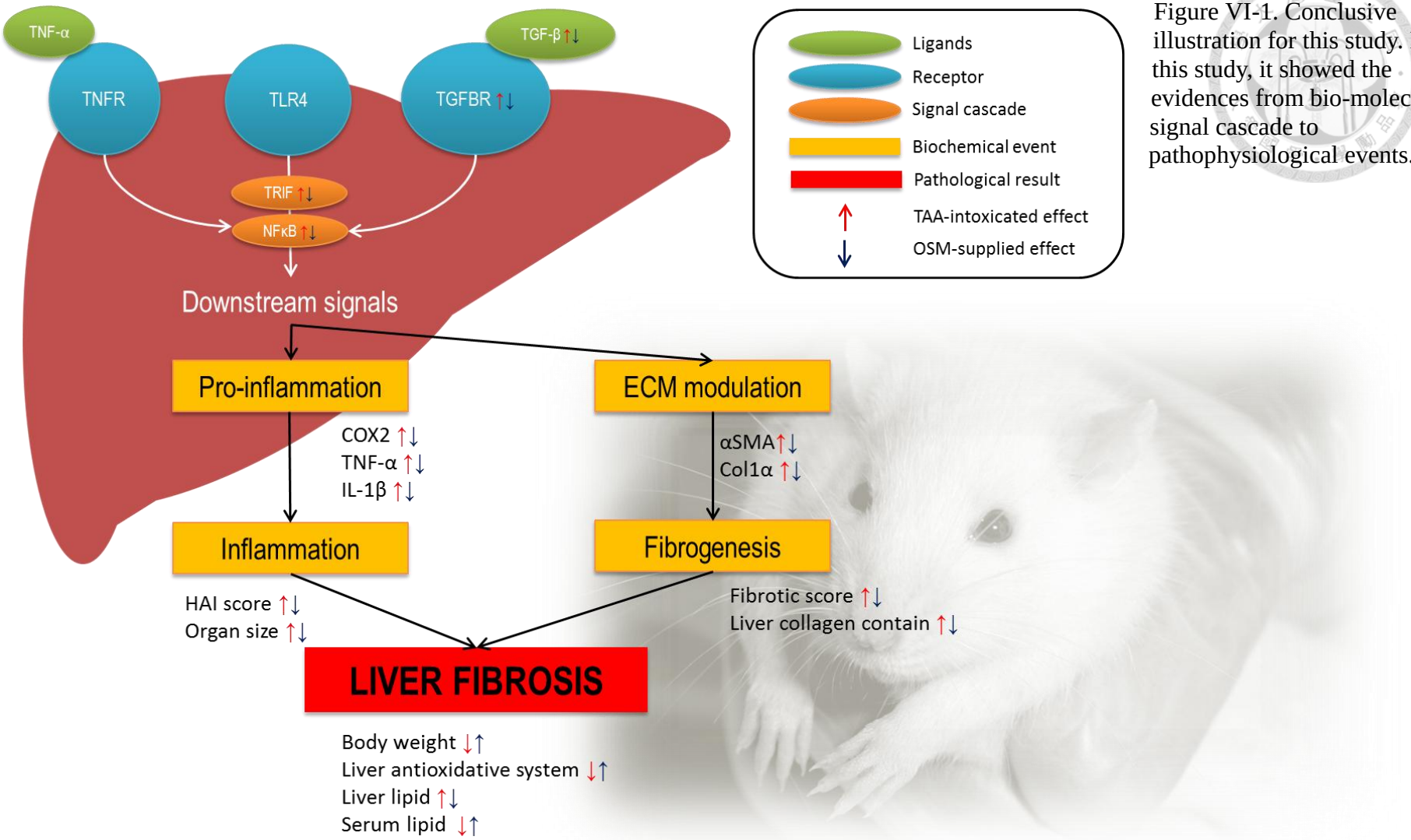


Figure VI-1. Conclusive illustration for this study. In this study, it showed the evidences from bio-molecular signal cascade to pathophysiological events.

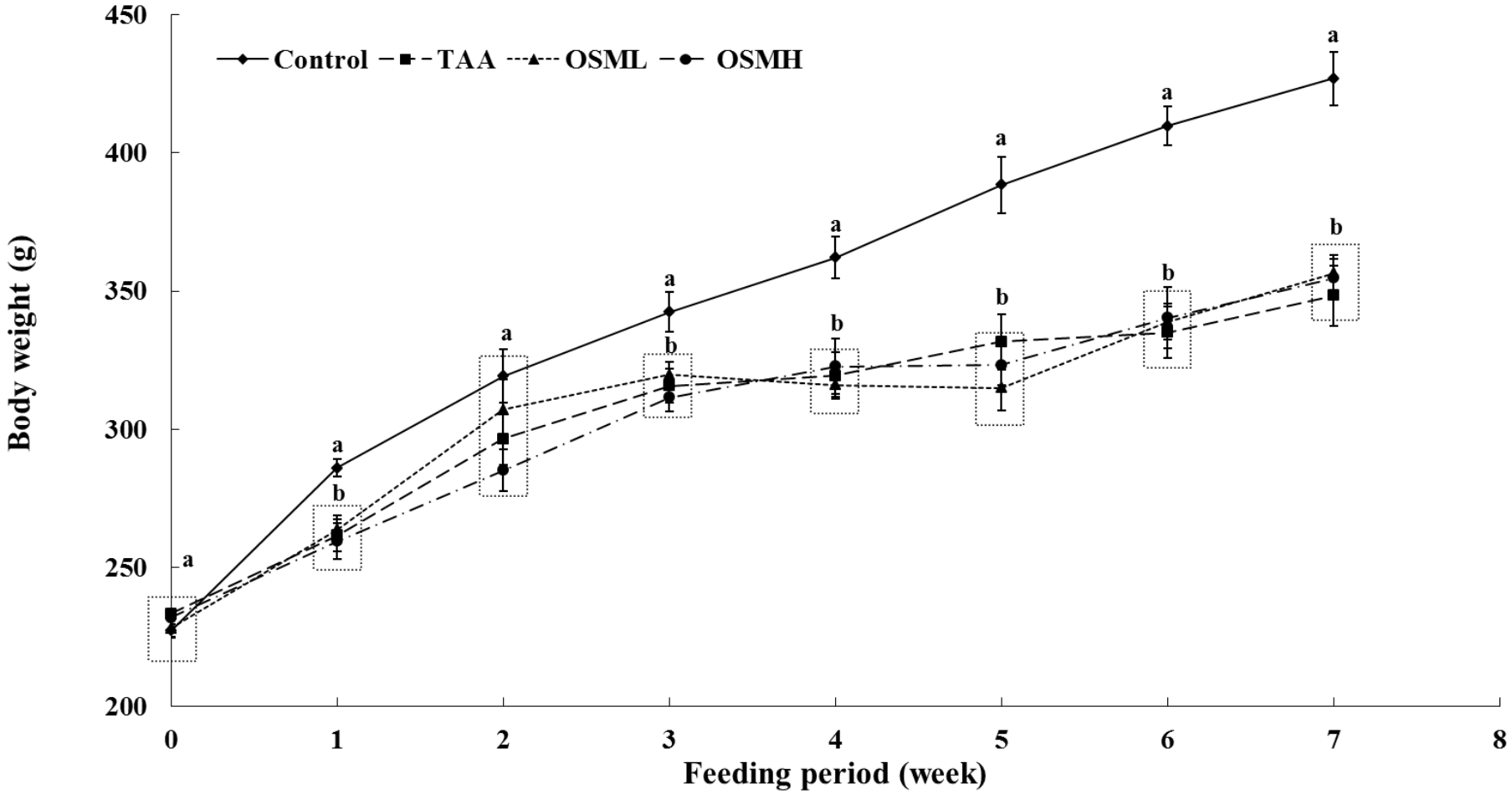


Figure IV-1. Body weight changes of experimental rats during the feeding period. The data are given as means + SEM (n=12). Different letters on data points in each feeding period indicate significant differences (p <0.05).

Table IV-1. Average food and water intakes, organ sizes, serum albumin and liver lipids

Parameter	Treatment			
	Control	TAA	OSML	OSMH
Food intake (g/rat/day)	26.77±0.69a	24.31±1.32a	24.81±0.73a	26.46±1.36a
Water intake (mL/rat/day)	54.29±1.94a	43.24±1.95b	45.51±1.57b	43.89±1.65b
Organ size (mg/100g BW)				
Heart	297.38±6.83a	311.47±9.80a	322.78±8.30a	316.82±9.66a
Liver	2459.16±61.59c	3093.84±76.10a	2855.06±48.00b	2914.79±107.01ab
Kidney	557.86±17.06c	654.01±19.27a	604.47±12.95bc	627.93±18.36ab
Serum albumin (g/dL)	4.00±0.10a	4.10±0.10a	4.10±0.10a	4.30±0.10a
Liver lipid (mg/g liver)				
Triglyceride	10.29±0.07b	11.56±0.29a	10.12±0.15b	10.06±0.28b
Cholesterol	2.44±0.07c	4.51±0.08a	2.76±0.11b	2.70±0.09b

* The data are given as means ± SEM (n=12). Mean values with different letters in each testing parameter were significantly different ($p< 0.05$).

Table IV-2. Antioxidative capacity and enzymatic activities of liver tissues

Parameter	Treatment			
	Control	TAA	OSML	OSMH
MDA (nmol/mg protein)	0.45±0.02c	2.38±0.04a	1.62±0.05b	1.63±0.06b
GSH (nmol/mg protein)	60.67±1.87a	60.55±1.62a	60.91±1.36a	63.44±1.37a
TEAC (nmol/mg protein)	100.18±3.11a	83.51±3.04b	108.87±3.66a	109.36±4.98a
SOD (unit/mg protein)	11.12±0.41b	4.35±0.09c	14.15±0.19a	10.64±0.26b
CAT (unit/mg protein)	19.72±0.39b	17.38±0.36c	20.83±0.43ab	20.97±0.38a
GPx (nmol NADPH oxidized/min/mg protein)	36.16±0.79a	23.54±0.54d	28.21±0.55c	30.71±0.59b

* The data are given as means ± SEM (n=12). Mean values with different letters in each testing parameter were significantly different ($p< 0.05$).

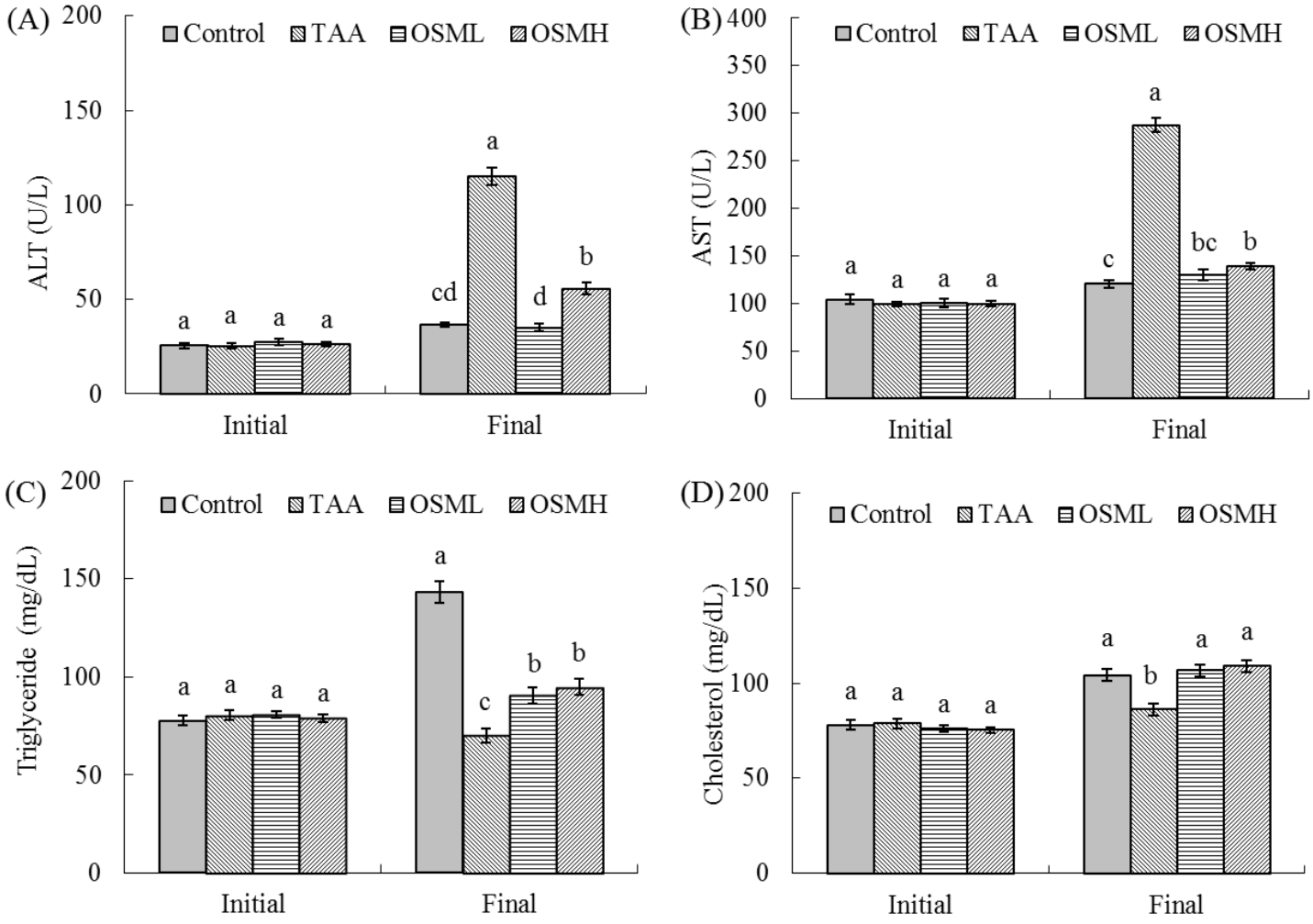
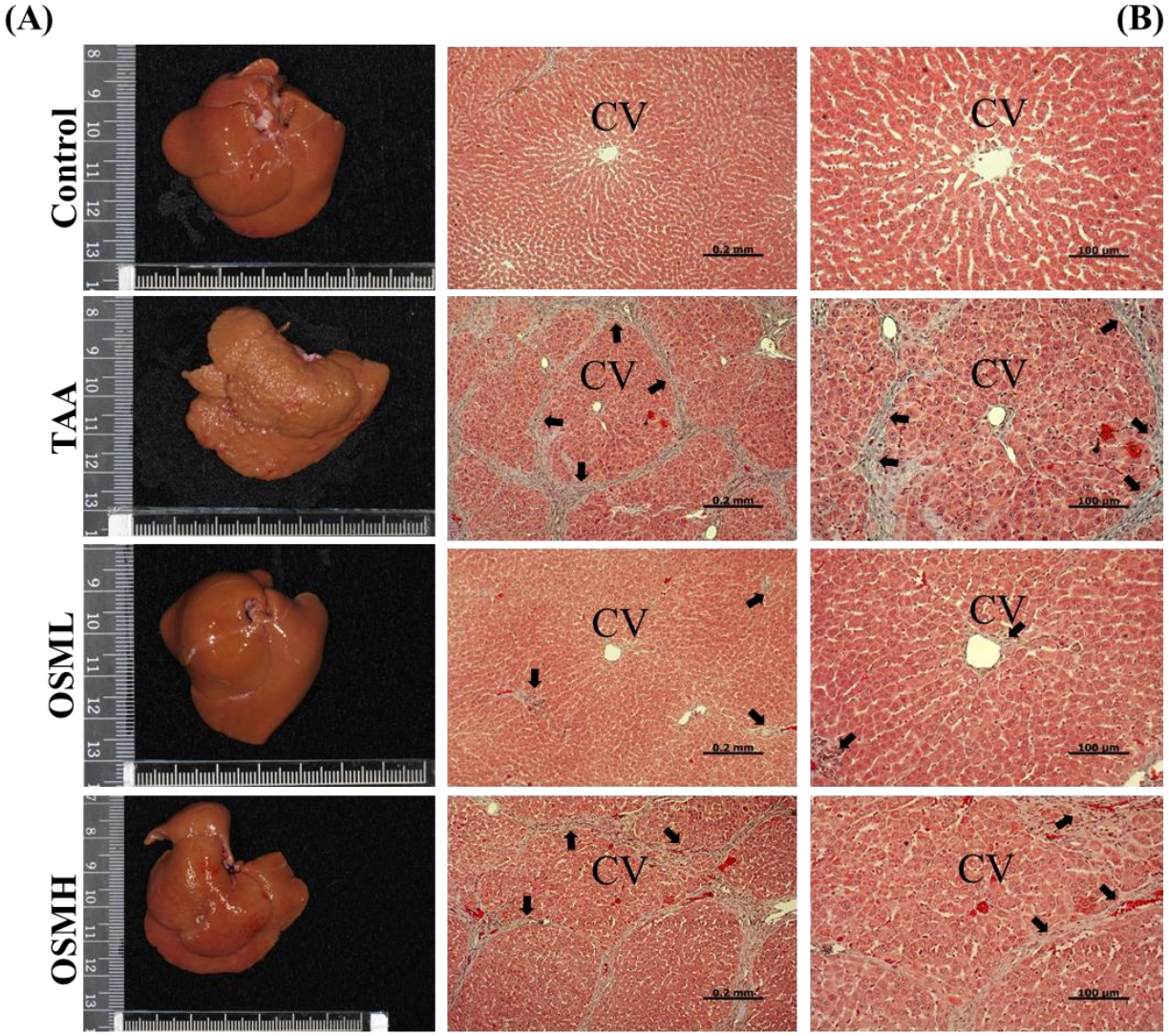


Figure IV-2. Serum triglyceride (A), cholesterol (B), AST (C), and ALT (D) levels of serum. The data are given as means ± SEM (n=12). Different letters on data points in each feeding period indicate significant differences ($p<0.05$).

Table IV-3. Liver collagen and cytokine contents

Parameter	Treatment			
	Control	TAA	OSML	OSMH
Cytokine (pg/mg protein)				
Interleukin-1β	3.28±0.12b	5.03±0.12a	3.68±0.20b	3.73±0.19b
Tumor necrosis factor-α	18.40±0.98c	26.00±0.85a	22.72±0.84b	22.02±0.71b
Liver collagen content (g/g liver)	0.29±0.14c	0.43±0.01a	0.36±0.01b	0.35±0.01b

* The data are given as means ± SEM (n=12). Mean values with different letters in each testing parameter were significantly different ($p< 0.05$).



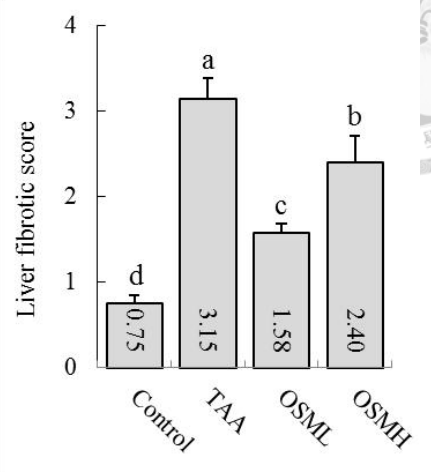


Figure IV-3. Masson's trichrome staining for liver tissues. (A) The gross appearance of representative liver in animals. The scale is shown in 200 (100X) and 100 (200X) μm. CV is abbreviated names of central vein, and the black arrows were toward to the collagen fibers. (B) The score of liver fibrosis. Values were expressed as means ± SEM.

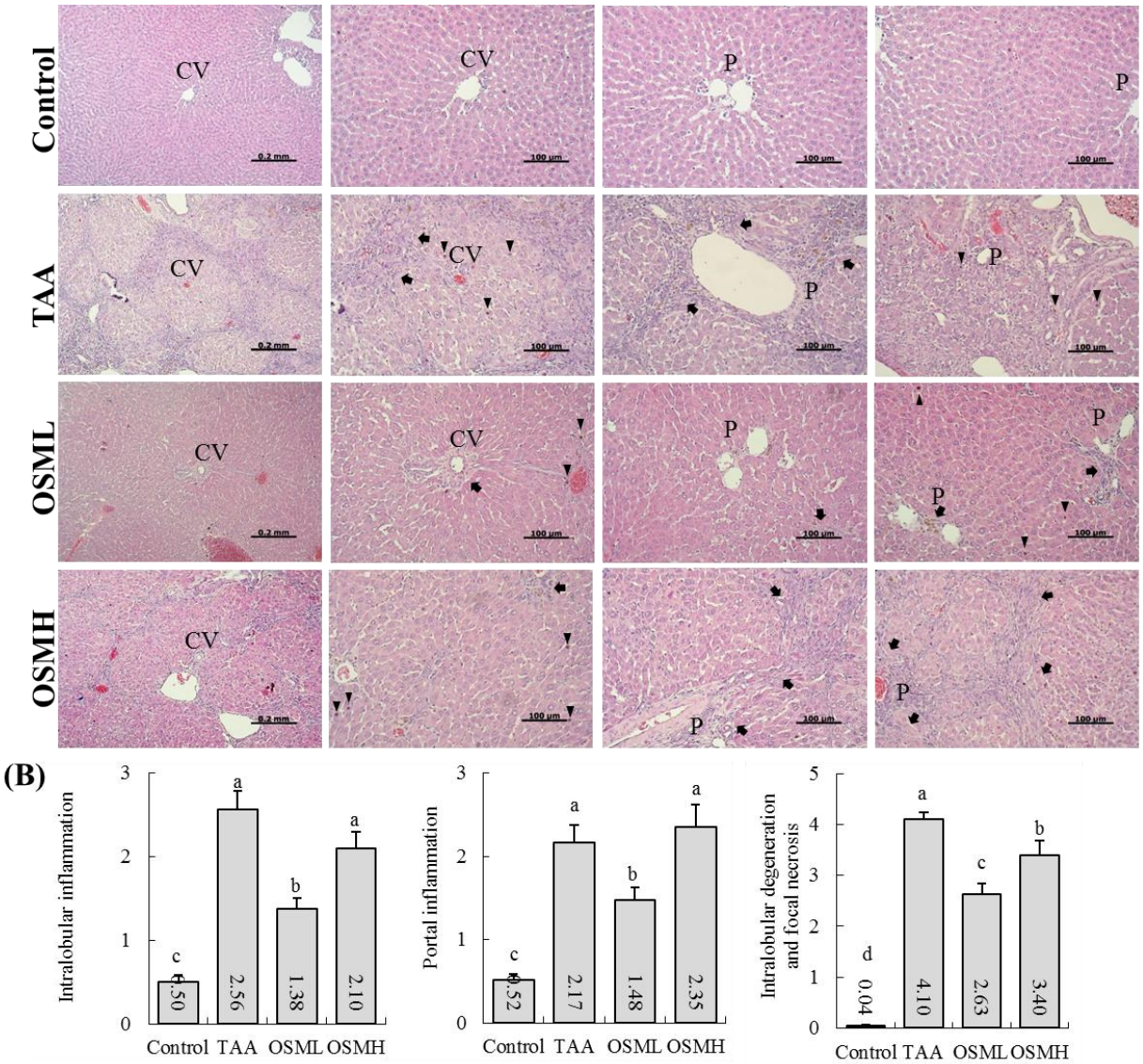


Figure IV-4. HE staining for liver tissues. (A) The scale is shown in 200 (100X) and 100 (200X) μm. CV and P are abbreviated names of central vein and portal tracts; the black arrows and triangles are toward to inflammation area and necrotic cells. (B) The score of HAI values were expressed as mean ± SEM.

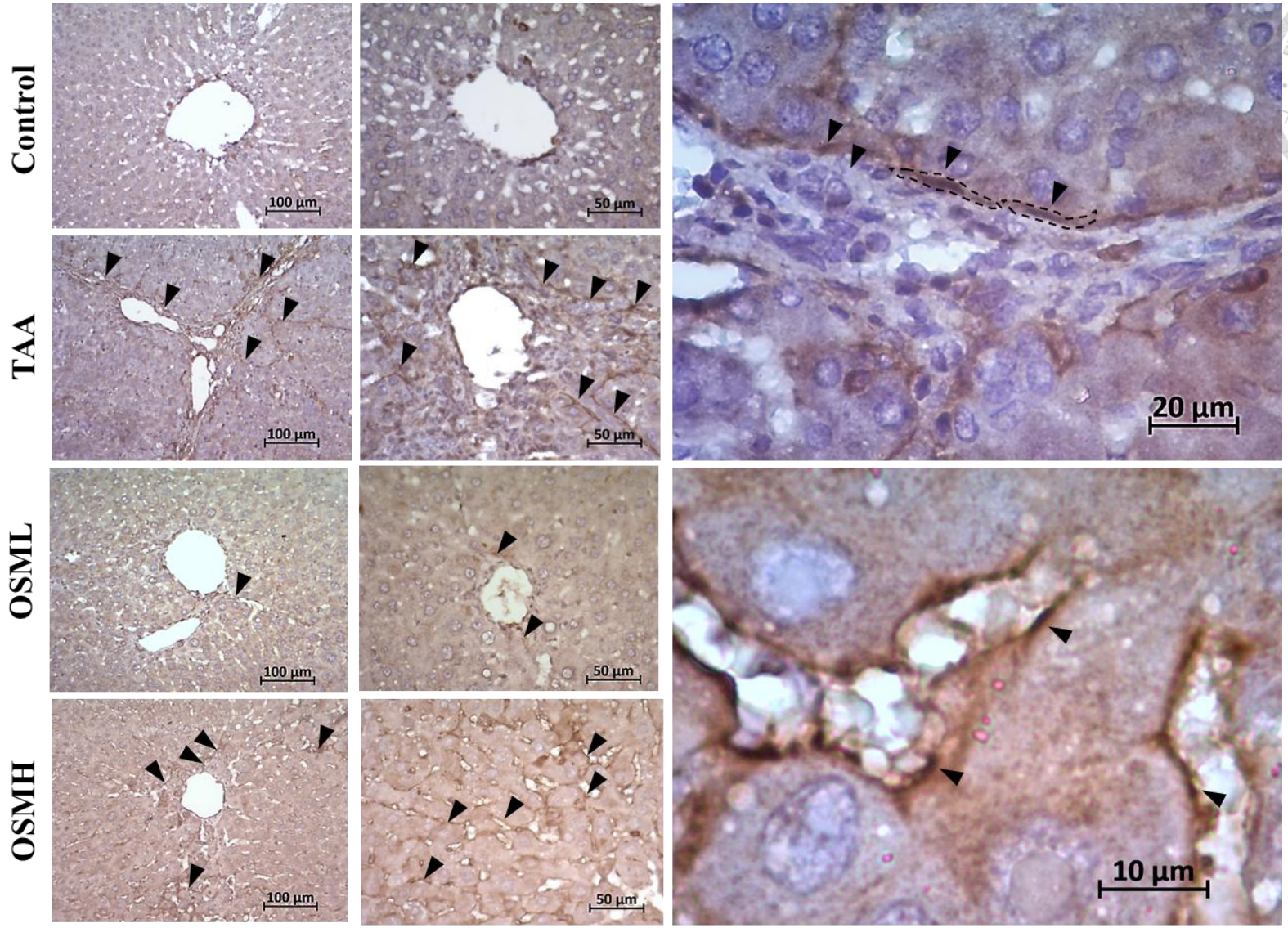


Figure IV-5. Immunochemical analysis: αSMA protein expression. The scale is shown in 100 (200X, first column in left hand) and 50 µm (400X, second column), and the right one demonstrated the expression area (arrowhead) and cell type (circle in dotted line; long spindle shape). Slide was stained with primary antibody and hematoxylin. The positive are in bown color, which may indicate to myofibroblast-like cells.

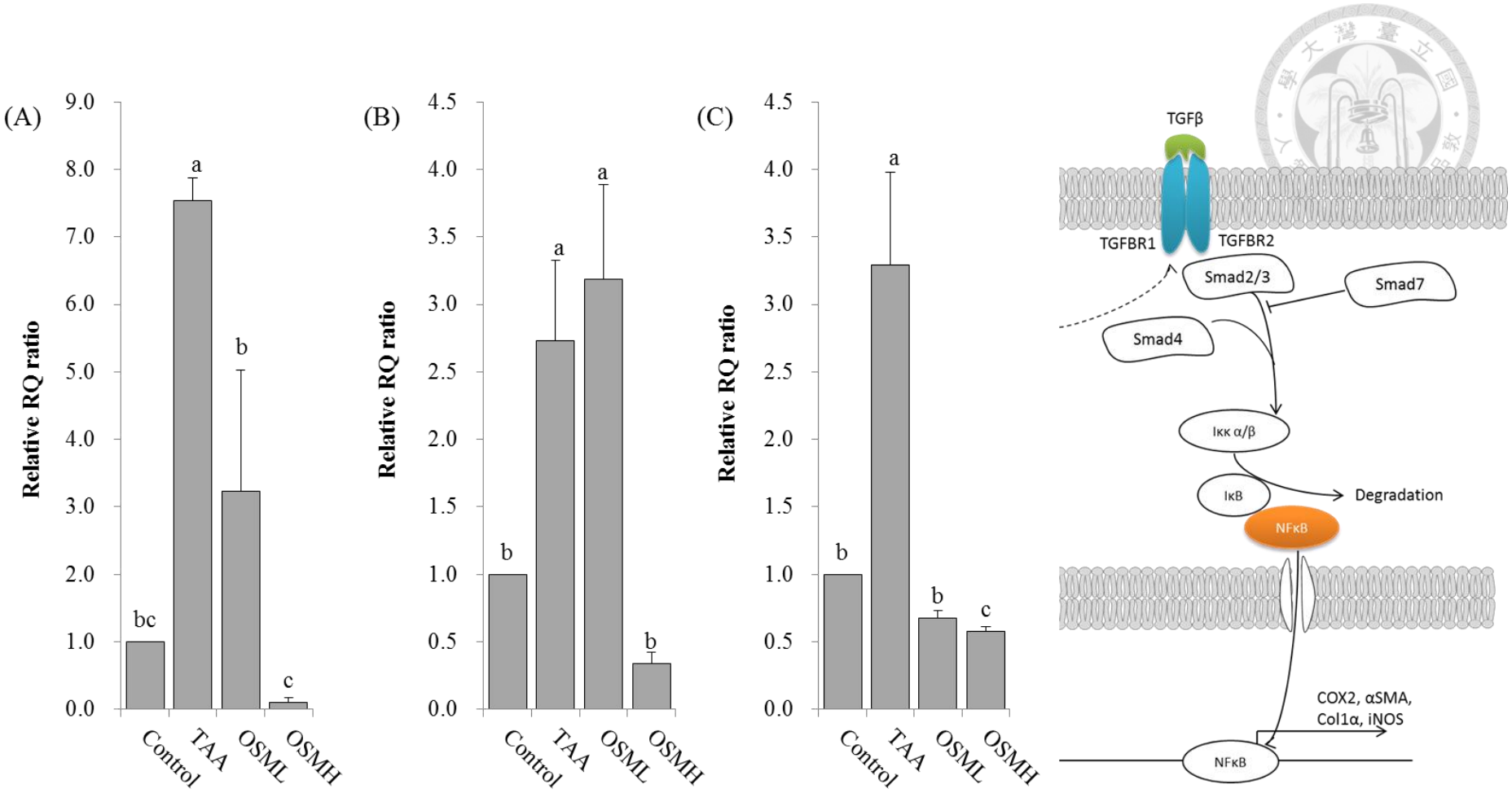


Figure IV-6. TGF-β related gene expressions. mRNA expression of (A) TGF-β, (B) TGFBR1, and (C) TGFBR2 were detected by SYBR Green system . The data are given as means ± SEM (n=12). Different letters on data bars indicate significant differences ($p<0.05$).

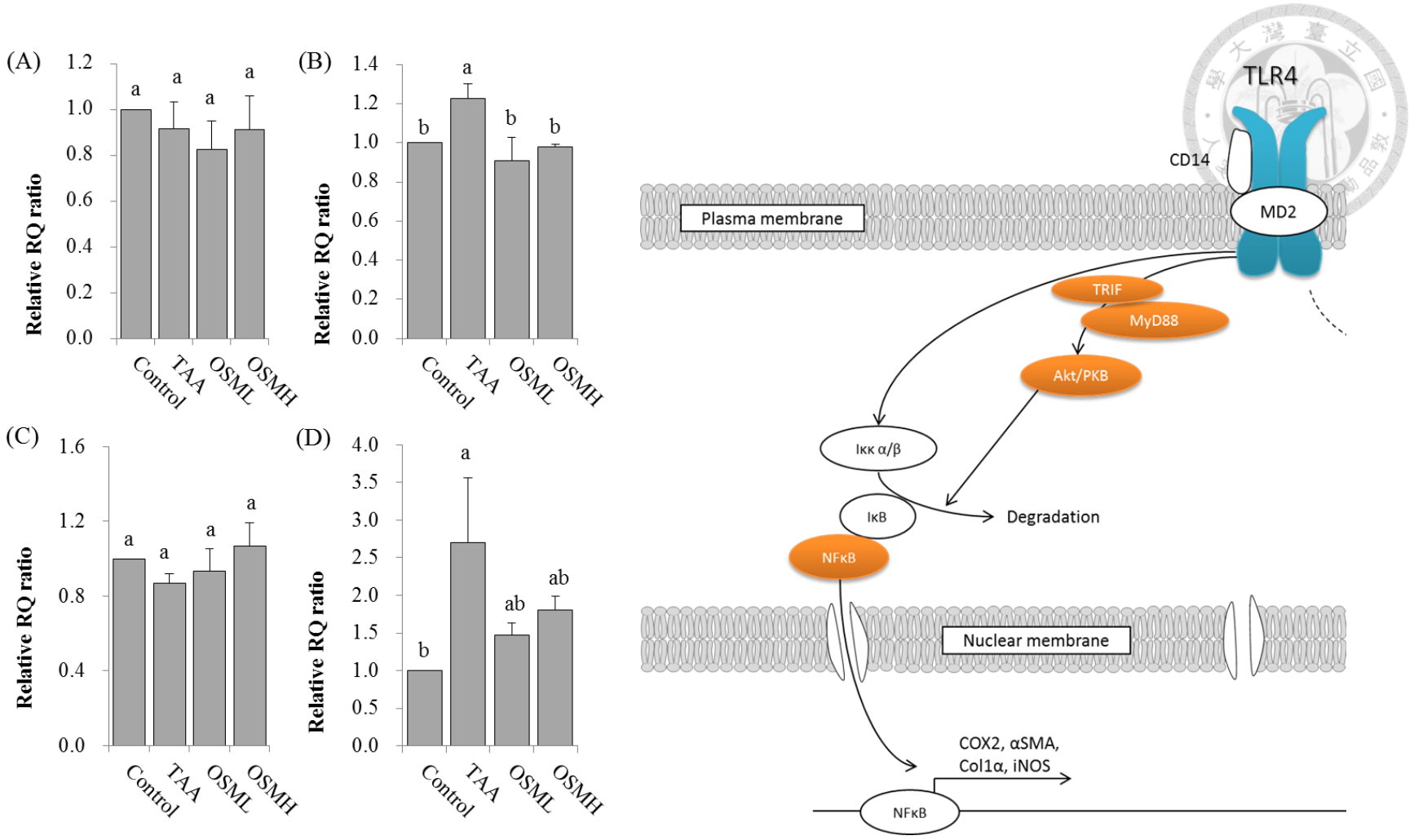


Figure IV-7. TLR4 related gene expressions. mRNA expression of (A) TLR4, (B) TRIF, (C) MyD88, and (D) Akt were detected by SYBR Green system . The data are given as means ± SEM (n=12). Different letters on data bars indicate significant differences ($p<0.05$).

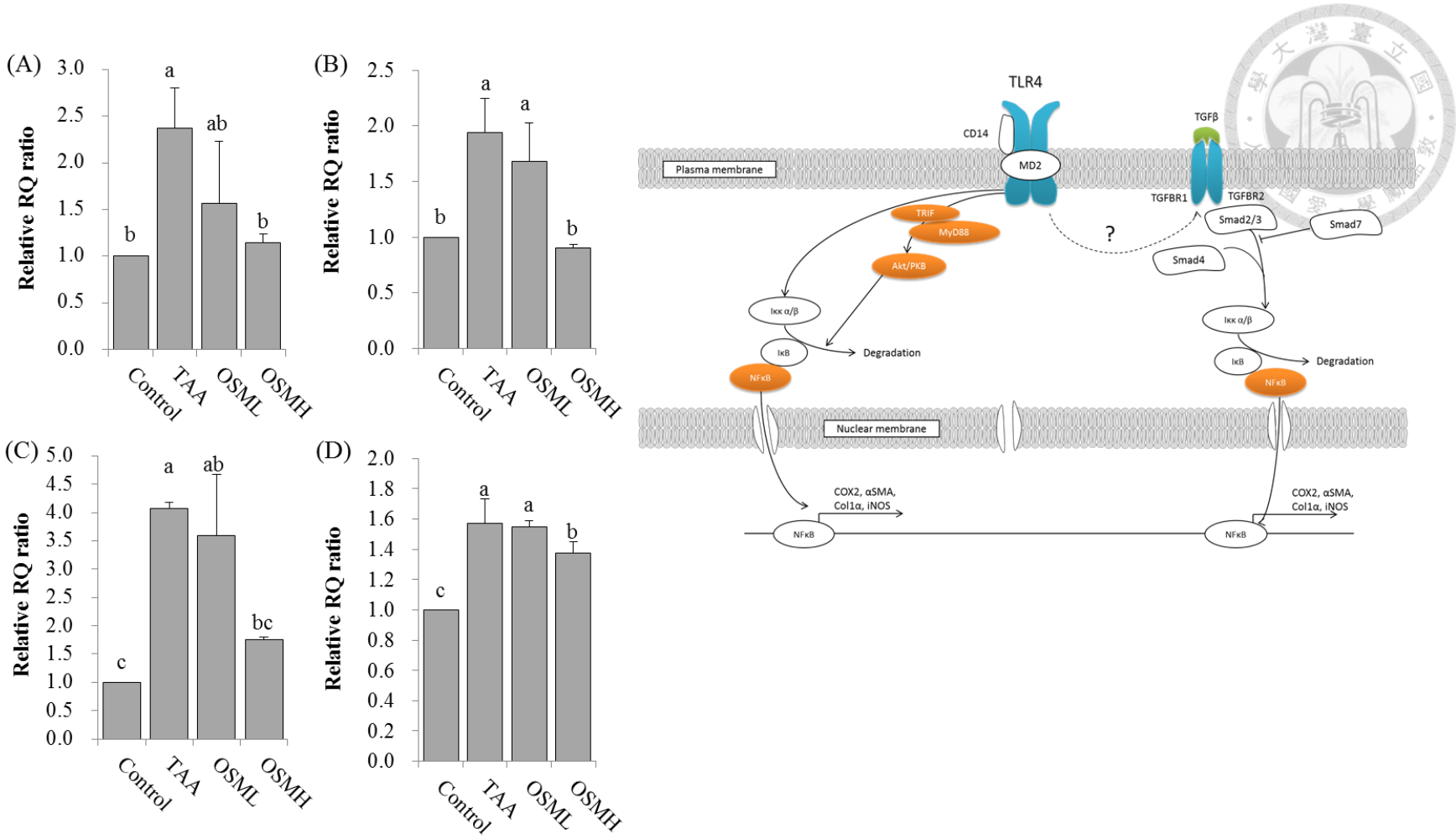


Figure IV-8. Hepatic fibrosis related gene expressions. mRNA expression of (A) αSMA, (B) Col1α, (C) NFκB and (D) COX2 were detected by SYBR Green system. The data are given as means ± SEM (n=12). Different letters on data bars indicate significant differences ($p<0.05$).

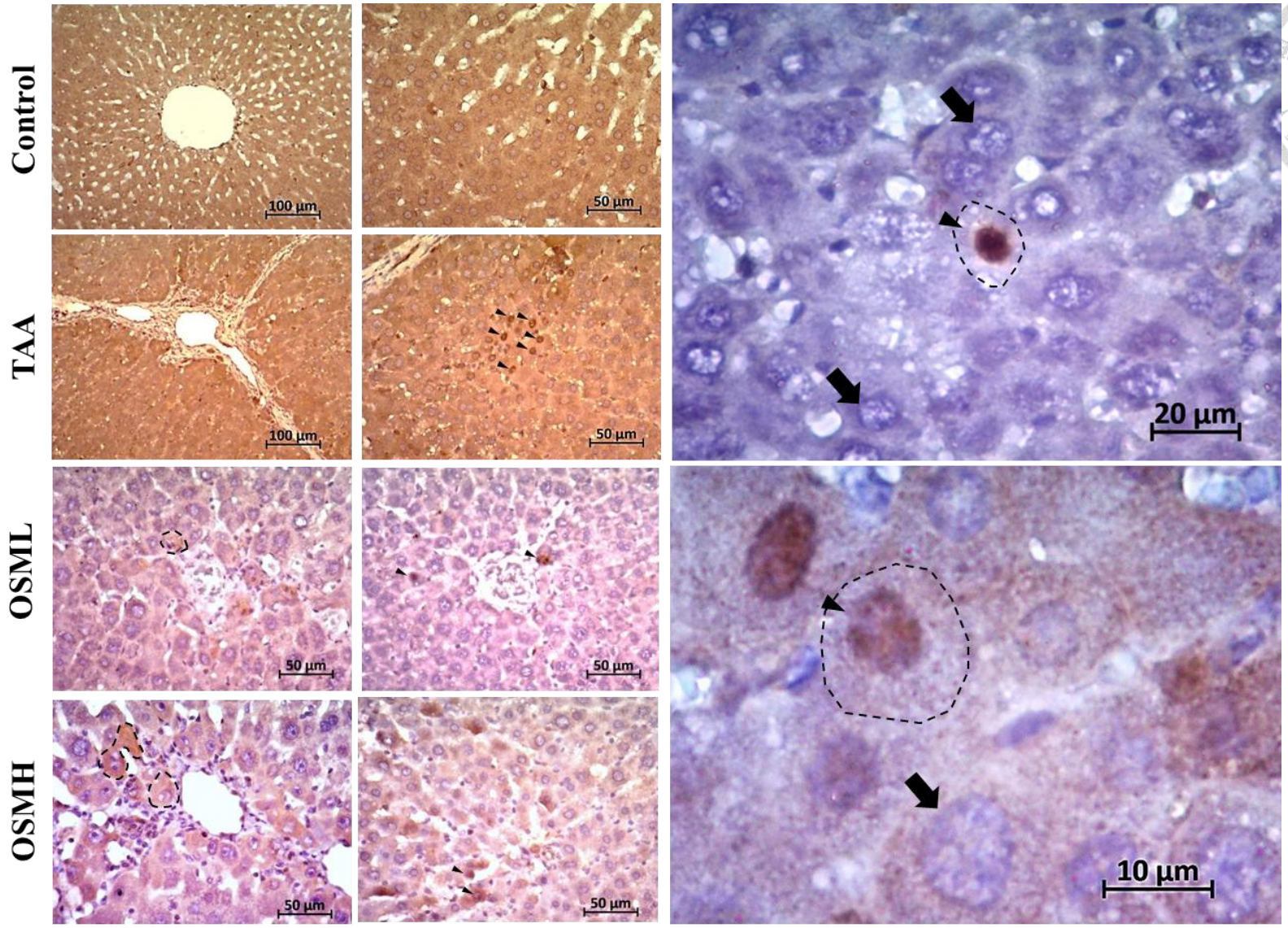


Figure IV-9. Pathway detection by Immunohistochemistry analysis: NFκB protein expression. The scale is shown in 100 (200X, first column in left hand) and 50 µm (400X, second column), and the right one demonstrated the expression area (arrowhead) and cell type (circle in dash-line; round with pig nuclei). NFκB is a key transcription factor of this study, and the entrance of nuclei means the activation of it.

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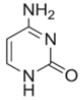
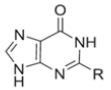
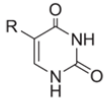
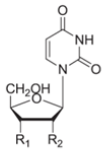
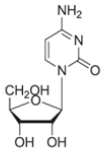
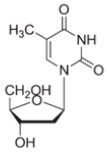
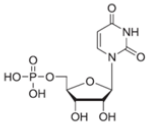
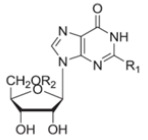
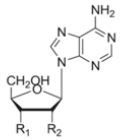
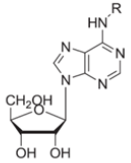
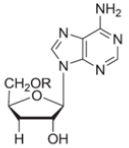
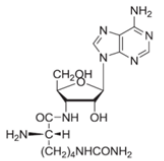
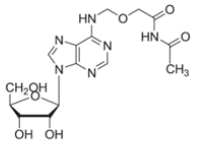
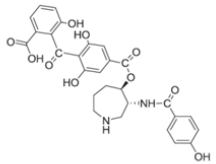
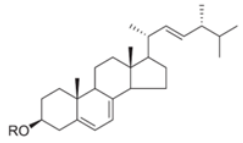
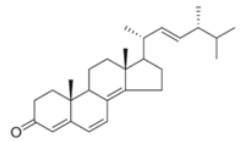
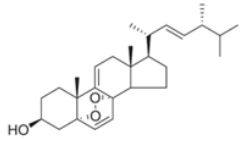
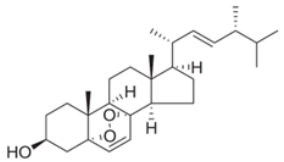
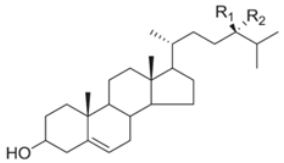
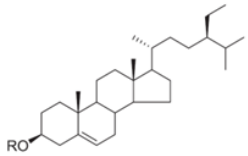
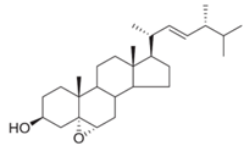
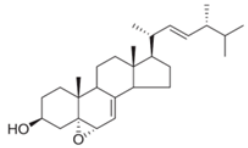
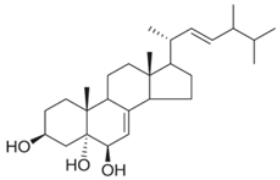
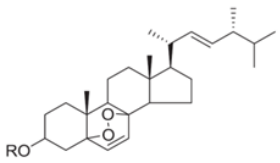
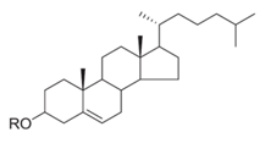
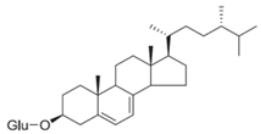
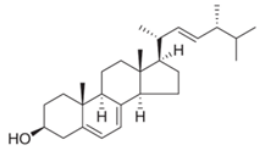
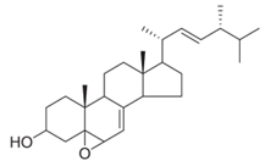
APPENDIX

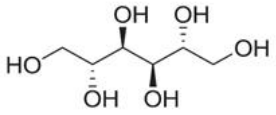
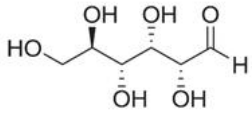
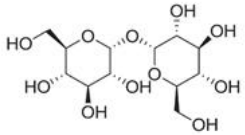
Supplement 1. List of major chemicals and their structures found in OS (Zhao *et al*., 2013)

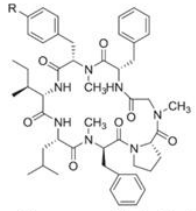
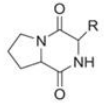
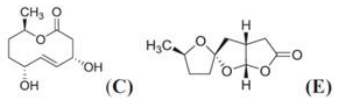
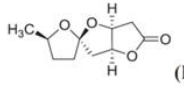
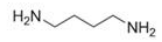
No.	Structure	No.	Structure	No.	Structure
Nucleosides, bases and their analogues					
1	 Adenine	2	 Cytosine	3-4	 Guanine: R=NH_2; Hypoxanthine: R=H
5-6	 Thymine: R=CH_3; Uracil: R=H	7-9	 Uridine: R_1=OH, R_2=OH; 2'-deoxyuridine: R_1=OH, R_2=H; 3'-deoxyuridine: R_1=H, R_2=OH	10	 Cytidine
11	 Thymidine	12	 Uridine-5' -monophosphate	13-15	 Inosine: R_1=H, R_2=H; Guanosine: R_1=NH_2, R_2=H; Guanosine-5' -monophosphate: R_1=NH_2, R_2=HPO_3^-
16-19	 Adenosine: R_1=OH, R_2=OH; 2'-deoxyadenosine: R_1=OH, R_2=H; 3'-deoxyadenosine: R_1=H, R_2=OH; 3'-amino-3' -deoxyadenosine: R_1=NH_2, R_2=OH	20-21	 N^6-hydroxyethyl -adenosine: R=CH_2OH N^6-methyladenosine: R=CH_3	22-23	 Adenosine-5' -monophosphate: R= HPO_3^-; $O^{5'}$-acetylcordycepin: R=$COCH_3$
24	 3'-homocitrullyl-amino-3'-deoxyadenosine	25	 N^6-[β-(acetylcarbamoyloxy)ethyl] adenosine	26	 Ophicordin

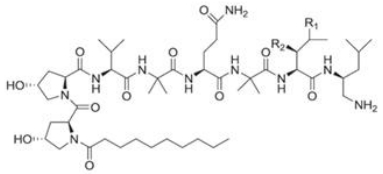
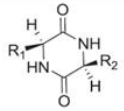
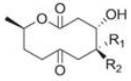
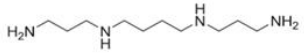
No.	Structure
Sterols and their analogues	
1-2	 Ergosterol: R=H Ergosteryl-3-O-β-D-glucopyranoside: R=glu
7	 Ergosta-4,6,8(14),22-tetraen-3-one (ergone)
10	 5α, 8α-epidioxy-22E-ergosta-6,9(11),22-trien-3β-ol
13	 Ergosterol peroxide
16-17	 Campesterol: R_1=CH_3, R_2=H Dihydrobrassicasterol: R_1=H, R_2=CH3

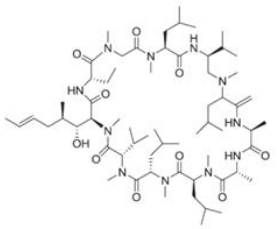
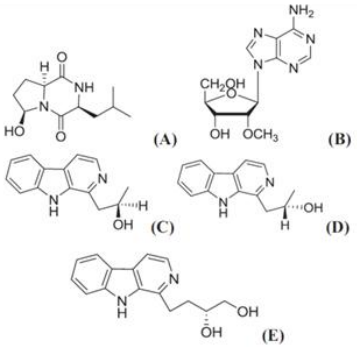
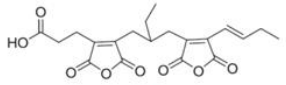
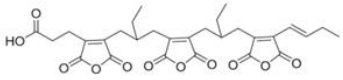
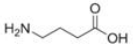
No.	Structure
3-4	 β-sitosterol: R=H Daucosterol: R=Glu
8	 5a,6a-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol
11	 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol
14	 Ergosta-7,22-diene- 3,5,6-triol
18-19	 5,8-epidioxyergosta-6,22-dien-3-ol : R=H 5,8-epidioxyergosta-6,22-dien-3-O-β-D -glucopyranoside: R=glu

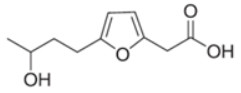
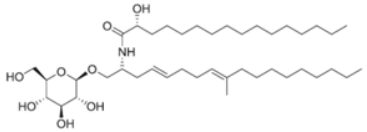
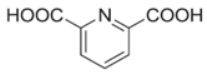
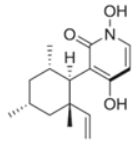
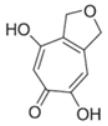
No.	Structure
5-6	 Cholesterol: R=H Cholesteryl palmitate: R=$CO(CH_2)_{14}CH_3$
9	 22-dihydroergosteryl-3-O-β-D-glucopyranoside
12	 δ-3-Ergosterol
15	 5,6-epoxyergosta-7,22-dien-3-ol

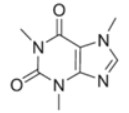
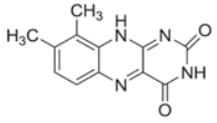
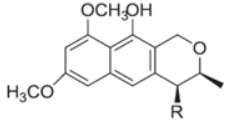
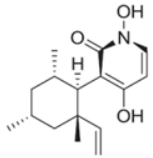
No.	Structure	No.	Structure	No.	Structure
Carbohydrates					
1	 Cordycepic acid: Mannitol	2	 Glucose	3	 Trehalose
4	SCP-I: (1→4)-α-D-glucan backbone	5	CPS : (1→4)- and (1→3)-α-D-glucan backbone	6	CPS50-I: (1→3)-xylose and (1→2)-mannose and galactose backbone
7	WIPS: (1→4)-α-D-Glcp backbone	8	AIPS: Linear D-glucans with a backbone of (1→4)-α-D-Glcp (>60%).	9	AEPS-1: (1→3)-α-D-Glcp backbone
10	EPS-1A: (1→6)-α-D-glucose and (1→6)-α-D-mannose backbone	11	CBHP: (1→4)- and (1→3)-α-D-Glcp backbone	12	O-linked heterogalactomannans: α-(1→6)-mannose backbone.
13	CPS-2: (1→4)-α-D-glucose and (1→3)-α-D-mannose backbone	14	CPMN Fr III: β-(1→6)-galactoglucomannan.	15	CM-hs-CPS2: α-glucan.
16	CBP-1: (1→4)-α-D-mannose backbone	17	CPS1: (1→2) and (1→4)-mannose, (1→3)-galactose and (1→) and (1→3, 6)-glucose backbone	18	CS-Pp:1, 3-β-D-glucan backbone
19	P70-1: (1→6)-β-D-mannopyranosyl backbone	20	Mannoglucan: (1→4)- and (1→3)-α-D-glucan backbone	21	APS: α-(1→, →5) -α-(1→, →4)-Galp-(1→ and →4)-GalAp-(1→ backbone
22	CT-4N: (1→6) - and (1→2)-α-D-mannopyranosyl backbone	23	CMB: (1→6)-Man, (1→6)-Glc, (1→4)-Glc and (1→4)-Gal backbone	24	CPS-1: (1→2) mannose, (1→4) xylose and (1→2) or (1→3) galactose backbone
25	CPS-2: Unknown	26	CPS-3: α-D-glucan	27	CO-1: (1→3)-β-D-glucopyranosyl backbone
28	CI-P, CI-A: (1→6)-α-D-mannose backbone	29	C-3: (1→2)- and (1→6) -α-D- mannopyranosyl backbone	30	EPS: β-D-glucan backbone
31	CS-F10: α-D-glucopyranosyl backbone	32	PCA I: 1,4-mannopyranosyl backbone	33	PCA II: Nonlinear linked heterogeneous oligosaccharide
34	PCB I: 1, 4-mannopyranosyl	35	PCB II: Unknown	36	CS- I: 1, 2-D-mannofuranosyl
37	CPSN: (1→2)-D-mannopyran	38	EPS: (1→3)-β-D-glucan backbone		

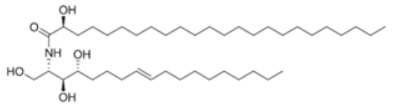
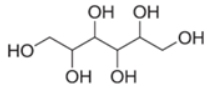
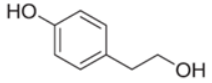
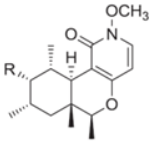
No.	Structure
Miscellaneous	
1-2	 Cordyheptapeptide A: R=OH Cordyheptapeptide B: R=H
6-8	 Cyclo-(L-glycyl-L-prolyl): R=H Cyclo-(L-leucyl-L-prolyl): R=$CH_2CH(CH_3)_2$ Cyclo-(L-valyl-L-prolyl): R=$CH(CH_3)_2$
19-21	 (C) (E)  (F) Cepharosporolides C-F
27	 Putrescine
30	 Cadaverine

No.	Structure
3-4	 Cicadpeptin I: R_1=CH, R_2=H Cicadpeptin II: R_1=H, R_2=CH_3
9-13	 Cordycedipeptide A: R_1= $CH_2CH_2NH_2$, R_2= $CH_2CH(CH_3)_2$; Cyclo-(L-threonyl-L-leucyl): R_1= CH(OH) CH_3, R_2= $CH_2CH(CH_3)_2$; Cyclo-(L-alacyl- L-leucyl): R_1= CH_3, R_2= $CH_2CH(CH_3)_2$; Cyclo-(L-alacyl-L-valyl): R_1= CH_3, R_2= $CH(CH_3)_2$; 3,6-di(4-hydroxy)benzyl-2,5-dioxopiperazine: R_1= R_2= 4-hydroxybenzyl
22-24	 10-membered macrolides 1: R_1=H R_2=OMe; 10-membered macrolides 2: R_1=OMe R_2=H; 10-membered macrolides 3: R_1=H R_2=OH
28	 Spermine
31	Cordymin: The N-terminal sequence is AMAPPYGYRTPDAAQ

No.	Structure
5	 Cyclosporin
14-18	 Cordysinin A-E
25-26	 Cordyanhydride A  Cordyanhydride B
29	 1,3-diaminopropane
32	 γ-aminobutyric acid

No.	Structure
60	 2-carboxymethyl-4-(3'-hydroxybutyl)-furan
63	 Cerebroside B
66	 Pyridine-2,6-dicarboxylic acid
70	 Cordypyridones A
73	 1-deoxo-cordytropolone

No.	Structure
61	 Caffeine
64	 7,8-dimethyl-iso-alloxazine
67-68	 Monomers 1: R=H Monomers 2: R=OH
71	 Cordypyridones B
74	Fatty acids and their esters

No.	Structure
62	 N-(2'-hydroxy-tetracosanoyl)-2-amino-1,3.4 -trihydroxy-octadec-8E-ene
65	 D-allol
69	 4-(2-hydroxyethyl) phenol
72	 Cordypyridones C: R=H Cordypyridones D: R=OH
75	Amino acids