Phyllanthus Urinaria Treatment in Experimental Model of Non-alcoholic Steatohepatitis

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Abstract

Non-alcoholic steatohepatitis (NASH) results from excessive accumulation of hepatic fat (steatosis) and oxidative stress. Therefore, inhibition of fatty acid cytotoxicity and liver inflammtary change is an important goal in the treatment of NASH. Phyllanthus urinaria, a herbal medicine, has been reported to have potential anti-oxidant property. We tested the effects of Phyllanthus urinaria on nutritional steatohepatitis both *in vitro* and *in vivo*, and determined the mechanism of its action.

Immortalized normal hepatocytes AML-12 or primary hepatocytes were cultured in control, and the methionine and choline deficient (MCD) culture medium in the presence or absence of Phyllanthus urinaria for 24 hours. Hepatocyte triglyceride contents, release of alanine aminotransferase, lipoperoxides and reactive oxygen species production were determined in the cell culture study. Age-matched wild-type C57BL/6 and diabetes db/db mice were fed control or MCD diet for 10 days with or without Phyllanthus urinaria. The levels of Hepatic steatosis, necroinflammation, triglycerides and oxidative stress were investigated. Hepatic expression of inflammatory factors and lipid regulatory mediators were assayed. The results demonstrated that Phyllanthus urinaria reduced steatosis and alanine aminotransferase (ALT) levels in culture of hepatocytes in a dose-dependent manner. Phyllanthus urinaria protected the livers against MCD-induced hepatic fat accumulation and steatohepatitis in mice. This effect was associated with repressed levels of hepatic lipid peroxides, reduced expression of cytochrome P450 (CYP) 2e1, pro-inflammatory tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), dampened activation of inflammatory C-jun N-terminal kinase (JNK) and nuclear factor κB (NF-κB),

increased expression of lipolytic Cyp4a10 and suppressed transcriptional activity of lipogenic CCAAT/enhancer binding protein β (C/EBPβ). Hepatic acyl co-enzyme A oxidase (ACO) that regulated hepatic beta-oxidation of fatty acid and other lipid regulators were not affected by Phyllanthus urinaria.

Our study indicated that Phyllanthus urinaria effectively prevented MCD-induced steatohepatitis. This effect were probably mediated through dampening oxidative stress, ameliorating inflammation and decreasing lipid accumulation. Phyllanthus urinaria deserves further evaluation for its potential therapeutic effect on NASH in humans.

摘要

脂肪變性和氧應激及其產生的肝臟損傷和炎症細胞浸潤是非酒精性脂肪肝炎(NASH)最主要的病理生理表現。由此，治療非酒精性脂肪肝炎的重點在於抑制脂肪酸引起的細胞毒性和肝臟的炎性改變。自古至今，傳統草藥常被用於治療慢性肝臟疾病。其中，研究發現草藥葉下珠具有抗氧化劑的功用。本試驗利用肝細胞和動物模型探討葉下珠治療 NASH 的可行性及其作用機理。

在永生化肝細胞株 AML-12 或原代肝細胞體外培養的研究中，同樣的肝細胞分別置於正常的對照培養液，蛋氨酸膽鹼缺乏(MCD)培養液，和 MCD 培養液加入不同濃度的葉下珠溶液中，溫箱孵育 24 小時。收集的細胞和培養液進行肝細胞甘油三酯含量，丙氨酸轉氨酶(ALT)水準，脂質過氧化物及活性氧分子(ROS)產物的檢測。在細胞培養的基礎上，本試驗進一步研究葉下珠對 C57BL/6 及糖尿病 db/db 小鼠餵養 MCD 飲食進展為 NASH 的預防治療作用。小鼠在 10 天的實驗期間，進食加入蛋氨酸膽鹼對照鼠糧，MCD 鼠糧及 MCD 鼠糧中加入不同含量的葉下珠粉末。餵養結束後，取出小鼠的肝臟組織，進行脂肪變性和炎症組織病理學觀察評分，並對肝臟甘油三酯，氧應激，炎性因數及脂質代謝的調節基因進行測定。

試驗結果顯示葉下珠有效地保護肝臟抵抗 MCD 引起的肝細胞內脂肪沉積和脂肪肝炎症損害，明顯改善了肝臟切片的病理表現。深入探究其預防脂肪肝炎和保護肝臟的作用機理，研究發現葉下珠抑制了肝臟內脂質過氧化物的產生，顯著降低了參與氧應激-脂質過氧化損傷的基因 CYP2e1，與炎性反應相關的細胞因數 TNF-α，IL-6 的表達，同時衰減了炎症相關因數 JNK 和 NF-κB 的活性。在改善肝臟脂肪代謝調解機制方面，葉下珠一方面增強了脂肪分解因數 CYP4a10 表達，另一方面抑制了參與脂肪合成的基因 C/EBPβ。在本試驗中，參與游離脂肪酸β-氧化作用的基因 ACO 及其他一些調節脂肪代謝的基因在肝臟內的表達未發現受葉下珠治療的影響出現明顯

升高或降低。

綜上所述，傳統草藥葉下珠可保護肝臟免受 MCD 誘發的肝臟脂肪代謝障礙，脂質變性和過氧化，及炎症，有效地預防脂肪肝炎的形成，為臨床預防和治療 NASH 提供了新的思路。

Publications

Journal Articals

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Poster Presentation

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Abbreviations

ABC	Avidin–biotin complex
ALT	Alanine aminotransferase
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
DBA	Diaminobenzidine
DCF	Dichlorofluorescein
DCFH	dichlorodihydrofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DGAT2	Diacylglycerol acyltransferase 2
DMEM/F12	Dulbecco's modified Eagle's medium and F12 1:1 mixture
DMSO	Dimethyl sulfoide
DTT	Dithiothreitol
EBS	Enzyme Dispersion Solution
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	[Ethylene-bis(oxyethylenenitrilo)]tetraacetic acid
FAS	Fatty acid synthesis
FasL	Fas ligand
FFA	Free fatty acid
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HDL	High-density lipoproteins

HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]
HPLC-MS/MS	High-performance liquid chromatography tandem mass spectro mass spectrometry
ICAM-1	Intercellular adhesion molecule-1
IKK-β	Inhibitor kappa kinase beta
IL	Interleukin
IRS	Insulin receptor substrates
JNK	Jun N-terminal kinase
LCAD	Long-chain Acyl-CoA dehydrogenase
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCD	Methionine and choline deficient
MEM	Minimum essential medium eagle
MRC	Mitochondria respiratory chain
MRM	Multiple reaction monitoring
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-kB	Nuclear factor kappa B
O_2^-	superoxide anion radical
·OH	hydroxyl radicals
8-OHdG	8-hydroxydeoxyguanosine
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl fluoride
P. urinaria	Phyllanthud Urinaria Koreanis
PPAR	Peroxisome proliferator-activated receptors
PVDF	Polyvinylidene difluoride

real-time PCR	Real-time polymerase chain reaction
ROS	Reactive oxygen species
RT	Reverse transcriptional
SCD1	Stearoyl-CoA desaturase-1
SDS	Sodium dodecyle sulfate
SDS-PAGE	Sulphate-polyacrylamide gel
SREBP1c	Sterol regulatory element-binding proteins
TBARS	thiobarbituric acid reactive substances
TBA	Thiobarbituric acid
TBS	Tris buffered saline
TBS-T	1 × TBS, 0.1% Tween-20
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TGF-β1	Transforming growth factor beta 1
TMP	1,1,3,3-tetramethoxypropne
TNF-α	Tumor necrosis factor-alpha
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoproteins

Contents

CHAPTER 1. BACKGROUD

1.1 Definition of NAFLD and NASH

The term 'non-alcoholic steatohepatitis' was first described in 1980 by Ludwig et al. on the biopsy findings among patients with steatohepatitis in the absence of significant alcohol intake. [Ludwig, 1980] After extensive debate during the next two decades, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have been widely recognized as a distinct disease entity instead of a diagnosis by exclusion of other liver diseases.

Most people adopt the definition of NAFLD and NASH as suggested by the Asia-Pacific Working Party on NAFLD [Farrell GC, 2005] and the American Association for Study of Liver Diseases (AASLD). [Neuschwander-Tetri BA, 2003] Non-alcoholic fatty liver disease (NAFLD) is more comprehensive than NASH to cover the full spectrum of histologic abnormalities of liver, including steatosis (fatty change), steatohepatitis with or without fibrosis and cirrhosis. NASH is the term used to describe liver injury with steatohepatitis not resulting from alcohol and is characterized by the presence of macro- and micro-vesicular steatosis along with scattered lobular inflammation, Mallory body and/or pericellular fibrosis. There is some controversy on the maximum alcohol intake to be qualitied as "non-alcoholic". Most authorities accept 20g to 40g ethanol/day or up to 140g and 210g ethanol/week for women and men, respectively. [McCullough AJ, 2005] The duration of alcohol intake is another

important consideration. In fact, low levels of regular alcohol intake is benefit for improving obesity, insulin resistance, type 2 diabetes mellitus (T2DM) and dyslipidemia including hypertriglyceridemia, high free fatty acid (FFA), low high-density lipoproteins (HDL), which are risk factors associated with the development of NASH. [Farrell GC, 2006]

1.2 Epidemiology

NAFLD is becoming a major public health problem due to the rapid change in lifestyle and the rising prevalence of obesity and T2DM worldwide. The Third National Health and Nutritional Examination Survey (NHANES III) found 3-23% prevalence of NAFLD in North America by elevation of serum aminotransferase levels. [McCullough AJ, 2005] Recent data using more sensitive methods to detect fatty liver disease has indicated that the prevalence of NAFLD and NASH have been underestimated in previous studies. Using proton magnetic resonance spectrometry, approximately 30% of the U.S. population was found to have increasing hepatic triglyceride content. [Browning JD, 2004] In the East, there is growing interest in this disorder as the prevalence of NAFLD has been found to range from 12% to 29% in different Asian areas (Table 1.1).

It is particularly worrying that NAFLD is prevalent not only in adults but also in children and adolescents. Fraser A. et al. reported a 3.6% prevalence of NAFLD as defined by an elevation of alanine aminotransferase (ALT) among USA adolescents 12-19 years old in 1999-2004. [Fraser A, 2007] Several studies in

Europe, Asia, South America and North America have been conducted in cohorts of children selected for overweight and obesity and found that the prevalence of NAFLD in these groups ranges from 12% to 80%. [Dimitrios Papandreou, 2007] A population-based study of pediatric NAFLD in Japan determined the prevalence was 2.6% in children aged 4-12 years. [Tominaga K, 1995] Recently, investigators in China demonstrated the prevalence of NASH is approximately 44% in obese children. [Fu JF, 2006]

Table 1.1 **Prevalence of NAFLD in surveys from Asian countries**

Study locations (reference)	Years	Number	Participants	NAFLD
Shanghai, China (Fang JG, 2005)	2002-2003	3175	Community survey of adults	15.35%
Hong Kong, China (Yiu DC, 2004)	2003	1627	Adults (age range 20-65ys) to hospital outpatient clinic	15.9%
Guangdong, China (Zhu YJ, 2007)	2005	3543	Population survey in 6 urban and rural areas	15%
Korea (Park SH, 2006)	2003	6648	Community of urban residents	18.7%
Manila, Philippines (De Lusong MA, 2008)	1999-2004	1102	Patients in hospital	12.2%
Japan (Jimba S, 2005)	2002-2003	1950	Adults in healthy examination program	29%
Taiwan (Yang CC, 2006)	2006	3245	Community survey of rural residents	21.2%
Mumbai, India (Amarapurkar D, 2007)	2007	730	General population	18.9%

1.3 Histological progression of NAFLD and NASH

Since there is no non-invasive surrogate marker for the assessement of severity of liver disease in NAFLD, liver biopsy plays a pivotal role in the diagnosis and assessment of this condition in clinical practice.

1.3.1 Steatosis

When the lipid content in the liver exceeds 5% of the liver weight, we make the diagnosis of hepatic steatosis (fatty liver). Fatty liver has fat droplets mainly as large single macrovesicular droplets that displace the nucleus to the periphery of the liver cell. In other circumstances, microvesicular fat may be seen as large numbers of smaller droplets surrounding a central nucleus. In early or mild NAFLD, the fat is seen in zone 3 hepatocytes. Patients with simple steatosis have minimal risk of progression to liver fibrosis in a series of report up to 16 years of follow-up. [Hui AY, 2005; Adams LA, 2005; Adams LA, 2007] Though simple steatosis is reversible and has an excellent prognosis, some investigators reported that patients with pure steatosis still have risk to progress to liver cirrhosis. [Neuschwander-Tetri BA, 2003]

1.3.2 Steatohepatitis

Steatohepatitis referes to the presence of both fatty change and hepatocyte injury with necroinflammatory activity. Hepatocyte injury can be in the form of ballooning degeneration, hepatocyte necrosis or apoptosis. In most cases, steatohepatitis is accompanied with Mallory bodies and varying degrees of

fibrosis. Steatohepatitis was found in 18.5% of markedly obese patients and 2.7% of lean patients. [Wanless IR, 2005] NAFLD patients with metabolic syndrome have a higher prevalence and severity of necroinflammatory activity, compared to subjects with pure fatty liver. [Pagano G, 2002] Patients with steatohepatitis are at higher risk of severe fibrosis as compared to patients with only simple steatosis.

1.3.3 Fibrosis and cirrhosis

During liver injury and inflammation, de novo cytokines releasing triggers the activation of hepatic stellate cells which cause the formation of fibrosis. Approximately one third of patients with NASH will have progressive fibrosis over 3 to 4 years, whereas over half will progress when followed up for 6 years. [Hui AY, 2005] In a clinical series, approximately 20% of patients with NASH progress to cirrhosis with 18 years follow-up. [Matteoni CA, 1999] Nowaday, NAFLD is a well recognized factor associated with an increased risk of death particularly among patients with T2DM and liver cirrhosis.

1.3.4 Hepatocellular carcinoma (HCC)

The mounting evidence demonstrates that patients with NASH may eventually develop cryptogenic cirrhosis and even HCC. Obesity and diabetes are recognized as significant risk factors for the development of HCC. Investigators found the incidence of HCC among obese patients was 4.0%, slightly higher than lean patients who had cirrhosis ($P = 0.013$). [Nair S, 2003] In recent

population-based case-control study from the USA, the results showed threefold increase of risk for HCC among patients who had diabetes, and there was 43% proportion of HCC patients with diabetes compared with controls (19%). With the rising incidence of metabolic symdrome which is associated with the prevalence of NASH, the risk of NASH to HCC appears higher (13% to 23%). [Hui JM, 2003; Marrero J, 2002; Hassan M, 2002] Case report in Japan showed that two cases of patients with NAFLD progressed to NASH-related cirrhosis, eventually developing HCC with 26 years follow-up. [Tsutsumi K, 2007] The available studies suggest that NASH associated with development of HCC may be of increasing important issue in the coming years.

1.4 Pathogenic mechanisms of NASH

There is increasing information in the literature on the key issues in the pathogenisis of NASH, such as the nature of insulin resistance, FFA accumulation, the mechanisms for inflammatory infiltration and perpetuation, the biochemical basis of oxidative stress, the cell biological basis of hepatocyte injury and the pathogenesis of fibrosis. In 1998, Day and James proposed the 'two hit' hypothesis to describe the progression of NASH. [Day CP, 1998] Hepatic steatosis, the 'first hit', develops when the balance between hepatic triglycerides synthesis and export is altered such that synthesis exceeds the export capacity. As the severity of steatosis increases, which is paralleled by the development of lipotoxicity, the liver increases its sensitivity to oxidative stress -'the second hit'. De novo triglyceride synthesis, insulin resistance, obesity,

T2DM, and hyperlipidemia are the most important factors for the development and progression of steatohepatitis. It has been repeatedly demonstrated that steatosis always precedes the development of hepatic insulin resistance in a rat model fed with short-term high-fat diet. [Kraegen EW, 1991; Samuel VT, 2004] Steatosis is sometimes regarded as an epiphenomenon of the injurious mechanisms rather than a true 'first hit'. The key candidates for the second hits are oxidative stress, which is associated with lipid peroxidation, and cytokines, principally, tumor necrosis factor-alpha (TNF-α). All these factors may be related to an increase of reactive oxygen species (ROS) (Figure 1.1).

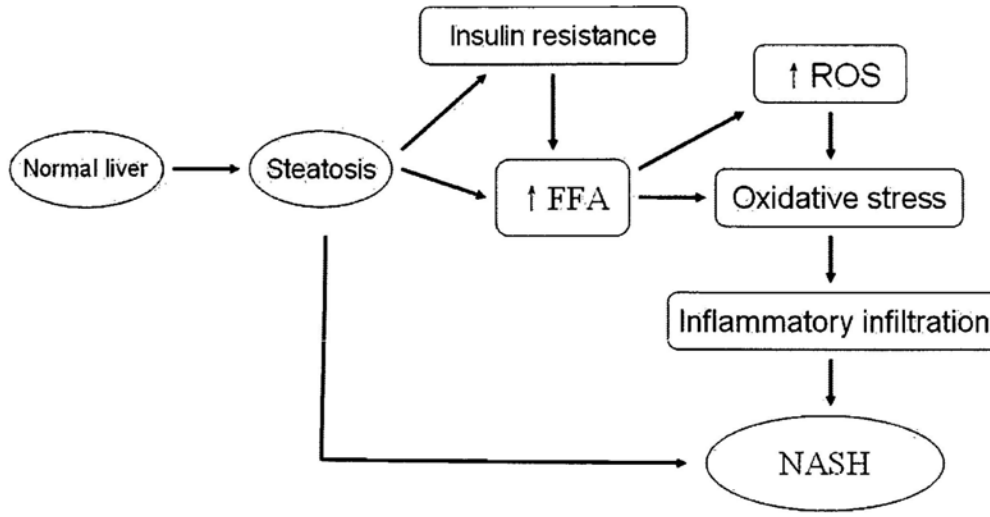

Figure 1.1 **Pathogenesis of NASH.** Increased fatty acid accumulation and lipid beta-oxidation generates ROS, oxidative stress and lipid peroxidation which promotes the production of cytokines, growth factors and causes mitochondrial dysfunction. These contribute to hepatocellular injury, apoptosis, infiltration of inflammatory cells, activation of stellate cells, and fibrogenic reaction.

1.4.1 Free fatty acid and insulin resistance

Long-chain fatty acids can be bound or attached to other molecules, such as triglycerides (TG) or phospholipids. When they are in a simple non-esterified form, they are known as FFA. Under the condition of increased FFA influx into the hepatocyte, a number of consequences occur. Fatty acids are increasingly directed towards TG synthesis. When this exceeds the capacity of the liver cell to assemble and/or export triglyceride-rich very low-density lipoproteins (VLDL) particles, hepatocellular steatosis develops. Excessive accumulation of TG in hepatocytes is the hallmark of NAFLD, which is strongly associated with insulin resistance. Recent studies have favored the concept that accumulation of intrahepatic lipids precedes the development of insulin resistance. In the insulin-resistant state, there is a disproportionately greater influx of FFA from visceral adipose tissue to the liver via the portal circulation. In the liver, these FFA not only damage the function of insulin to suppress hepatic glucose output, but also inhibit the tricarboxylic acid cycle and enter the mitochondria for oxidation, which produces an oxidative stress in the liver. [Arun J. Sanyal, 2005] Though many investigators support for the hypothesis that the accumulation of lipid in non-adipose tissue is correlated with the severity of insulin resistance, [Kahn BB, 2000; Savage DB, 2006] there is still some debate on the causal relationship between steatosis and insulin resistance. Recently, researchers have found that hepatic TGs themselves are not toxic and may in fact protect the liver from lipotoxicity by buffering the accumulation of fatty acids. Yamaguchi et al. showed in mice with steatosis induced by methionine and choline deficient

(MCD) diet that inhibition of TG synthesis led to the improvement of liver steatosis but increased hepatic fatty acids, generation of ROS and oxidative stress. [Yamaguchi, 2007] In genetically determined mouse model, overexpression of diacylglycerol acyltransferase 2 (DGAT2) significantly induced hepatic steatosis but it failed to induce any abnormalities in the hepatic glucose production or insulin tolerance. These results suggested that the development of hepatic steatosis may not necessarily be associated with insulin resistance. [Monetti M, 2007; Catherine Postic, 2008]

1.4.2 ROS generation and mitochondrial injury

Intracellular pro-oxidant species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and superoxide anion radical (O_2^-), collectively called ROS. High intracellular levels of ROS can lead to impairment in mitochondrial function, DNA modification, lipid peroxidation and elevation of cytokine production. Under normal physiological circumstances, FFAs either undergo oxidation in the mitochondria or are esterified to triglyceride, phospholipids and cholesteryl esters. Patients with NASH have high levels of hepatic FFA which enhanced mitochondrial fatty acid β-oxidation. Mitochondrial FFA oxidation causes increased generation of ROS which oxidize fatty acids to release lipid peroxidation products. These lipid peroxidation products react with mitochondrial DNA (mtDNA) and proteins to partially block the flow of electrons in the respiratory chain. [Fromenty B, 2005; Mantena SK, 2008] It is suggested FFA oxidation may be an important cause of ROS production in

NASH. [Cortez-Pinto H, 1999] An in vitro study demonstrated that incubating hepatocytes with FFA increased their ROS formation. The impairment of FFA transportation into mitochondrial and the dysfunction of activity of mitochondria respiratory chain (MRC) complexes have been demonstrated during the development of NASH. Decreased activity of all five oxidative phosphorylation complexes of MRC was found in liver biopsies from human patients with NASH compared to normal liver. [Perez-Carreras M, 2003] Similarly, decreased respiratory complex activities were also found in the livers of ob/ob mice. [Garcia-Ruiz C, 2006] This evidence strongly implicates that increase mitochondrial FFA is a critical source of ROS in fatty liver disorders, and impairment of the mitochondrial β-oxidation pathway contributes to the pathogenesis of NAFLD.

1.4.3 Oxidative stress and lipid peroxidation

The development of steatohepatitis requires the insult by oxidative stress that can be generated by multiple pathways within the hepatocytes. The intrahepatic lipid peroxidation products may create and amplify the oxidative stress and initiate a cascade of reaction that eventually leads to the activation of the transcription factor nuclear factor kappa B (NF-kB). NF-kB will result in the increased transcription of various inflammatory cytokines, chemokines, adhesion molecules, growth factors and death ligands by hepatocytes and non-parenchymal cells. [Ribeiro PS, 2004] Human and animal studies have provided persuasive evidence that oxidative stress plays a central role in the

progression of NASH. The magnitude of oxidative stress actually correlates with the disease severity in patients with NASH and in animal models of steatohepatitis. [Leclercq IA, 2000; Seki S, 2002; Albano E, 2005] In line with these observations, at least four oxidative stress associated genes (Eh, Gclc, HO-1, and Nqo1) have been shown to undergo transcriptional upregulation during experimental NASH. [Andrew J, 2007] Additional evidence for oxidative stress playing a key role in NASH has come from the detection for the hepatic expression of 8-hydroxydeoxyguanosine (8-OHdG), a good marker of oxidative DNA damage, which was more frequently detected in cases with NASH. [Nomoto K, 2008] Moreover, the induction of Cytochrome P450 2e1 (CYP2e1) has been found to be another important factor in the pathogenesis of NASH, probably because of the associated increase in oxidative stress. Evidence has been demonstrated from human studies as well as in experimental models that CYP2e1 is overexpressed in steatohepatitis. [Weltman MD, 1998, Chalasani N, 2003] More recently, studies of insulin receptor signaling intermediates indicate that the operation of hepatic insulin resistance in the MCD model is most likely caused by CYP2e1-induced oxidative stress. [Schattenberg JM, 2005]

On the other hand, lipid accumulation favors increased concentrations of FFA that may be directly toxic to the hepatocytes. It has recently been proposed that such 'lipotoxicity' in NASH results in insulin resistance and generation of ROS-mediated lipid peroxidation.

1.4.4 Genes involved in hepatic fatty acid metabolism

Hepatic steatosis represents an excess accumulation of triglycerides in the hepatocytes of the liver. The underlying cause of fat accumulation in steatosis is mostly due to the synthesis of fatty acids and inhibition of fatty acid oxidation. Tanscription factors (sterol regulatory element-binding proteins (SREBP1c), peroxisome proliferator-activated receptors gamma (PPARγ)), genes of fatty acid uptake (liver X receptor (LXR) α, LXRβ), fatty acid synthesis (FAS), and esterification stearoyl-CoA desaturase-1(SCD1) pathways that favor import of lipid into the liver. [Browning, 2004; Anania, 2005; Shimano, 1996; Shimano, 1997; Shimomura, 1998; Yahagi, 2002] PPARγ is a transcriptional regulator that modulates adipocyte differentiation, fat metabolism and inflammation (Hevener, 2003; Matsusue, 2003). Moreover, PPARγ has been reported to play a key role in determining insulin sensitivity. [Sharma AM, 2007] SCD-1, which converts saturated FFA to monounsaturated FFA, is critical for the hepatic synthesis of triglyceride and the development of steatosis. [Cohen P, 2002] SREBP-1 is a key player in lipogenesis in the adipose cell, which activates genes required for fatty acid synthesis and storage of triglycerides. [Al-Hasani H, 2005] The up-regulation of SCD-1 and FAS enzymes catalyzes the synthesis of monounsaturated fatty acids. [Browning, 2004; Anania, 2005; Shimano, 1996] More recently, the CCAAT/enhancer binding protein (C/EBP), a family of transcription factors, has emerged as an important regulator of hepatic lipogenesis. Two groups of investigators have reported that C/EBPβ deletion in mice would limit the development of obesity and diabetes, reduce hepatic

steatosis and increase fatty acid oxidation. [Rahman SM, 2007; Schroeder-Gloeckler JM, 2007]

Fatty acids oxidation in the liver occurs via mitochondria and peroxisomes β-oxidation and CYP4A-catalyzed ω-oxidation. [Rao, 2004; Reddy, 2001] Acyl-CoA oxidase (ACO) and long-chain Acyl-CoA dehydrogenase (LCAD) are the key enzymes of these three fatty acid oxidation systems in liver and are regulated by the lipolytic transcription factor PPARα. [Rao, 2004; Reddy, 2001; Anania, 2005] PPARα serves as a sensor of increased intracellular FFA in the hepatocyte. It is activated principally by unsaturated and polyunsaturated fatty acids. Cytochrome P450 4a (CYP4a) and ACO are involved in fatty acid turnover that are regulated by PPARα. PPARα$^{-/-}$ mice fed a MCD diet develop severe steatohepatitis compared with wild-type littermates. [Ip E, 2003] Conversely, activation of PPARα by PPARα agonist (Wy-14,643) in wild-type mice attenuates steatohepatitis mainly through massively up-regulating CYP4a and ACO, which stimulate hepatic β-oxidation of fatty acids. Thus in a situation of steatosis, stimulation of lipid combustion depletes the substrates for lipid peroxidation and thereby decreases oxidative stress. [Ip E, 2003]

1.4.5 Recruitment of hepatic inflammatory response

As the severity of steatosis increases and lipotoxicity develops, the liver becomes more insulin resistant. Elevated levels of circulating FFA enters into liver causes injury to hepatocytes by inducing apoptosis. Recent studies reported that

hepatocyte apoptosis is an important mode of cell death in NASH mediated by the death receptors including TNF-α, tumor necrosis factor related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL). [Harmeet Malhi, 2007] It is widely acknowledged that TNF-α expression is increased significantly in the presence obesity, simple steatosis and NASH. Increased level of TNF-α will induce insulin resistance through activating inhibitor kappa kinase beta (IKK-β) in adipocytes and hepatocytes, which further reduces the tyrosine phosporylation of the insulin receptor substrates (IRS) 1 and 2. [Yuan M, 2001] TNF-α also attracts inflammatory leucocytes to the liver and is involved in the activation of Jun N-terminal kinase (JNK) as well as the IKKβ/NF-κB pathway. [Shoelson SE, 2006; Christine CK, 2007] IKKβ activation leads to translocation of NF-κB to the nucleus, resulting in a feed-forward loop that promotes the synthesis of cytokines such as TNF-α, interleukin (IL)-1β and IL-6, transforming growth factor beta 1 (TGF-β1) and cyclooxygenase-2 (COX-2) that subseaquently leads to liver injury. [Leclercq IA, 2004; Yu J, 2006 Hepatology] NF-κB also plays an important role in regulating the expression of cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and recruiting inflammatory cells to the site of inflammation. [Kumar S, 2007]

Other cytokines and mediators of inflammation including IL-6 and COX-2, have also been shown to play a significant role in the development of NASH. Though the pathogenic mechanism of IL-6 still remains obscure, serum IL-6 levels are

elevated in animal models and in patients with NASH and are correlated with the severity of liver histology. [Haukeland JW, 2006; García-Galiano D, 2007] Several studies have shown that the liver is the major target for IL-6 actions where it is responsible for lipid overloading, inhibits insulin signalling and sensitizes hepatocytes to apoptosis. [Klover PJ, 2003]

COX-2, a rate-limiting enzyme, is present at extremely low levels in normal liver. However, hepatic COX-2 activity is markedly upregulated in a metabolic form of steatohepatitis. Our laboratory has previously demonstrated that COX-2 plays an important role in the initiation of liver inflammatory infiltration in NASH [Jun Yu, 2006 & 2007]

1.5 Management of NAFLD

Simple steatosis usually has a benign clinical course. In contrast, patients with NASH are more likely to progress to liver cirrhosis, liver failure and even hepatocellular carcinoma. Metabolic syndrome, obesity and T2DM are the most common risk factors associated with disease progression. [Chan HL, 2007] The management plan for NAFLD should start with lifestyle modification followed by pharmacological therapy. Briatric surgery should be considered for those who are mobidly obese and fail the more conservative treatments.

1.5.1 Lifestyle adjustments for NASH

Most NAFLD patients are obese as well as insulin resistant. A logical approach to treat this population is to promote gradual weight loss through diet and exercise as the first-line management. Physical exercise itself can significantly increase insulin sensitivity and may modify the liver fat content. [Siebler J, 2006] Thus, moderate- to high-intensity exercise (30 min 3–5 times/week) such as walking and jogging should be recommended to patients with NAFLD. [Méndez-Sánchez N., 2007] Rapid weight loss (greater thatn 1 kg/week) which may lead to worsened liver function and accelerate hepatic fibrosis is not advisable. [Farrell GC. Larter CZ, 2006; Powell EE, 1990; Caldwell SH, 2002] Weight reduction from diet and exercise improve insulin sensitivity, decrease hepatic fat content, but has little effect on fibrosis and inflammation.

1.5.2 Pharmacological therapy

At this time, there is no treatment to cure NASH. Treatment of NASH in clinic is still experimental and unproven. Medications are available in the market to assist weight reduction. These medicines can be used in obese individuals. The most popular medications are orlistat, an oral intestinal lipase inhibitor that induces fat malabsorption, and silbutramine, a serotonin reuptake antagonist that decreases the appetite and food intake. They are currently approved to be effective to reduce body weight for at least 6 months treatment. [Zelber-Sagi S, 2006]

The other option for the treatment of NAFLD is insulin sensitizers. Metformin and thiazolidinediones (rosiglitazone and pioglitazone) have been tested in

several small studies. [Chavez-Tapia, 2006; Angelico, 2007] Most studies on metformin have shown significant improvement in liver biochemistry but the benefit on liver fibrosis is less certain. [Nair S, 2004; Duseja A, 2007] In a placebo-controlled, randomized trial using 6-month pioglitazone treatment in NASH, there was significant improvement in the insulin sensitivity and histologic necroinflammation in the treatment group as compared to the control group, but again the improvement in liver fibrosis just fell short of statistical significance. [Belfort R, 2006] One major limitation of most of these studies is the small sample size and short study period. Larger scaled, placebo-controlled studies using histologic liver fibrosis as the primary endpoint is needed before insulin sensitizers can be recommended for the general use to treat NASH in clinical practice.

Antioxidant therapy is a logical remedy to prevent the progression of NASH. Vitamin E has been evaluated to have beneficial effect by suppressing oxidative stress, and reducing insulin resistance and PPARα expression in NASH. [Chang CY, 2006; Yakaryilmaz F, 2007] However, data on the benefit of vitamin E on liver histology is lacking and it cannot be recommended for routine use in patient with NASH.

1.5.3 New molecules and targets

The PPARα agonist Wy-14,643 has been studied in animal models of NASH. Activation of PPARα by Wy-14,643 can ameliorate nutritional steatohepatitis

and fibrosis, presumably due to regulate genes involved in fatty acid oxidation, including upregulation of CYP4a and β-oxidation enzymes, and mediation of fatty acid transport proteins. [Ip E, 2004] However, PPARα agonists are potent hepatocarcinogens in rats and mice. [Gonzalez FJ, 2007] Although, there is no evidence that humans chronically administered PPARα agonists have increased cancer risk, the safety and the role of pharmacologic modulation of hepatic lipid turnover through activating PPARα in the treatment of human NASH is worthy of further investigation.

Inhibiting the expression of COX-2 is another potential treatment target of NASH. COX-2 plays an important role in mediating proinflammatory activation. Selective COX-2 inhibitors (celecoxib and NS-398) have been shown to partially prevent the development of steatohepatitis induced by MCD diet in wild-type C57BL6/N mice. [Yu J, 2006 & 2007].

A novel compound, NO-1886 has been discovered to increase activity of lipoprotein lipase (LPL) which plays a crucial role in the removal of triglycrides and elevation of HDL. [Tsutsumi K, 1993] In recent MCD dietary mouse model, NO-1886 exhibited potential therapeutic effect on NASH by acceleration of fatty acid oxidation, reduction of lipid peroxidation and suppression of pro-inflammatory factors. [Yu J, 2007]

Pentoxifylline, an inhibitor of TNF-α, is also a potential candidate for the treatment of NASH. When pentoxifylline was administrated to rats fed with high-fat diet, it could significantly improve the liver biochemistry, lower TNF-α level and reduce hepatic inflammatory infiltration. [Yalniz M, 2007] One study in humans with 12-month pentoxifylline treatment has shown sustained benefit in the liver biochemistry and histology. [Satapathy SK, 2007] Pharmacological inhibition of TNF-α may be beneficial to patients with NASH, but require further large scaled, randomized, controlled trials before their use can be recommended.

1.6 Phyllanthus species

Phyllanthus species is common in tropical and subtropical regions of both hemispheres. Since the mid-1960s, phyllanthus has been the subjects of many researches to determine the active constituents and their medicinal uses. [Figure 1.2]

1.6.1 Active constituents of phyllanthus

Phyllanthus is a rich source of plant chemicals, including many which have been found only in the Phyllanthus genus. Many of the "active" constituents are attributed to biologically active lignans, glycosides, flavonoids, alkaloids, ellagitannins, and phenylpropanoids found in the leaf, stem, and root of the plant. [Leslie Taylor, 1996] The extracts and purified lignans, including hexane extract, lignan-rich fraction, lignans phyltetralin, nirtetralin, and niranthin, exhibit marked anti-inflammatory properties. [Kassuya CA, 2005] The tannins

and flavonoids found in the plants are both suggested to have antioxidant activities. [Huang YL, 1998]

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Figure 1.2 **Picture of Phyllanthus urinaria Linnea from www. plant.ac.cn.** One of the herbal plants belong to genus Phyllanthaceae with erect growth habit. The plant has alternately arranged leaves disposed in two ranges and small inconspicuous star-shaped flowers arising from the area between the leaflets and the central axis of the leaf.

1.6.2 Pharmacological activities of phyllanthus in liver diseases

In Asian countries, such as India, China and Korea, phyllanthus species have been widely used for medical purposes, especially in the treatment of liver disorders. [Bagalkotkar G, 2006, Chan HLY, 2003] Many studies have shown that phyllanthus species protect the liver from damages by antagonizing oxidative stress, [Bhattacharjee R, 2006 & 2007] preventing lipid peroxidation [Chatterjee M, 2006] and suppressing inflammation. [Kiemer AK, 2003; Rao YK, 2006] Moreover, phyllanthus species have been proven to be effective in protecting against alcohol-induced liver injuries *in vitro* and *in vivo.* It also enhances liver cell recovery by reduction of liver enzymes, TNF-α and IL-1β levels. [Sailaja R, 2006; Pramyothin P, 2006] Cell culture experiments have revealed that phyllanthus exhibited antifibrogenic effects by decreasing proliferation and inducing apoptosis of hepatic stellate cells. [Lee, Simon Kwok-ying, 2006] Phyllanthus has also been reported to have direct antiviral activity in human, animal, and cell culture studies against the hepatitis B virus. However, the results have been inconsistent, due to the general low methodological quality and the variations of the herb. Further larger trials are needed to evaluate the antiviral effect of phyllanthus species in chronic hepatitis B. [Liu J, 2001; Dhiman RK, 2005]

1.7 Study objectives

At present, there is no effective treatment for NAFLD/NASH. Whether phyllanthus species would benefit NASH has not been assessed. In the present

study, we evaluated the effect and the underlying mechanism of phyllanthus on the prevention of steatohepatitis both *in vitro* and *in vivo*. The project will be divided into the following four parts--

1. To set up two rodent hepatocyte cell lines of immortalized hepatocytes AML-12, and primary hepatocytes for in vitro steatohepatitis experiment.
2. To establish two NASH animal models of C57BL/6 and db/db mice.
3. To explore the potential benefits of phyllanthus in prevention of the development of NASH.
4. To investigate the underline mechanisms of phyllanthus protection against the development of NASH.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1 Traditional medicine – *Phyllanthus Urinaria Koreanis* (*P. urinaria*)

P. urinaria was purchased from Chen Hwang Pharmacy, 209-1 Songsan-dong, Mapo-gu, Seoul, Korea. Three major ingredients in Phyllanthus tablet include corilagin, flaronoids and polysaccharides. Each tablet contains 480mg of Korean phyllanthus urinaria extracts (Figure 2.1).

Figure 2.1 ***Phyllanthus Urinaria Koreana.***

2.2 Chemical analysis of methionine and choline in *P. urinaria*

2.2.1 Apparatus and materials

2.2.1.1 Apparatus

HPLC-MS analysis was performed on a Perkin-Elmer series 200 liquid chromatograph connected to a QTRAP LC/MS/MS system (AB Applied Biosystem, MDS, SCIEX).

2.2.1.2 Chemicals and solvents

L-methionine and choline were purchased from Sigma Chemical Co (St. Louis, MO, USA). All solvents used for analysis were HPLC grade. Double deionized water was used for all aqueous solutions and HPLC mobile phase. Two samples, namely *P. urinaria* and control diet, were tested by high-performance liquid chromatography tandem mass spectrometry- mass spectrometry (HPLC-MS/MS).

2.2.2 HPLC analysis of *P. urinaria*

2.2.2.1 HPLC-MS/MS conditions

The analysis was performed on a reversed-phase C18 column (Agilent Zorbax, 4.6×250 mm, 5μm, Agilent , Santa Clara, CA) coupled with a C18 guard column

and were detected using multiple reaction monitoring (MRM) mode. The optimized parameters are shown in Table 2.1.

Table 2.1 **Summary of performing parameter for HPLC-MS/MS**

Parameter	**Methionine**	**Choline**
Mobile phase	water / methanol (9:1)	NH4OAc (50 mM) / methanol (9:1)
Flow rate	0.5 ml/min	0.5 ml/min
ESI	positive	Positive
Nebulizing gas	50 psi	50 psi
Auxiliary gas	50 psi	50 psi
Curtain gas	50 psi	30 psi
DP	16 V	46 V
EP	9.5 V	11 V
CEP	12 V	12 V
CXP	4V	12 V
MRM (m/z) (precursor→product)	→150/61	→104/58

2.2.2.2 Calibration curves

Methanol stock solution containing methionine and choline were prepared and diluted to appropriate concentration ranges for the construction of calibration curves. The injection volume was 30μl. The calibration curves were constructed by peak area against concentration of the corresponding analysis.

2.2.2.3 Sample preparation and analysis

The above mentioned sample preparation method was used for the study. Control diet and *P. urinaria* were weighted precisely and dissolved with 1 ml water (50 mg/ml). The mixture was centrifuged at 2,300 × g for 10min, the resultant solution was filtered, and the filtrate (30μl) was subjected to HPLC-MS analysis.

2.3 Cell culture

2.3.1 Primary hepatocytes and AML-12 cell line

Primary hepatocytes were isolated from male Wistar rats at the age of 6-8 weeks with weight of 250-300g, breed by Laboratory Animal Services Centre, the Chinese University of Hong Kong.

Immortalized AML-12 hepatocyte cell line was bought from American Type Culture Collection (ATCC), Manassas, VA. AML-12 cell line was established from hepatocytes from a human TGF alpha transgenic mouse.

2.3.2 Hepatocyte isolation

2.3.2.1 Materials for hepatocyte isolation

Buffers and solutions are prepared in Milli Q water, sterilized by passing through 0.22μm filters, and stored in 4°C refrigerator. The containing of perfusate and enzyme buffer was listed in table 2.2 and table 2.3.

Table 2.2 **Perfusate (1L), pH 7.2**

Chemical*	Mass (g)	Anhydrous Mass (g)
NaCl		8.000
KCl		0.400
$NaH_2PO_4.2H_2O$	0.078	0.0599
$Na_2HPO_4.12H_2O$	0.151	0.0598
N-[2-hydroxyethyl]piperazine -*N'*-[2-ethanesulfonic acid] (HEPES)	2.380	
Phenol Red	0.006	
[Ethylene-bis(oxyethylenenitrilo)] tetraacetic acid (EGTA)		0.190
$NaHCO_3$		0.350
Glucose	0.900	

* All chemicals were bought from USB Corperation, Cleveland, Ohio.

Table 2.3 **Enzyme buffer: Dulbecco's modified Eagle's medium and F12 1:1 mixture (DMEM/F12) (1L), pH 7.2**

Chemical	Concentration	Manufacturers
DMEM/F12 powder	15.600g	Sigma-Aldrich, Inc. St. Louis, MO
$NaHCO_3$	0.350g	USB Corperation, Cleveland, Ohio
10 000 U/ml Penicillin-10 mg/ml streptomycin, liquid	10ml	Invitrogen, Carlsbad, CA

3. Enzyme perfusate/solution (Prepared prior to use)

a) High concentration of pronase solution (Pronase HC), pH 7.4

0.4g Pronase (Roche, HK) dissolved in 100ml DMEM/F12 solution

b) Collagenase solution, pH 7.2

0.025g Collagenase NB 4 (Serva Electrophoresis GmbH, HK) dissolved in 225ml DMEM/F12 solution

c) Enzyme dispersion solution (EBS)

0.030g pronase dissolved in 100ml enzyme buffer.

d) DNase I solution, pH 7.2

0.006g DNase I (Roche, Hong Kong) dissolved in 30ml enzyme buffer

4. Culture Media

a) Minimum essential medium eagle (MEM): the amount of MEM powdered medium (Sigma-Aldrich, Inc. St. Louis, MO) required for 1L, 2.38g HEPES, 2g $NaHCO_3$, 10ml Penicillin-Streptomycin dissolved in 1L Milli Q water, adjust to pH 7.2.

b) Hepatocyte culture medium: 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, with GlutaMAX™-1, 2.438 g/L Sodium Biocarbonate (Invitrogen, Carlsbad, CA), Insulin-Transferrin-Selenium, 100× (Invitrogen, Carlsbad, CA), 40 ng/ml dexamethasone (Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and 0.2% Primocin (Invivogen, San Diego, CA).

6. Other solution

- 100ml 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for peristaltic

pump sterilization

- 2ml 5% Pentobarbital (Sigma-Aldrich, St. Louis, MO) for rat anesthesia

7. Other requirement (all sterile):

- Sugical tools: Operating scissors, (blunt *1, sharp*1), Pean, curve *1, Dressing forceps (coarse*1; fine*2; tissue*1, 1×2 teeth), Sewing thread
- 500 ml conical flask
- Magnetic stirrer
- Funnel with two layer of clothes
- Angiocath 18G×1.88" (Becton Dickinson, Guangzhou, China)
- Instrument: Masterflex Consle Drive (Cole-Parmer, Vernon Hills, Illinois)

2.3.2.2 Methods

I. Preparation

1. Solutions and buffers including perfusate, DMEM/F12 medium, Pronase HC, collagenase and EBS were kept in 37°C water bath.

2. Peristaltic pump tube was rinsed repeatedly with 3% hydrogen peroxide for about 30 minutes.

3. Peristaltic pump tube was rinsed repeatedly with sterile 250ml MilliQ water for 2 times.

4. Rat was anesthetized using approximately 0.8ml 5% pentobarbital (0.2ml/100g rat)

II. Perfusion

Rat must be kept alive with heart-beating and blood unclotted before pronase HC perfusion.

1. Rat was dissected on tray. Hepatic portal vein was identified and inserted by 18G catheter. Catheter was kept in place and tightened using sewing thread.

2. Rat liver was first washed with at least 100ml perfusate under peristaltic pumping at 15 ml/min until blood was washed away and liver turned pale and yellow. During this and further perfusion steps, the liver should keep warm at 37°C.

3. Once injection was started, severe abdominal artery to allow blood lost.

4. If perfusion is proceeding normally, preparation of collagenase solution should be started at this point.

5. After preperfusion steps, 100ml pronase HC solution was inject at 6 ml/min.

6. The liver was then perfused with 100ml collagenase solution at around 6 ml/min. The duration of this step lasted for a maximum of 20min. During this step, softening of the tissue gradually appeared as well as marbling, indicating that dissociation is proceeding efficiently.

III. Hepatocyte isolation and washing

1. At the end of the collagenase perfusion, the liver sample was removed from rat into a sterile dish and transferred into a laminar flow hood. Operating in the culture hood, attached non-liver tissue was removed (eg. fats and muscles). Liver was gently disrupted with forceps and scissors. The homogenate of liver was transferred into sterile 100mm culture dish and complemented with 4ml 4°C DNase I solution and 30ml Pronase LC solution. 4°C MEM solution was added

into the homogenate to 100ml and shaken at 160 rpm for 30 minutes at 37°C water bath.

2. The homogenate was filter through a nylon filter (250 meshes) and the filtrate is distributed into 50mL centrifuge falcons. The filter is washed twice with approximately 200ml of MEM solution to collect the hepatocytes that are trapped in the undissociated tissue homogenate.

3. Tubes were centrifuged at 60 × g for 8min at room temperature.

4. After centrifugation, the supernatant was discarded and the pellet, represented hepatocytes, gently resuspended in 3ml DNase I and 45ml 4°C MEM to each falcon with pipetting up and down. The falcons were centrifuged at 60 × g for 8min at room temperature.

5. Step 3 and 4 are repeated twice. At the end of the last centrifugation, the yield of the preparation may be roughly estimated by measuring the volume of the pellet: 1mL of pellet represents approximately 10E8 cells.

6. At the end of the last washing, the pellet is resuspended in an culture medium: 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, with GlutaMAX^{TM}-1, 2.438 g/L Sodium Biocarbonate, 100×Insulin-Transferrin-Selenium, 40 ng/ml dexamethasone, 10% fetal bovine serum, and 0.2% Primocin antibiotics.

7. Yield and viability of cells are classically evaluated by examination under a microscope using the Trypan-blue exclusion test. In our hands, the cell viability, on average, was 85%.

2.3.3 General culture media

AML-12 cell line and primary hepatocytes were cultured in the 1:1 mixture of DMEM and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, and 10% fetal bovine serum (Table 2.4).

Table 2.4 **Reagents of general culture media**

Reagents	Manufacturers
Penicillin/streptomycin (5 ml/L)	Invitrogen, Carlsbad, CA
Fetal bovine/calf serum (FBS)	Invitrogen, Carlsbad, CA
Phosphate Buffered Saline (PBS)	Sigma, St. Louis, MO
Trypsin-EDTA	Invitrogen, Carlsbad, CA
Insulin-Transferrin-Selenium, 100×	Invitrogen, Carlsbad, CA
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, MO
Dexamethasone, 40 ng/ml	Sigma, St. Louis, MO
DMEM/F12 liquid, with GlutaMAX^{TM}-1, 2.438 g/L Sodium Biocarbonate	Invitrogen, Carlsbad, CA
D-MEM/F12 without methionine and choline (MCD) liquid	Invitrogen, Carlsbad, CA

2.4 Animal models construction

2.4.1 P. urinaria administration

The dosage of P. urinaria recommended by Chen Hwang Pharmacy is 6.4 mg/kg body weight (BW) daily for liver protection purpose, while the maximal dosage of treatment for people with hepatitis and liver diseases is 51.2 mg/kg BW per day (assuming 75kg in weight). The dosage of P. urinaria use in animal was calculated according to the guidelines issued by Food and Drug Administration, US Department of health and Human Service. According to the guidelines the conversion of human equivalent dose to animal dose was equal to multiplication by a factor of 12.3 in mouse. Thus the dosages of 78.7 mg/kg BW/day to 629.8 mg/kg BW/day in mice is equivalent to the therapeutic dosages in human. We used low dose, 500 ppm, 1000 ppm and 2000 ppm (equal to 50, 100, 200 mg/kg BW per day, respectively), to assess the preventive potential of P. urinaria on NASH.

2.4.2 Animal models of NASH

Wild-type C57BL/6 mice, male, 8-week old and diabetes db/db (defective long-form leptin receptor) mice, female, 8-week old were all breed by Laboratory Animal Services Centre, the Chinese University of Hong Kong, housed in a 22°C-controlled room under a 12-hour light-dark cycle, with free access to water. They were allowed to adapt to their food and environment for 1 week before starting the experiment.

The C57BL/6 mice were divided into 5 groups (5/group) and fed with control diet containing 2 g/kg choline bitartrate and 3 g/kg DL-methionine (ICN, Biochemicals, Aurora, OH), Methionine and choline deficient (MCD) diet (ICN) or MCD diet supplemented with P. urinaria (Chen Hwang Phamacy, Korea) at 500, 1000 or 2000 ppm, respectively. A separate experiment was performed on db/db mice from the C57BL/6 background with 3 groups feeding with control diet, MCD diet and MCD diet supplemented with Phyllanthus at 1000 ppm. Body weight, physical appearance and food consumption were recorded every day during the experiment period.

After 10 days experiments, mice were sacrificed under anethesia. Blood was collected by cardiac puncture and livers were rapidly excised and weighed. Aliquots of liver were fixed in 10% formalin for histological analysis or snap frozen in lipid nitrogen and kept at -80°C until required.

All animal experiments and procedures were performed by holders of animal experimentation license issued by Hong Kong Government and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

2.5 Histological staining and grading

2.5.1 Fixation and processing of liver tissue for paraffin sections

Liver tissues were fixed in 10% formalin at room temperature for 8 hours but not to exceed 24 hours. The formalin-fixed tissues were dehydrated on Shandon Pathcentre® Tissue Processor (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then tissues were embedded with paraffin in waxes. Paraffin-embedde liver tissues were cut into 4μm thick sections on a Leica RM2135 microtome (Wetzlar, Germany) and float on 40°C water bath containing distilled water. The sections were transferred onto superfrost slides. Allow the slides to dry overnight and store slides at room temperature for histological staining.

2.5.2 Hematoxylin and Eosin (H&E) staining

1. Reagents for H&E

- Xylene: Laboratory grade (Anapath , Cheyenne, Wyoming)
- Acid Ethanol: 1 ml concentrated HCl + 400 ml 70% ethanol (Sigma, St. Louis, MO)
- Hematoxylin: Harris hematoxylin with glacial acetic acid (Sigma, St. Louis, MO)
- Eosin: Eosin Phloxine stain (Sigma, St. Louis, MO)

2. Protocol for H&E staining

- slides containing paraffin sections were placed in a metal slide holder
- Sections were deparaffinized and rehydrated:

  3 x 3′ Xylene (excess xylene was blotted before going into ethanol)

  3 x 3′ 100% ethanol

1 x 3′ 95% ethanol

1 x 3′ 80% ethanol

1 x 5′ deionized H2O

- Hematoxylin staining

  1 x 10′ Hematoxalin

  Rinsed in deionized water

  1 x 5′ Tap water

  Dip 1 to 3 sec Acid ethanol to destain

  Rinsed 2 x 1′ under tap water

  Rinsed 1 x 2′ in deionized water

- Eosin staining and dehydration:

  1 x 10′ Eosin

  3 x 5′ 95% ethanol

  3 x 5′ 100% ethanol (excess ethanol was blotted before going into xylene)

  3 x 15′ Xylene

- Coverslip slides using permount
- Dried overnight in the hood.

2.5.3 Histological grading

H&E stained slides were examined by by 2 independent liver pathologists blinded to the study and were scored for hepatic steatosis (Table 2.5) and necroinflammation as: 0, none; 1, mild; 2, moderate; 3, severe (Table 2.6). [Pauline de la M. Hall, 2005] Images of liver tissues were also photographed

using a Leica DM RXA_2 microscope (Wetzlar, Germany) in combination with a Leica DCS500 digital camera (Wetzlar, Germany) under objective x10 and x 20.

Table 2.5 **Grading of steatosis**

Grade	**Steatosis**
Grade 0	No fat droplets in hepatocytes
Grade 1	Fat droplets in 1-25% hepatocytes
Grade 2	Fat droplets in 26-50% hepatocytes
Grade 3	Fat droplets in 51-75% hepatocytes
Grade 4	Fat droplets in >75% hepatocytes

Table 2.6 **Grading of necroinflammation**

Grade	**Lobular inflammation**	**Portal inflammation**
Grade 0	Absent	None
Grade 1	Polymorphs and mononuclear cells, mild and scattered	None, or mild
Grade 2	Polymorphs and mononuclear, moderate	Mild, or moderate
Grade 3	Polymorphs concentrated in areas of ballooning, marked	Not severe, or severe

2.6 ALT testing

Cell-free culture media were collected to test ALT activity with Sigma diagnostics transaminase reagents (Sigma, St. Louis, MO). 1 ml Alanine-α-KG substrate was pipetted into a 2ml test tube and warmed in a 37°C water bath. Exactly after 0.2 ml media was added into and mix with Alanine-α-KG gently, 1ml Sigma coloring reagent was added and shook gently to mix at room temperature. After twenty minutes, 10ml 0.4N sodium hydroxide solution was added to the sample and mixed by inversion. 5 minutes later, three samples at each of 300μl from the mixture were pipetted into a 96 well microplate and absorbance determined at wavelength 490 nm was measured by a microplate spectrophotometer VICTOR3TM multilabel counter (Perken Elmer, Waltham, MA). The ALT activity expressed in international units per liter (U/L) was determined from the calibration curve according to the manufactory's protocol.

2.7 Analysis of hepatic steatosis

2.7.1 Oil Red O dye

1. Reagents

- Oil Red O powder (Sigma, St. Louis, MO)
- Oil Red O stock solution: 0.5 g Oil Red O was mixed with 100 ml isopropanol. The mixture was warmed in a water bath at 56°C for 1 hour, then cooled and filtered through filter paper.

- Oil Red O working solution: deionized water was added into and mixed well with Oil Red O stock solution at the ratio 4: 6. The mixture stood at room temperature for 10mins, and then was filtered through filter paper. The solution can be kept at room temperature for 24hs.

2. Protocol for Oil Red O staining

- Cells were seeded on coverslip in 6-well plate to 70% confluence (about 1.0×10E5 cells).
- Media were change to treated media including control, MCD, and MCD medium with 0.5 mg/ml or 1 mg/ml P. urinaria.

Post-fixation

- Media were removed and the cells in wells were rinse with PBS.
- The coverslips with cells were post-fixed in 4% paraformaldehyde for 1 hour.
- Rinsed with PBS. Cells can be store in PBS at 4°C for at most 1 week at this step.

Neutral fats staining

- Cells were rinsed in deionized water.
- Cell were rinsed in 50% isopropanol for 5mins.
- Oil Red O working solution was added into wells and stained cells for 30mins, then removed.
- Cells were rinsed in 50% isopropanol for several seconds to remove excess stain.
- Cells were rinsed in deionized water.

Nuclei stain

- Cells were stained in hematoxylin solution for 3mins.
- Rinsed in deionized water.
- Rinsed in Acid alcohol for 1 second.
- Rinsed in deionized water.
- Cells on the coverslip were immersed in tap water for 3mins.
- Rinsed in deionized water.
- Rinsed in 70%ethanol
- Coverslips were half dried and transferred from wells to slides.

Images of cells stained with Oil Red O were obtained with a Leica DCS500 digital camera at x100 and x 200 magnifications.

2.7.2 Hepatic triglycerides testing

1. Preparation of E-test reagents: Wako triglyceride E-test kit was purchased from Wako Pure Chemical Industries, Osaka, Japan. One vial of chromogen substrate was solved in 105ml buffer for working solution according to the company protocol. The solution can be stored for up to 10 days at 4°C.

2. Extration of liver lipids: 10% (w/v) liver tissue (about 30mg liver tissue) was homogenized in water and was kept on ice. Or hepatocyte pellet was resuspended in 50µl water on ice box. 50µl liver homogenate or total hepatocyte suspension was added into 250µl premixed solution (6ml ter-butyl alcohol, 2ml

methanol and 2ml triton X-100) on a vortex, and then centrifuged under 1000 rpm for 5mins at 4°C.

3. Reaction: 10μl extract (in triplicate), 5μl and 10μl standard (supplied in kit, Glycerol 31.2 mg/dL, corresponding to 3 mg/ml Triolein) were added into 1.2ml working solution. The mixtures were warmed in 37°C water bath for 5mins. The absorbance of the blue color was measured at 600nm.

2.8 Assays for oxidative stress

2.8.1 Dichlorofluorescein (DCF) examination

The DCF assay was performed to measure the formation of ROS production. Non-polar, non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) can easily pass lipid membrane and undergo deacetylation by cellular esterases to form the polar, nonfluorescent dichlorodihydrofluorescein (DCFH). The DCFH product, by reacting with ROS, can be oxidized to form the fluorescence derivative dichlorofluorescein (DCF). Carboxy-DCF H-DA used as DCF probe was purchased from Invitrogen. Stock solutions of DCFH-DA (5.0mM) were prepared in dimethyl sulfoide (DMSO) and kept frozen at -20°C.

After isolation and washing, viable primary hepatocytes (1×10E4 cells/well) were seeded into 96-well plate. 24h later, cells were treated with control, MCD, MCD + 0.5 mg/ml P. urinaria and MCD + 1 mg/ml *P. urinaria* media, 8 wells repeated respectively. After 24h's incubation, the cells in the plates were washed

with phosphate sodium buffer and incubated with 100μM DCFH-DA in the DMEM/F12 medium and 1% FBS for 30min at 37°C. The formation of oxidized fluorescent derivative (DCF) was detected by VICTOR3TM multilabel counter at an excitation wavelength of 485nm and an emission wavelength of 535nm. The percentage increase in fluorescence per well was calculated by the formular $(Ft_{30}- Ft_0)/ Ft_0 \times 100\%$, where Ft_0 = fluorescence at time 0 min and Ft_{30} = fluorescence at time 30 min. [Wang H., 1999]

2.8.2 Thiobarbituric acid reactive substances (TBARS) testing

The following method is based on that of Ohkawa et al. 1979, and given some modification.

- A 10% (w/v) liver (30 to 40mg) homogenate or 100μl cellular suspension was prepared in 1.15% KCl.
- 15ml Falcons were set up to contain 100μl liver homogenate or cellular suspension in triplicate.
- Another series of tubes containing 0, 5, 10, 15, 20, 25, 30, 40, and 50nmol 1,1,3,3-tetramethoxypropne (TMP) (Sigma, St. Louis, MO) in duplicate for a standard curve.
- 1.5ml 20% acetic acid (pH of acid adjusted to 3.5 with NaOH) (Sigma, St. Louis, MO), 1.5ml 0.8% thiobarbituric acid (TBA, Sigma, St. Louis, MO) (400mg TBA mixed with 5ml DMSO and 45ml distilled water) and 0.9ml distilled water were added into the tubes prepared before and mixed by inverting.

- The mixture was heated in a boiling water bath for 1 hour.
- The heated tubes were cool under tap water.
- 1ml distilled water and 5ml n-butanol/pyridine (15:1, v/v, Sigma, St. Louis, MO) were added in to the reaction and mixed vigorously on the vortex.
- The tubes were centrifuged at 4000 rpm for 10mins at room temperature.

The absorbance with pink color was determined at 532nm.

- The amount of TBARS in nmol was determined from the standard curve.

2.9 mRNA expression analysis

2.9.1 mRNA extraction

1. Homogenization: Frozen liver tissues were homogenized in 1ml Trizol reagent (Invitrogen, Carlsbad, CA) using homogenizer in 1.5ml microcentrifuge tubes.

2. Phase separation: The homogenized samples were incubated for 5mins at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes, then added with 0.2ml of chloroform was added per 1ml of Trizol reagent. The tubes were shaked vigorously by hand for 15 seconds and incubated for 3mins at room temperature. Then the samples were centrifuged at 12,000 × g for 10mins at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol used for homogenization.

3. RNA precipitation: The aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by mixing with 0.7ml isopropanol. The mixture was incubated at room temperature for 10mins, then cemtrifuged at 12,000 × g for 10mins at 4°C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tubes.

4. RNA wash: The supernatant was removed and the RNA pellets were washed once with 75% ethanol, at least 1ml of 75% ethanol added. Mixed the samples by vortexing and centrifuged at no more than 7,500 × g for 5 minutes at 4°C. At the end of the procedure, the RNA pellets were air-dried for 5-10 minutes. The RNA pellets were re-suspended with 20-50μl RNase-free water and stored at -80°C. The concentration of the sample was determined with UV-spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The quality of RNA was evaluated by A260/280 value.

2.9.2 Reverse transcriptional (RT) reaction

The reaction was performed according to the protocol provided by Reverse Transcription System (Promega, Madison, WI).

1. RNA was placed in a microcentrifuge tube and thawed on ice and combined (up to 1 μg) with the cDNA primer in Nuclease-free water for a final volume of 5μl per reaction. Reagents were added to the reaction in the following list:

Experiment reactions

Component	**Volume**
RNA template (1 μg)	1.0 μl
Random primer	1.0 μl
Nuclease-Free water	3 μl
Final volume	5 μl

3. The tubes was placed into a preheated 70°C heat block for 10 minutes, then immediatedely chilled in ice for at least 5 minutes. Each tube was spinned for 10 seconds in a microcentrifuge to collect the condensate and maintained the original volume. The tubes were kept closed and on ice until the RT reaction mix is added.

4. The RT reaction mix was prepared by combining the following components. Vortexed gently to mix, and kept on ice.

Experiment reactions

Component	**Volume**
Reverse transcription 5× buffer	4.0 µl
$MgCl_2$, 25 mM (6mM final conc.)	4.8 µl
dNTP mix (10mM)	1.0 µl
Recombinant RNasin Ribonuclease Inhibitor (1 u/µl, optional)	0.5 µl
M-MLV reverse transcriptase	1.0 µl
Nuclease-Free water	3.7 µl
Final volume	15 µl

5. 5µl of RNA and primer mix was added to each reaction, given a final reaction volume of 20µl per tube.

6. The RT reaction was carried out in an anneal-extend-inactivate reverse transcriptase program. The temperature was set at 25°C for 10mins and 42°C for 60mins. The action tubes then were heated at 95°C for 5 minutes to stop the reaction. The cDNA product was incubated at 4 °C.

2.9.3 Real-time polymerase chain reaction (real-time PCR)

Real- time PCR was used to quantitate differences in mRNA expression among the liver tissues of groups. Related gene primers for Real- time PCR reaction were designed with Primer Express software (Applied Biosystems, Foster, CA), and diluted at 10µM in Nuclease-Free water (Table 2.7). 2µl of each cDNA

product at 100 × dilution was prepared for experiment reaction and SYBR Green Master Mix (Applied Biosystems, Foster, CA) was used in the procedure of the assay according to the manufacturer's instructions. A no-template-control and standard samples were prepared for each pair of primers. The contents of the reactions were transferred to a MicroAmp optical 96-well reaction plate which then was sealed with a MicroAmp optical adhesive cover (Applied Biosystems, Foster, CA). The PCR reactions were performed in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster, CA). Each Ct value was normalized to housekeeping gene β-actin.

Experiment reactions	
2 ×SYBR Green Master Mix	12.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Template (diluted cDNA)	5 µl
Nuclease-Free water	6.5 µl
Total volume	25 µl

Table 2.7 **Primer Sequences used for amplification of mRNA by real time PCR**

mRNA	Accession No.	Forward Primer	Reverse Primer
SCD1	NM009127	5'-GTACCGCTGGCACATC AACTT-3'	5'-GGCTAAGACAGTAGCC TTAG-3'
LXRα	NM013839	5'-TCAGCATCTTCTCTGCA GACCGG-3'	5'-TCATTAGCATCCGTGG GAACA-3'
LXRβ	NM009473	5'-AAGCTGGTGAGCCTGC GC-3'	5'-CGGCAGCTTCTTGTCCT G-3'
ACO	NM015729	5'-GGAAGACTTCCAATCA TGCGATAG-3'	5'-GACAACAAAGGCATGT AACCCG-3'
PPARγ	NM011146	5'-CAGGCTTGCTGAACGT GAAG-3'	5'-GGAGCACCTTGGCGAA CA-3'
LCAD	U21489	5'-CAGAGAAACATGGCGG CA-3'	5'-AGCCAGCGCGTGTGCA ATT-3'
FAS	NM007987	5'-GCAAACCAGACTTCTA CTGCG-3'	5'-TTTGTATTGCTGGTTGC TGTG-3'
PPARα	NM011144	5'-TGGGGATGAAGAGGGC TGAG-3'	5'-GGGGACTGCCGTTGTC TGT-3'
CYP2E1	BC042693	5'-AGTGTTCACACTGCAC CTGG-3'	5'-CCTGGAACACAGGAAT GTCC-3'

Table 2.7 **Primer Sequences used for amplification of mRNA by real time PCR (continued)**

CYP4A14	NM007822	5'-GCCAGAATGGAGGATA GGAACA-3'	5'-ATGAATGTGTCCACCT CTGCAC-3'
CYP4A10	NM01001	5'-ACACTGCTCCGCTTCG AACT-3'	5'-CAAGTCGGGCTAAGGG CA-3'
IL-6	NM031168	5'-ACAACCACGGCCTTCC CTACTT-3'	5'-GTGTAATTAAGCCTCC GACT-3'
TNF-α	NM013693	5'-CGTGCTCCTCACCCAC AC-3'	5'-GGGTTCATACCAGGGT TTGA-3'
IL-1β	NM008361	5'-TCAGGCAGGCAGTATC ACTCA TT-3'	5'-GGAAGGTCCACGGGAA AGA-3'
TGF-1ß	NM011577	5'-CAACTTCTGTCTGGGA CCCT-3'	5'-TAGTAGACGATGGGCA GTGG-3'
ICAM-1	NM010493	5'-CCTGCCTCTGAAGCTC GGAT-3'	5'-ACAGGAACTTTCCCGC CACC-3'
β-actin	NM031144	5'-TACTGCCCTGGCTCCTA GCA-3'	5'-TGGACAGTGAGGCCAG GATAG-3'

2.10 Protein expression analysis

2.10.1 Materials and antibodies

Table 2.8 **Materials for protein manipulation**

Materials	**Manufacturers**
Acrylamide/Bis-acrylamide	Beohringer Mannheim, Mannheim, Germany
Aprotinie	Sigma, St. Louis, MO
Bovine serum albumin (BSA)	Sigma, St. Louis, MO
Bromophenol blue	Bio-Rad, Hercules, CA
Cytobuster	Merck KGaA, Darmstadt, Germany
Dihiothreitol	Sigma, St. Louis, MO
Dithiothreitol (DTT)	Sigma, St. Louis, MO
Enhanced chemiluminescence (ECL) western blotting detection	Arnersham Biosciences, Buckinghamshire, UK
Glycerol	Sigma, St. Louis, MO
Glycine	Sigma, St. Louis, MO
Methanol	Lab-scan, Patumwan, Bangkok
NaCl	USB Corperation, Cleveland, Ohio
Nonidet P-40	USB Corperation, Cleveland, Ohio

Table 2.8 **Materials for protein manipulation (continued)**

Materials	Manufacturers
Paraformaldehyde	Sigma, St. Louis, MO
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, St. Louis, MO
Polyvinylidene difluoride (PVDF) membrane	Amersham Biosciences, Buckinghamshire, UK
protease inhibitor	Promega, Madison, WI
Proteins molecular weight marker full range rainbow marker	Bio-Rad, Hercules, CA
Sodium orhovanadate	Sigma, St. Louis, MO
Sodium dodecyle sulfate (SDS)	USB Corperation, Cleveland, Ohio
Tris-HCL	USB Corperation, Cleveland, Ohio
Triton	USB Corperation, Cleveland, Ohio
Tween-20	USB Corperation, Cleveland, Ohio
X-ray film	Amersham Biosciences, Buckinghamshire, UK
avidin–biotin complex (ABC)	Dako A/S, Glostrup, Denmark
horseradish peroxidase	Dako A/S, Glostrup, Denmark
Diaminobenzidine (DBA) substrate kit	Becton Drive, Franklin Lakes, NJ USA

Table 2.9 **Antibodies for immunoblotting and immunohistochemistry**

Antibodies	**Manufactures**	**Cat. No.**	**Diluted rates**
Anti-Phosphorylated C-jun N-terminal kinase (P-JNK)	Cell Signaling, Danvers, MA	9255	1:2000
Anti-GAPDH	Abcam, Hong Kong, China	ab9484	1:8000
Rabbit Polyclonal Antibodies			
Anti-CCAAT/enhancer binding protein β (C/EBPβ)	Cell Signaling, Danvers, MA	3080	1:2000
Anti-CYP2e1	Abcam, Hong Kong, China	Ab28146	1:2000
Anti-NF-κB subunit p65	Santa Cruz Biotechnology, Santa Cruz, CA	sc-109	1:50
Secondary Antibodies			
Anti-Mouse IgG_1-HRP	Cell Signaling, Danvers, MA	7076	1:6000
Anti-Rabbit IgG-HRP	Cell Signaling, Danvers, MA	7074	1:5000

2.10.2 Western blotting

This technique was used to detect protein expression involving the process from protein separation, protein transfer, to immunoblotting with antibodies.

2.10.2.1 Preparation of protein extract

20μg frozen liver tissue was homogenized in 400μl Cytobuster with 4μl protease inhibitor and incubated in room temperature for 30mins. After centrifugation at 10,000 rpm for 10mins at 4°C, the supernatant (protein extract) was allocated to several microcentrifuge tubes and proceeded with analysis or stored at -80 °C.

2.10.2.2 Preparation of nuclear protein extract

A. Buffers and solutions

1. Buffer A: Contain 10 mM HEPES, adjust to pH 7.9. 10mM KCl, 2mM Mg Cl_2, 0.1mM ethylenediaminetetraacetic acid (EDTA) in HEPES solution. Prepare stock solution 1M DTT.Before using, add DTT, adjust concentration to 0.5mM and protease inhibitor to buffer A.

2. Buffer B: 10mM HEPES, adjust to pH 7.9. 10mM KCl, 2mM Mg Cl_2, 0.1mM EDTA and 0.1% Triton X-100 in HEPES solution. Before using, 0.5mM DTT and protease inhibitor were added to Buffer B.

3. Buffer C: 10mM HEPES, adjust to pH 7.9. 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA and 25 % (v/v) Glycerol in HEPES solution. Before using, 0.5mM DTT and protease inhibitor were added into Buffer C.

B. Nuclear protein extraction

1. add about 50mg tissue in 0.5ml ice-cold **Buffer A**, homogenize, incubate on ice for 10mins, spin at 2500 rpm, 3mins;
2. Remove supernatant completely;
3. Add 3X pellet volume of **Buffer B** (ice-cold) (100~150ul, depending on the pellet size), tap or flick to resuspend the pellets, incubate on ice for 15mins;
4. Add 50ul 2% NP-40, vortex 10 x 1 sec (or homogenize cells with 1cc syringe and 27 G ½ needle, 10 times);
5. Spin at 3500 rpm, 5mins;
6. Collect the supernatant which contains cytoplasmic protein into a 1.5 Eppendorf tube and freeze at -80°C; the pellet contains nuclear fraction;
7. Resuspend the pellet in 80ul **Buffer C**;
8. Vortex vigorously;
9. Incubate on ice for 30mins with occasional vortexing;
10. Spin at 13,000 rpm for 10mins at 4°C;
11. Save the supernatant (nuclear protein extract) at -80°C;
12. Perform protein assay (keep samples on ice in cold room during protein assay), aliquot into at least 3 separate tubes and store at -80°C.

2.10.2.3 Determination of the concentration of protein

Protein concentration was evaluated by DC protein assay (Bio-Rad, Hercules, CA). The standard curve was generated with BSA in water (0 – 1.5 mg/ml). 5µl of standards and sample proteins were added into 96-well plate (Becton Dickson

Labware, Franklin Lakes, NJ). After pretreated with 25µl mixture of DC reagent A and S (50:1), the proteins were added with 200µl DC B reagent. The mixtures were incubated for 15mins in room temperature before the absorbance of blue color was detected at 700nm and the concentration of proteins was determined from standard curve.

2.10.2.4 Gel electrophoresis and protein transfer

20µg protein samples were mixed with 6 × SDS loading buffer (62.5mM Tris-HCl pH 6.7, 100mM dithiothreitol, 2% w/v SDS, 0.01% w/v bromophenol blue, 10% glycerol). The protein was denatured by heating at 95°C for 10mins and cooled on ice. The protein sample per lane was resolved on a 10% sulphate-polyacrylamide gel (SDS-PAGE). Broad range markers were loaded to determine molecular weights.Electrophoresis was carried out at room temperature at 100V for one hour and a half.

After electrophoresis, the gel was immersed in pre-chilled transfer buffer (25mM Tris base, 0.2M glycine, 20%methanol, pH 8.5) at 4°C for 15mins. The proteins were electrotransfer to PVDF membrane using a Bio-Rad Mini Trans-Blot cell. The transfer sandwich was assembled with sponge on the bottom, followed by PVDF membrane, stacking gel, and sponge. The transfer was performed for 35-50mins at 15V.

2.10.2.5 Immunobloting

After transfer, the PVDF membrane was washed with 20ml Tris buffered saline (TBS) (24.2g Tris base, 80g NaCl, ajust pH to 7.6 with HCl) for 15mins at room temperature. Then the membrane was incubated in 20ml of blocking buffer (5% w/v BSA, 1 × TBS, 0.1% Tween-20) for one hour at room temperature. After blocking, the membrane was washed three time for 15, 5, 5mins, respectively, each with 15ml of washing buffer (1 × TBS, 0.1% Tween-20 (TBS-T)). For western blots, membrane was incubated with diluted primary antibody under gentle agitation overnight at 4°C. The next day, the membrane was washed three times for 5mins with 15ml of TBS-T, then incubated with HRP-conjugated secondary antibody under gentle agitation for one hour at room temperature. Following the three times TBS-T washing for 5mins, ECL reagent kit was added onto the membrane and exposed to x-ray films. An initial 10-second exposure should indicate the proper exposure time.

The primary and secondary antibodies were diluted in blocking buffer, and diluted rates of antibodies used in immunoblotting were described in Table 2.9.

2.10.3 Immunohistochemistry

A. Solutions and reagents

- Phosphate Buffered Saline (PBS)
- 0.5% hydrogen peroxidate （H_2O_2）solution in PBS
- Horseradish Peroxidase
- DAB Substrate Kit

- Hematoxylin
- Bluing Reagent
- Graded alcohols
- Xylene
- Blocking solution: 10% nonimmunized goat serum in PBS.
- ABC solution

B. Deparaffinization and dehydration

- Sections were incubated in three washes of xylene for 5mins each.
- Sections were incubated in two washes of 100% ethanol for 10mins each.
- Sections were incubated in two washes of 95% ethanol for 10mins each.
- Sections were washed twice in deionized water for 5mins each.

C. Staining

- Sections were incubated in 0.5% H_2O_2 solution for 10 minutes.
- Slides were rinsed 3x in PBS for 5mins each time.
- Blocked non-specific binding by incubating with blocking solution for 30-60 min at RT.
- Primary antibody was added with dilution in blocking solution. Incubated for 2hs at room temperature.
- Slides were rinse 3x in PBS, 5 minutes each time.
- The biotinylated secondary antibody diluted in blocking solution and ABC reagent were applied to the tissue sections on the slide and incubated for 30 minutes at room temperature.

- Removed secondary antibody solution and washed section three times with PBS for 5mins each.
- The Horseradish Peroxidase was added pre-diluted to the tissue sections on the slide and incubate for 30 minutes at RT.
- Rinsed slides 3x in PBS, 2 minutes each time.
- DAB substrate solution was prepared by adding 1 drop of DAB chromagen to every 1 ml of DAB buffer. (When using other substrates follow manufacturer's recommendations.)
- Drain PBS from slides and apply the DAB substrate solution. Allow slides to incubate for 5 minutes or until the desired color intensity is reached.
- As soon as the sections developed, immersed in water.

D. Counterstain slides:

- Slides were dipped twice in Hematoxylin.
- Slides were rinsed thoroughly in water.
- Slides were dipped twice in Bluing Reagent or dilute ammonia water.
- Slides were rinsed thoroughly in water.
- Dehydrated through 4 changes of alcohol (95%, 95%, 100% and 100%). Clear in 3 changes of xylene (or xylene substitute) and coverslip using mounting solution.

2.11 Statistical analysis

Data were expressed as mean ± SD. Comparisons between the different treatment groups were analyzed via the Student's *t*-test and one-way ANOVA

analysis (followed by Scheffé F test). A two-side P value of less than 0.05 was considered statistically significant.

CHAPTER 3. DETERMINATION OF METHIONINE AND CHOLINE IN *P. URINARIA* TABLET

3.1 Introduction

MCD medium cell culture and dietary animal model are standard methods used to study the pathogenesis and potential therapeutic strategies of NASH. The proposed mechanism responsible for MCD induced-NASH model may be attributable to the impairing synthesis of glutathione (GSH), which contributes to oxidative stress, lipid peroxidation and impaired mitochondrial β-oxidation [Yermolaieva O., 2004; Yao, Z. M.1988; Ghoshal AK,1995] MCD diet induced-NASH animal models appear to have equivalent pathological features of the second 'hit' in human NASH, including increased oxidative stress, decreased antioxidant capacity, kuffer cell-mediated inflammatory response and progression of NASH.

In our study, we tested the preventive effect of *P. urinaria* on the development of NASH induced by MCD. *P. urinaria* commercial herbal medicine is the extract of whole *phyllanthus urinaria* herbal plants (Chen Hwang Pharmacy, Korea). It is a rich souse of plant chemicals, and the active constituents of Phyllanthus have not been fully delineated. Hence, it is necessary to examine the methionine and choline contents in the tablets of *P. urinaria*.

3.2 Materials and methods

The methionine and choline in the *P. urinaria* tablet and the control diet which contain methionine and choline were measured using a combined HPLC-MS/MS system that consisted of a Perkin-Elmer series 200 liquid chromatograph connected to a QTRAP LC/MS/MS system (Applied Biosystem, MDS, SCIEX). The analysis was performed on a reversed-phase C18 column (Agilent Zorbax, 4.6 × 250 mm, 5μm, Agilent, Santa Clara, CA) coupled with a C18 guard column and were detected using multiple reaction monitoring mode. High-pressure liquid chromatography-grade L-methionine and choline were purchased from Sigma Chemical Co. Methanol stock solutions containing methionine or choline were prepared with gradient elution for the construction of calibration curves. Control diet or *P. urinaria* was weighted and dissolved with water to 50 mg/mL. The mixture was centrifuged at 2,300g for 10 minutes, and the resultant solution was filtered, and the filtrate (30L) was subjected to high-performance liquid chromatography-tandem mass spectrometry analysis.

3.3 Results

High-performance liquid chromatography-tandem mass spectrometry analysis method was successfully established. HPLC-MS/MS analysis was used for the quantification study due to the high sensitivity of the method. The calibration curves of methionine and choline were described by y=87696x+2000000 (R^2=0.9973) and y=207298x+518837 (R^2=0.9972) at the concentration ranges 1.39-20.9 and 0.65-6.50 μg/g respectively. The retention time of choline and methionine was 4.30mins (Figure 3.1) and 5.11mins (Figure 3.2), respectively.

The lower limit of quantification of 1.33 μg/g for methionine and 0.67 μg/g for choline was adequate for the analysis.

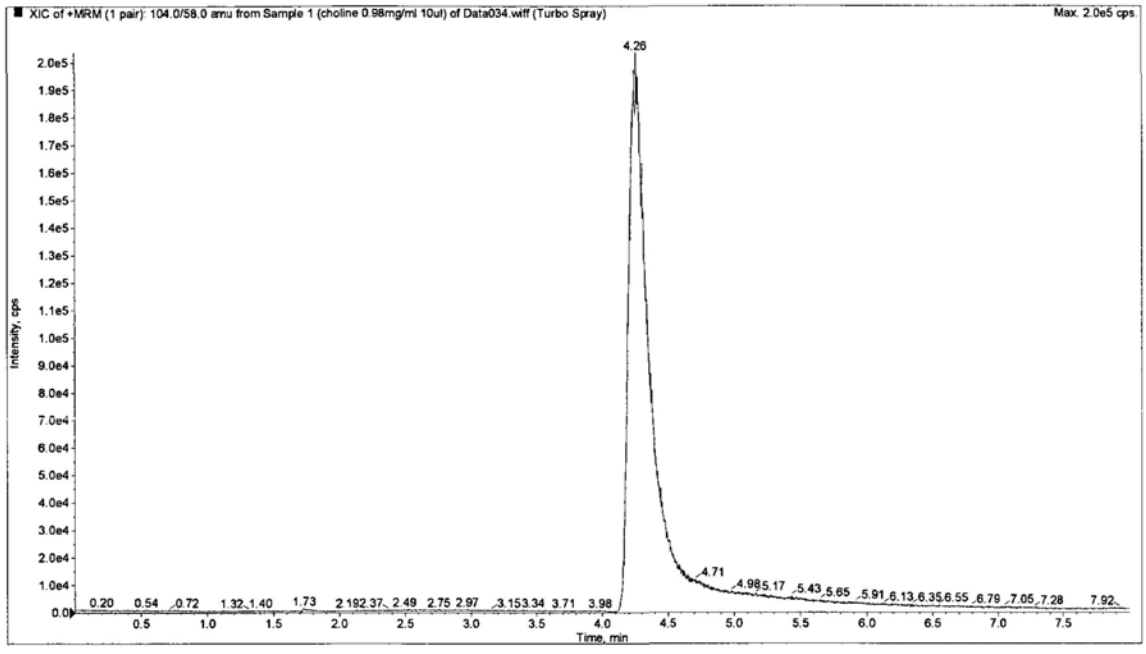

Figure 3.1 **HPLC chromatogram of authentic choline by HPLC-MS/MS analysis**

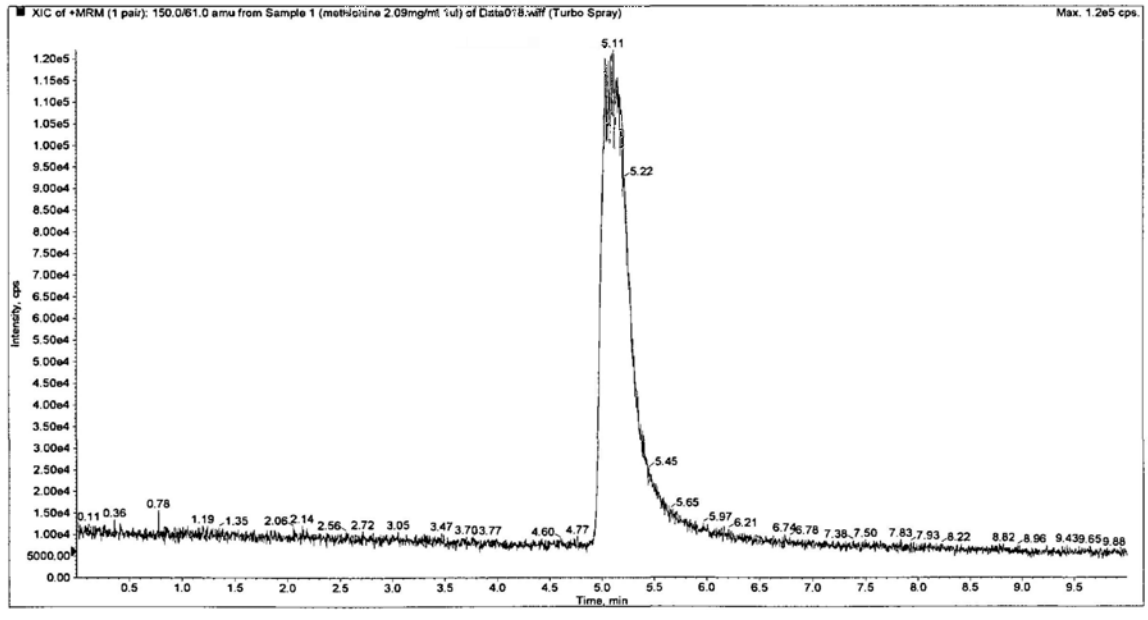

Figure 3.2 **HPLC chromatogram of authentic methionine by HPLC-MS/MS analysis**

HPLC-MS/MS results domenstrated that there was no detectable methionine, and a very small amount of choline in *P. urinaria* (0.7 μg/mg); the latter was undetectable when it was mixed in the MCD diet at dosage of 500, 1000, or 2000 ppm. The results were shown in table 3.1.

Table 3.1 **Contents of choline and methionine in control diet and *P. urinaria***

Sample	Choline (μg/mg)	Methionine (μg/mg)
Control diet	3.36 ± 0.05	3.07 ± 0.80
P. urinaria	0.7 ± 0.18	ND

ND: not determined

CHAPTER 4. HEPATOPROTECTIVE EFFECT OF *P. URINARIA* ON NASH IN VITRO STUDY

4.1 Introduction

Previous study has demonstrated that incubating cultured mouse hepatocytes with MCD medium for short term caused cellular steatosis and increased release of ALT, which was analogous to the early effects observed in MCD diet-fed mice. [Sahai A, 2006] Hence, we first investigated the effect of *P. urinaria* on NASH in vitro using MCD culture medium. In the present study, two types of hepatocyte lines were used. One is immortalized normal mouse hepatocyte (AML-12) cell line. The other was obtained through isolation of primary hepatocytes from a wild-type Wistar rats. We examined the short-term effect of exposing cultured hepatocytes to MCD culture medium with or without different concentration of *P.urinaria* by analyzing the development of steatosis, the release of ALT into the medium and the level of oxidative stress.

4.2 Materials and methods

4.2.1 Hepatocyte isolation and cell culture

Hepatocytes were isolated from male Wistar rats (approximately 250g) using the collagenase perfusion method. [Parkes JG, 2003] Briefly, the liver was perfused progressively with perfusate solution (pH 7.2), Dulbecco's minimum essential medium (DMEM)/Ham's F12 (1:1containing 0.05% (wt/vol) collagenase NB 4

(Serva, Heidelberg, Germany), 4.2 mmol/L NaHCO3, and 1% penicillin-streptomycin. The minced liver was disrupted with blunt forceps and filtered through 2 layers of cheesecloth. After washing in DMEM/F12 medium and 1% penicillin-streptomycin (P/S), hepatocytes were collected by 50g centrifugation for 5 minutes. Hepatocytes purity, measured by trypin blue, was greater than 85%.

Immortalized normal mouse hepatocytes (AML-12) or primary rat hepatocytes were grown in control medium, which is 1:1 mixture of DMEM and F12 (Invitrogen) with 10% fetal bovine serum (Invitrogen), 40 ng/mL dexamethasone (Sigma, St Louis, MO), and 1% P/S (Invitrogen). Equal amounts of cells (2 ×105) were seeded in 100-mm dishes. When the cultures reached 70%-80% confluence, serum-containing medium was replaced with serum-free medium for 24 hours' incubation. Then the quiescent cells were exposed to control medium, DMEM/F12 medium deficient in MCD or MCD medium containing different concentrations of *P. urinaria* (0.25, 0.5, or 1 mg/ml) for an additional 24 hours. [Sahai A, 2006]

4.2.2 Medium ALT and hepatocyte TG test

Cell-free culture media were collected to assess alanine aminotransferase (ALT) level which was determined using spectrophotometric assay kit (Sigma). ALT level expressed in U/L was determined from the calibration curve.

Hepatocytes were washed with cold PBS, scraped and centrifuged. The cell pellets were suspended in 100µl milli Q water and homogenized. Total hepatic triglycerides (TG) were estimated using Wako E-test triglyceride Kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instruction.

4.2.3 Oil Red O dye

AML-12 cell steatosis was analyzed histologically using Oil Red O dye. Briefly, the cells were seeded on coverslip and cultured in control, MCD, and MCD medium with 0.5 mg/ml or 1 mg/ml *P. urinaria* for 24hs. Cells were fixed in 4% paraformaldehyde and immerced in working solution of Oil Red O dye (0.5g Oil Red O in 100ml isopropanol). Hereafter, cells were washed with deionized water and were counterstained using hemotoxylin. Finally, coverslips were half dried, transferred from wells to slides and mounted for microscopic examination.

4.2.4 TBARS assay and DCF assay

For the determination of total cellular lipoperoxides, cultured hepatocytes were homogenized in 1.14% KCl. TBARS were measured in 100µl cell homogenate with 1,1,3,3-tetramethoxypropane as a standard (Sigma).

The rate of ROS production was determined by DCF assay. The 5- and 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) used as a DCF probe was purchased from Invitrogen.

4.3 Results

4.3.1 *P. urinaria* attenuated NASH in AML-12 cell line

4.3.1.1 Hepatocyte steatosis

ALM-12 hepatocytes were cultured in control medium, MCD medium in the presence or absence of *P. urinaria* for 24h. MCD medium-treated hepatocytes showed a marked increase in total cellular TG level compared with hepatocytes incubated with control medium. *P. urinaria* produced a dose-dependent decrease of TG content with a significant reduction in hepatocytes treated with *P. urinaria* at 0.5 and 1 mg/ml respectively compared with the MCD culture medium only (Figure 4.1).

Cells were fixed and stained with Oil Red O for measuring lipid accumulation morphologically. In parallel to the effect on TG content, the greatest amount of intracellular lipid accumulation was evidence in hepatocytes cultured in MCD medium. The obvious decrease of the percent positive staining of fat globules was observed in hepatocytes incubated in MCD medium with 0.5 mg/ml and 1 mg/ml *P. urinaria* (Figure 4.2).

4.3.1.2 Medium ALT level

ALT release in the cuture medium of hepatocytes exposed to MCD medium was (54.5 ± 7.92 U/L) siginificantly highter than that exposed to control medium (35.8 ± 3.76, $P < 0.01$). As shown in Figure 4.3, *P. urinaria* effectively blunted the increase in the ALT level. Mean ALT level was markedly reduced in the MCD medium in the presence of 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml *P. urinaria*. The maximum reduction in ALT level as compared to the MCD medium-cultured cells reached to 56% at the concentration of 1 mg/ml *P. urinaria* (Figure 4.3).

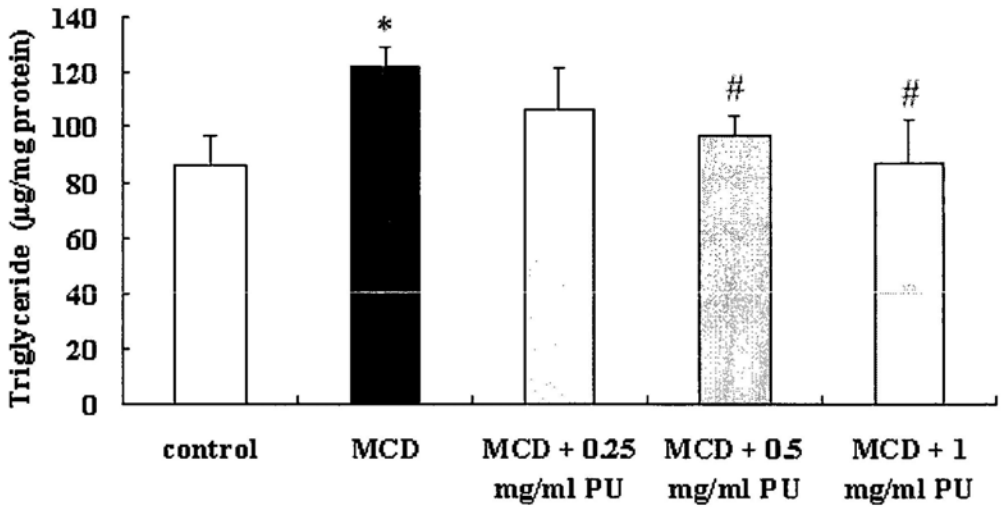

Figure 4.1 **The effect of *P. urinaria* (PU) on triglyceride accumulation of AML-12 hepatocytes.** AML-12 cells (1 x 10^6 cells) were exposed to control or MCD medium in the presence of PU (0, 0.25, 0.5, 1 mg/ml) for 24 h. The hepatocytes triglycerides measured as described in Materials and Methods section. *P<0.05, AML-12 cultured in MCD medium compared with control medium. #P<0.05, AML-12 in PU-treated MCD-medium compared with the MCD medium. Data are mean ± SD (n = 6).

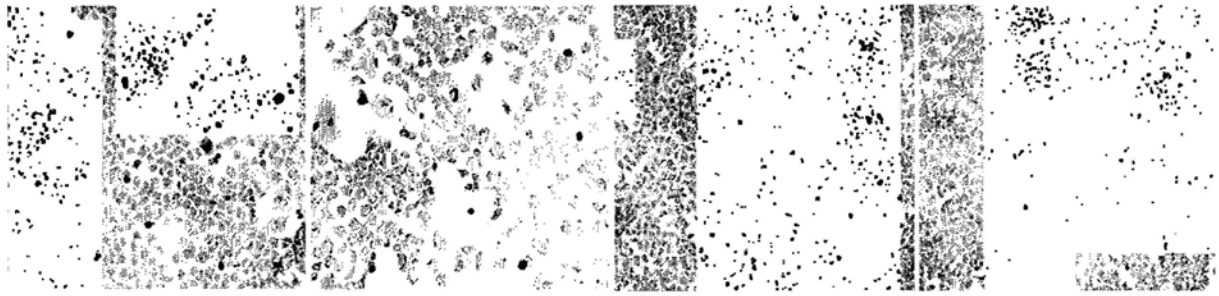

Figure 4.2 **The effects of *P. urinaria* (PU) on endogenous lipoid droplets in AML-12 hepatocytes by Oil Red O dye.** AML-12 cells were cultured in control or MCD medium in the presence of PU (0, 0.5, 1 mg/ml) on cover slips. After 24h treating, Oil Red O stain was used on fresh cells. Red droplets indicate neutral lipid staining. Slides are representative of 3 separate experiments (original magnification x 200).

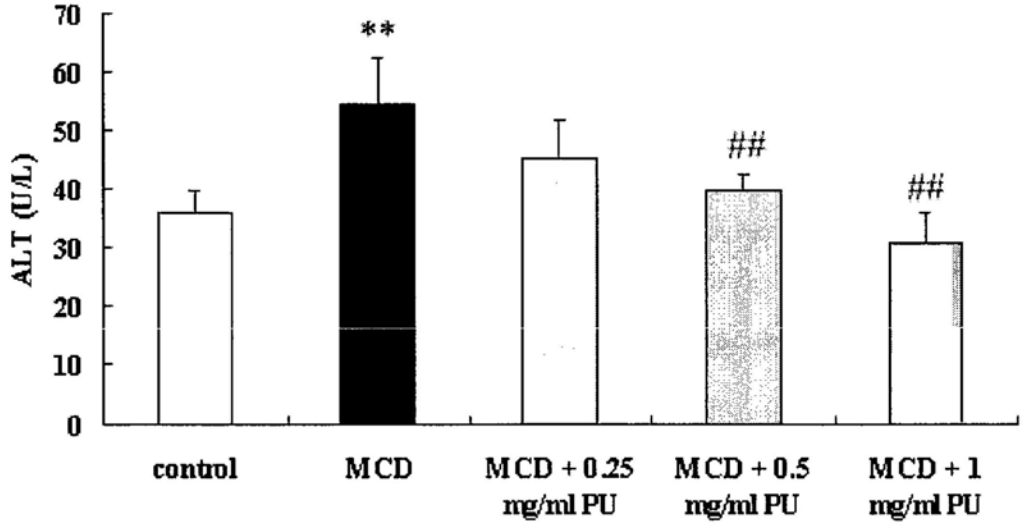

Figure 4.3 **The effects of *P. urinaria* (PU) on release of ALT by AML-12 hepatocytes.** AML-12 cells (1 x 10^6 cells) were exposed to control or MCD medium in the presence of *P. urinaria* (0, 0.25, 0.5, 1 mg/ml) for 24 h. ALT level in culture medium, were measured as described in Materials and Methods section. $^{**}P<0.01$, AML-12 cultured in MCD medium compared with control medium. $\#\#P<0.01$, AML-12 in PU-treated MCD-medium compared with the MCD medium. Data are mean ± SD (n = 6).

4.3.1.3 Measurement of oxidative injury by TBARS assay

TBARS were measured in ALM-12 hepatocytes exposed to control, MCD, or MCD medium containing *P. urinaria* (0.25, 0.5, and 1 mg/ml) for 24h to assess oxidative injury. The level of TBARs was significantly higher in ALM-12 exposed to MCD medium (52.7 ± 11.44 μmol/mg protein) compared with those exposed to control medium (24.1 ± 10.70 μmol/mg protein) ($P < 0.05$). TBARS were reduced in the ALM-12 cells treated with *P. urinaria* in a dose-dependent manner with significant reduction was observed in ALM-12 hepatocyted treated with *P. urinaria* at 1 mg/ml ($P< 0.001$) and 0.5 mg/ml ($P<0.046$), respectively (Figure 4.4).

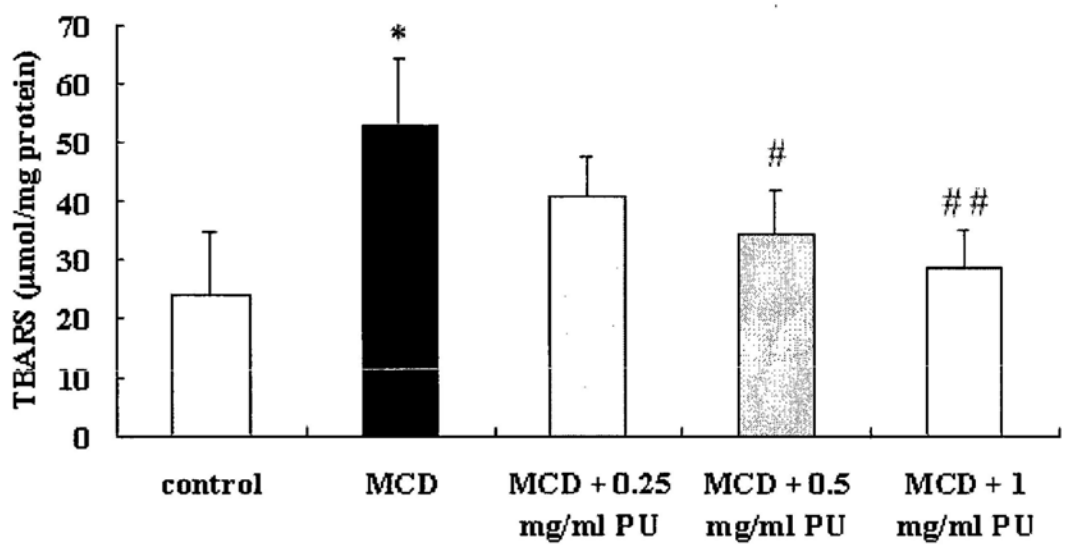

Figure 4.4 **The effects of *P. urinaria* (PU) on lipoperoxides levels of AML-12 hepatocytes.** AML-12 cells (1 x 10^6 cells) were exposed to control or MCD medium in the presence of *P. urinaria* (0, 0.25, 0.5, 1 mg/ml) for 24 h. Lipoperoxides were measured by TBARS test. **P*<0.05, ***P*<0.01, AML-12 cultured in MCD medium compared with control medium. #*P*<0.05, ##*P*<0.01, AML-12 in PU-treated MCD-medium compared with the MCD medium. Data are mean ± SD (n = 6).

4.3.2 *P. urinaria* in the prevention of NASH in primary hepatocytes

Primary hepatocytes were incubated in control medium, MCD medium or MCD medium with *P.urinaria* at 0, 0.5 or 1mg/ml. Hepatocytes steatosis and formation of ROS were determined by TG test and DCF assay as described in methodology section.

4.3.2.1 Hepatocyte steatosis

Primary hepatocytes cultured in MCD medium for 24h resulted in significant increase in total TG content compared with the control medium (1310 ± 240 μg/mg protein vs. 960 ± 100 μg/mg protein, $P < 0.05$). Hepatocytes incubated in MCD medium in the presence of 1 mg/ml *P. urinaria* significant lowered the cellular TG accumulation to normal level (950 ± 260 μg/mg protein) (Figure 4.5).

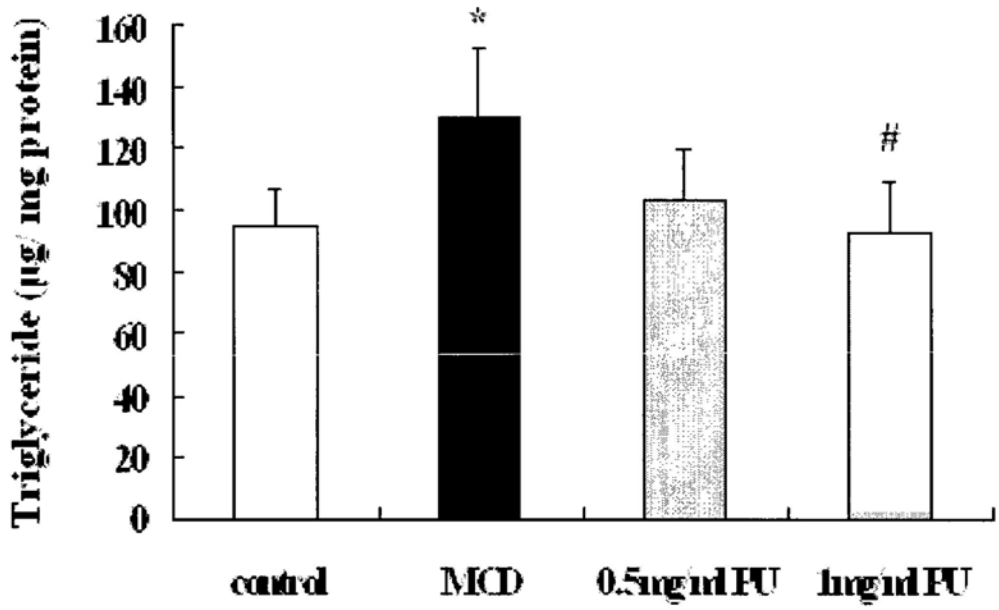

Figure 4.5 **The effect of *P. urinaria* (PU) on hepatocyte TG.** The level of TG was determined in primary hepatocytes cultured in control or MCD medium in the presence of *P. urinaria* (0, 0.5, 1 mg/ml) for 24 h. **P*<0.05, primary hepatocytes cultured in MCD medium compared with control medium. #*P*<0.05, hepatocytes in PU-treated MCD-medium compared with the MCD medium. Data are mean ± SD (n = 6).

4.3.2.2 Measurement of oxidative injury by DCF assay

DCF assay is routinely used in many laboratories to detect cellular radical formation. We used this assay to investigate the effect of *P. urinaria* on ROS formation in primary hepatocellular cultured in MCD medium. As shown in Figure 4.6, incubation with MCD medium leaded to a significant increase in DCF fluorescent signal, which indicated ROS formation. While hepatocytes cultured in MCD medium with 1 mg/ml *P. urinaria* exhibited a clear reduction of the DCF fluorescence compared with the MCD group, suggesting that *P. urinaria* inhibited the production of reactive oxidative species.

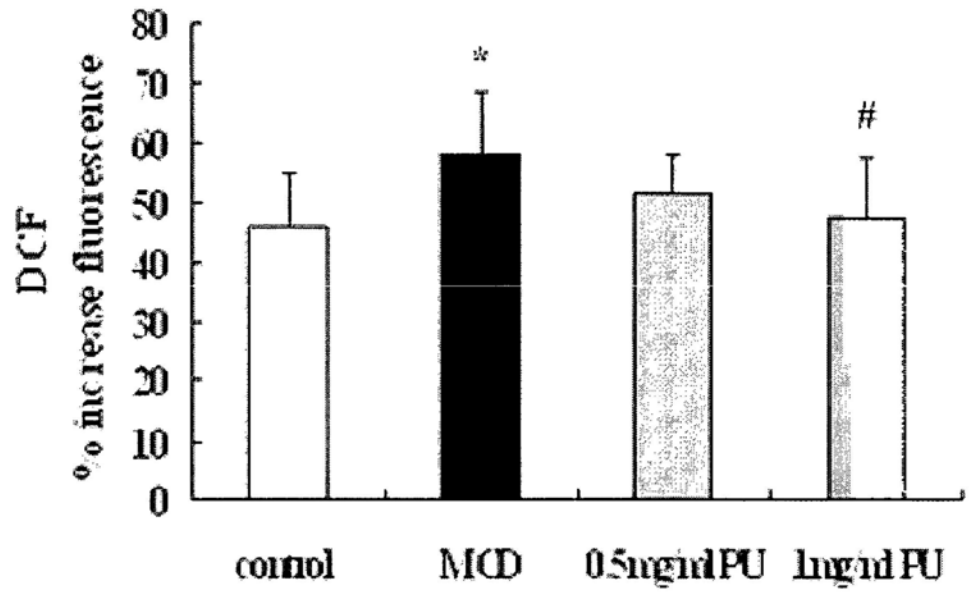

Figure 4.6 **The effect of *P. urinaria* (PU) on reactive oxygen species (ROS) in primary rat hepatocytes.** Primary hepatocytes were cultured in control or MCD medium in the presence of *P. urinaria* (0, 0.5, 1 mg/ml) for 24 h. ROS production was quantified by percentage increase of DCF fluorescence in hepatocytes. **P*<0.05, primary hepatocytes cultured in MCD medium compared with control medium. #*P*<0.05, hepatocytes in PU-treated MCD-medium compared with the MCD medium. Data are mean ± SD (n = 8).

CHAPTER 5. *P. URINARIA* AMELIORATED NASH IN ANIMAL MODELS

5.1 Introduction

Animal experiments were established to further evaluate the protective effect of *P. urinaria* against the development of NASH. Experiments were conducted with *P. urinaria* treatment in wild type mice and genetic db/db mice fed with MCD diet for 10 days. Hepatic steatosis and necroinflammatory infiltration were evaluated. Our data demonstrated that both wild type and db/db mice fed MCD diet developed NASH, treatment with *P. urinaria* lowered hepatic TG, decreased lipid peroxidation and inhibited inflammatory infusion.

5.2 Materials and methods

5.2.1 MCD diet induced NASH in murin models

8-week old C57BL/6 mice were ramdomly divided into 5 groups (5 mice per group) and fed with control diet, MCD diet or MCD diet supplemented with *P. urinaria* at 500, 1000, or 2000 ppm, respectively. The dosage of *P. urinaria* was chosen based on the daily human dose which was recommended by the company. Based on the results obtained from the C57BL/6 mice, a separate experiment was performed on age-matched db/db mice, with 3 groups feeding with control diet, MCD diet, and MCD diet supplemented with *P. urinaria* at 1000 ppm. After 10 days experiments, mice were sacrificed under anethesia. Blood was collected by cardiac puncture, and liver was weighed and fixed in 10% formalin for

histological analysis or snap frozen in lipid nitrogen followed by storage at -80°C freezer until required.

5.2.2 Histological examination

Hematoxylin & eosin-stained paraffin-embedded liver tissues isolated from mice (4μm thick) were graded for hepatic steatosis and necroinflammation as described previously (Yu J, 2006): hepatic steatosis (percentage of liver cells containing fat) was graded as 0: 0%, 1: 1-25%, 2: 26-50%, 3: 51-75%, 4: >75%. Necroinflammation was graded as 0: no inflammatory foci, 1: mild, 2: moderate, 3: severe. The investigator who scored the histological slides was blinded to the treatment assignment.

5.2.3 Hepatic TG assay

Total liver lipids were extracted from 30-50 mg of liver homogenate. Total hepatic TG was determined using Wako E-test triglyceride Kit according to the manufacturer's instruction.

5.2.4 TBARS assay

Frozen liver tissue was homogenized in 1.14% KCl solution. TBARS were measured in 100μl cell homogenate with 1,1,3,3-tetramethoxypropane as a standard.

5.2.5 Quantitation of hepatic mRNA expression levels

Total RNA was extracted from frozen liver using TRIzol reagent (Invitrogen). Five microgram total RNA for each sample was reversed transcribed into complementary DNA with 15T/18T primer and 100U MMLV reverse transcriptase (Promega, San Luis Obispo, CA). Real-time polymerase chain reaction was performed using SYBR Green Master Mix (Applied Biosystems, Foster, CA) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Each Ct value was normalized to β-actin. Transcript levels of inflammatory factors include interleukin (IL)-6, IL-1, tumor necrosis factor alpha (TNF-α), transforming growth factor beta-1 (TGF-β1), intercellular adhesion molecule-1 (ICAM-1), and lipid regulation factors acyl-coenzyme A oxidase (ACO), proliferator-activated receptor gamma (PPAR-γ), PPAR-α, cytochrome P450 2E1 (CYP2E1), CYP4A10, CYP4A14, long-chain acyl-coenzyme A dehydrogenase (LCAD), stearoyl coenzyme A desaturase-1 (SCD1), liver X receptors-alpha (LXR-α), LXRβ, fatty acid synthase (FAS).

5.2.6 Western blot analysis of hepatic proteins

Liver tissue was homogenized in Tris-HCl (pH 7.4) buffer containing a protease inhibitor cocktail (Roche, Indiapolis, IN). Total protein was extracted and concentration was measured by the Bradford method (DC protein assay, Bio-Rad Laboratories, Hercules, CA). Twenty-five micrograms protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. Membranes were

blocked using 5% skim milk for 1 hour, then incubated with specific antibodies against CYP2E1 (dilution 1: 5000, Abcam, Hong Kong, China), phosphorylated C-jun N-terminal kinase (P-JNK) (1:1000) (Cell Signaling Technology, Danvers, MA), or glyceraldehyde 3-phosphate dehydrogenase (1:5000) (Abcam, Hong Kong, China) overnight at 4°C. After incubation with secondary antibody, proteins were detected by enhanced chemiluminescence (Amersham Corp.).

5.2.7 Immunohistochemistry for nuclear factor κB (NF-κB) subunit p65

Paraffin-embedded liver sections were deparaffined, rehydrated, and pretreated with 0.5% hydrogen peroxidase in phosphate-buffered saline buffer for 20 minutes. After blocking with 10% nonimmunized goat serum, sections were incubated with antibody to nuclear factor kappa B (NF-B) subunit p65 (1:50, Santa Cruz Biotechnology) for 2 hours at room temperature. Then biotin-conjugated secondary antibody, avidin-biotin complex, and horseradish peroxidase were applied for 30 minutes at room temperature (Dako A/S, Glostrup, Denmark). Positive signals were visualized by diaminobenzidine and counterstained with hematoxylin. The number of p65 nuclear positive cells was counted per 1000 hepatocytes in 5 fields (magnification×100).

5.3 Results

5.3.1 Effect of *P. urinaria* on the development of NASH in C57BL/6 mice fed MCD diet

In light of the observed anti-steatosis and anti-oxidative injury effects of *P. urinaria* on the hepatocytes *in vitro*, we tested whether *P. urinaria* treatment could ameliorate NASH induced by MCD diet *in vivo*.

5.3.1.1 Physical appearances and histology

C57BL/6 mice had mean body weight loss up to 22% of starting body weight after fed the MCD diet, and supplement of *P. urinaria* did not rectify the weight loss. In addition, 3 of 5 mice fed MCD diet experienced hair loss which might come from malnutrition. Fair loss was also observed in 4 out of 5 mice fed MCD diet supplemented with 500 ppm *P. urinaria*. However, this hair loss was not seen in mice fed MCD diet supplemented with 1000 ppm and only one in 2000 ppm *P. urinaria* treated mice. Despite the weight changes and hair loss, all animals remained physically active throughout the experimental period.

As shown by hematoxylin & eosin staining, mice fed the control diet had normal liver histology (Figure. 5.1A), but mice fed with the MCD diet developed NASH with hepatocyte steatosis, scattered lobular inflammatory cells infiltration and inflammatory foli (Figure 5.1B). The MCD diet supplemented with 500 ppm *P. urinaria* failed to improve the liver histology (Figure 5.1C), while MCD diet supplemented with 1000 ppm and 2000 ppm *P. urinaria* clearly reduced the severity of hepatic steatosis and inflammatory infiltration (Figure 5.1D and 5.1E). Histological grading of liver sections confirmed that *P. urinaria* significantly ameliorated hepatic steatosis and necroinflammation (Table 5.1)

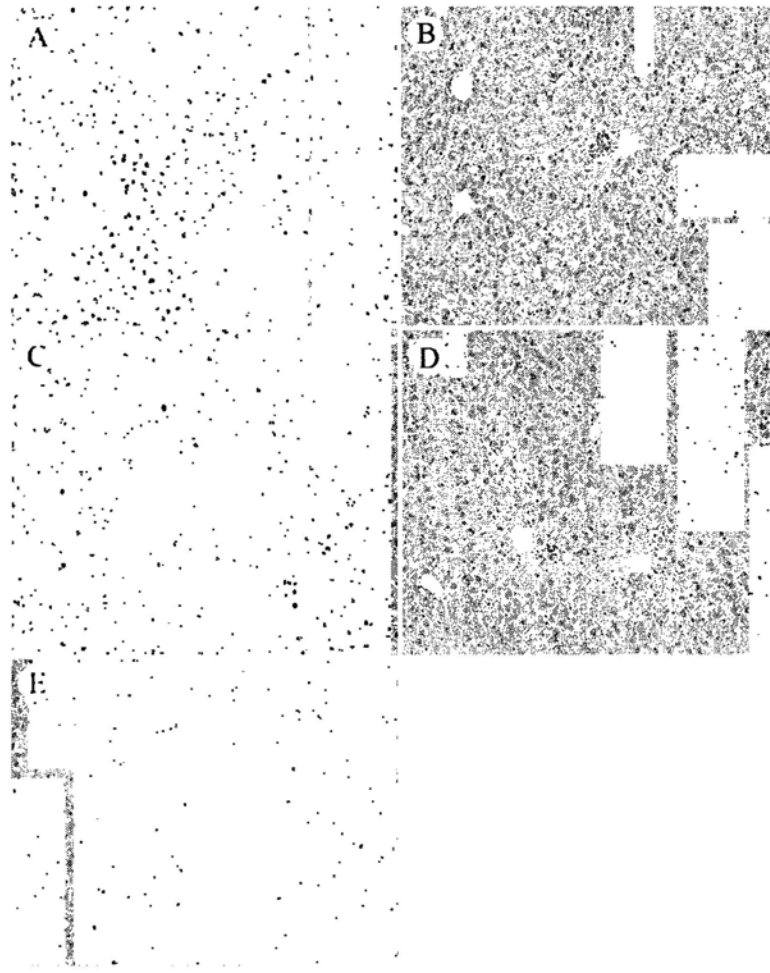

Figure 5.1 **Effect of *P. urinaria* on MCD diet-induced NASH in C57BL/6 mice.** Hematoxylin and eosin-stained liver sections from mice fed: (**A**) the control diet -appearances are normal. (**B**) MCD diet. Liver shows foci of necroinflammation and macrovesicular fat droplets. (**C**) In mice fed the MCD diet, treatment with *P. urinaria* (500 ppm), the severity of NASH was not significantly improved. (**D**) In mice fed the MCD diet, treatment with *P. urinaria* (1000 ppm) and (**E**) with *P. urinaria* (2000 ppm) largely ameliorated NASH. Experiment lasted for up to 10 days. Slides are representative of 5 separate

experiments (original magnification x 100). The group data for steatosis and inflammatory scores are presented in Table 5.1.

Table 5.1 **Effect of *P. urinaria* on Scores for Hepatic Steatosis and Necroinflammation**

Diet	Control	MCD	MCD+PU 500 ppm	MCD+PU 1000 ppm	MCD+PU 2000 ppm
Steatosis	0.0 ± 0.00	2.3 ± 0.45*	2.3 ± 0.27	1.2 ± 0.45##	1.0 ± 0.71##
Necroinfla-mmation	0.0 ± 0.00	1.8 ± 0.57*	1.7 ± 0.45	0.7 ± 0.45##	0.8 ± 0.57#

PU, *P. urinaria*. The severity of hepatic steatosis and necroinflammation were scored as described in Materials and Methods.

Duration of this experiment is 10 days.

Values of hepatic steatosis and necroinflammation are mean ± SD (n = 5/group).

*P<0.0001 control *vs* MCD diet, # P<0.05, ##P<0.01 MCD *vs* MCD+PU-treated mice.

5.3.1.2 Hepatic TG

Hepatic steatosis represents an excessive accumulation of triglycerides in the hepatocytes of the liver. The underlying cause of fat accumulation in steatosis is mostly due to the synthesis of fatty acids and inhibition of fatty acid oxidation. [Goldberg IJ, 2006] As shown in Figure 5.2, intake of the MCD diet resulted in a prominent increase in TG contents compared to that of the control diet. In contrast, the MCD diet supplemented with *P. urinaria* showed a dose-dependent reduction on the accumulation of hepatic triacylglycerol fractions. Significant reduction on the level of TG was observed in mice fed MCD treated with 2000 ppm *P. urinaria* compared with the mice fed with MCD diet only (Figure 5.2).

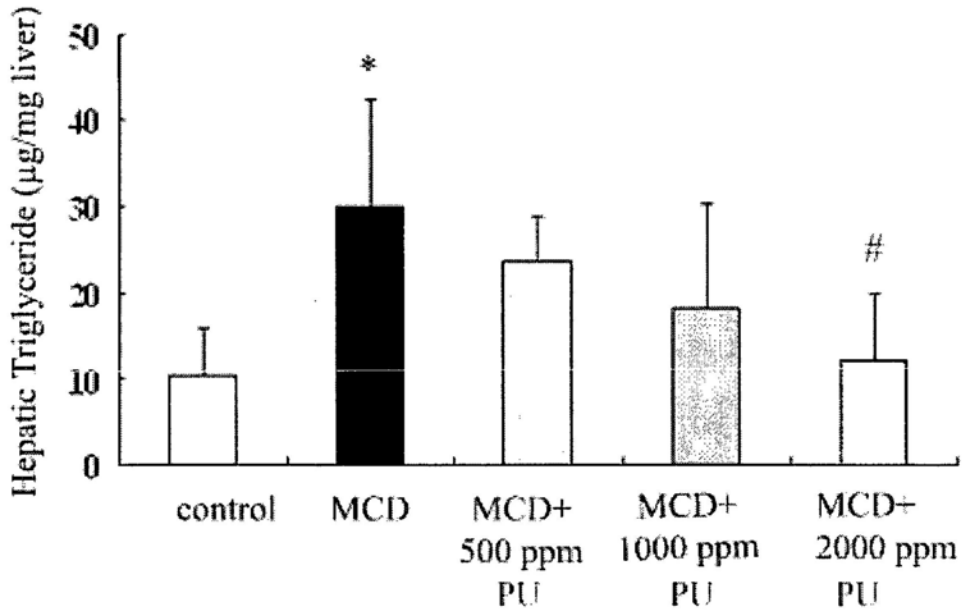

Figure 5.2 **Effects of the MCD diet and treatment with *P. urinaria* (PU) on hepatic TG content in C57BL/6.** Hepatic TG content was assessed in mice fed the control, MCD diet or MCD diet treated with *P. urinaria* (500, 1000, and 2000 ppm) for 10 days. Data are mean ± SD with 5 animals in each group. *$P<0.05$, MCD compared with control diet-fed mice. #$P<0.05$, *P. urinaria*-treated MCD-fed compared with the mice fed the MCD diet.

5.3.1.3 Hepatic lipid peroxidation

The measurement of TBARS was used to evaluate the extend of lipid peroxidation. Total hepatic TBARS levels showed 4-fold increase in the liver of MCD diet-fed mice compared to the control. While mice treated with 2000 ppm *P. urinaria*, hepatic TBARS concentration was 53 pecentage lower than that of the MCD group ($P < 0.05$). Though there was no statistical significance between TBARS levels of MCD diet-fed and MCD diet with 500 ppm or1000 ppm *P. urianria* treated mice, *P. urinaria* led to a dose-dependent reduction on TBARS formation (Figure 5.3). The results demonstrated that *P. urinaria* can protect liver against oxidative injury.

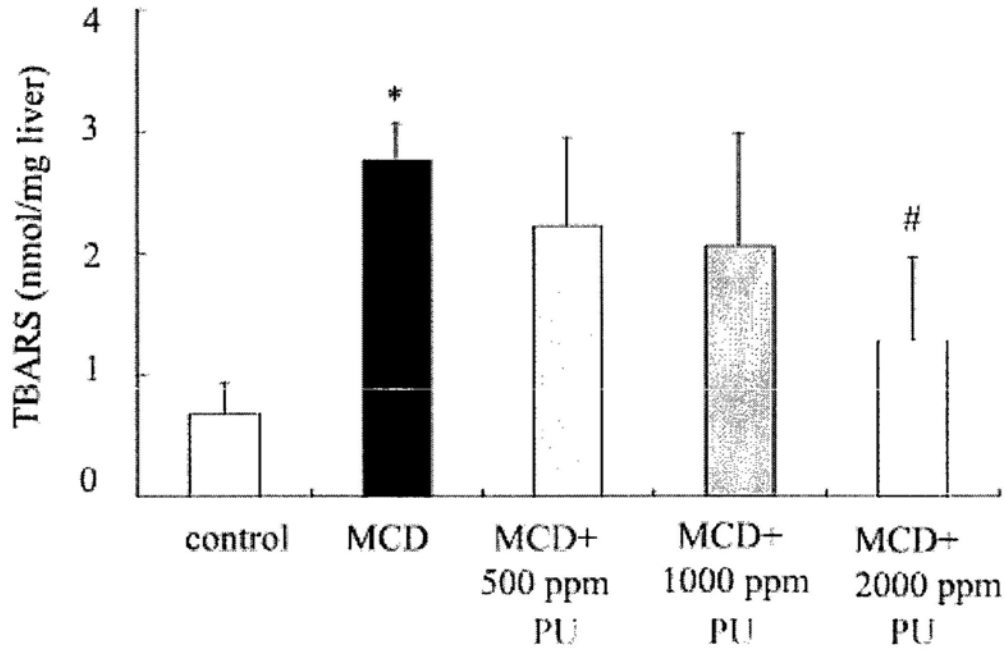

Figure 5.3 **Effects of the MCD diet and treatment with *P. urinaria* (PU) on hepatic lipoperoxide content in C57BL/6.** Lipoperoxide content was assessed in mice fed the control, MCD diet or MCD diet treated with *P. urinaria* (500, 1000, and 2000 ppm) for 10 days. Data are mean ± SD with 5 animals in each group. *$P<0.05$, MCD compared with control diet-fed mice. #$P<0.05$, *P. urinaria*-treated MCD-fed compared with the mice fed the MCD diet.

5.3.2 Effect of *P. urinaria* on the development of NASH in db/db mice fed MCD diet

To confirm that the effects observed with Phyllanthus in C57BL/6 mice, an additional experiment was performed on genetic db/db mice. In this genetic NASH model, mice fed with control diet, MCD diet and MCD diet supplemented with *P. urinaria* at 1000 ppm respectively. The effects of *P. urinaria* on histological scoring and oxidative stress were evaluated.

5.3.2.1 Physical appearances and liver histology

Db/db mice fed MCD diet had 5% body weight loss, but did not show hair loss.

In this genetic NASH model, db/db mice fed control diet for 10 days exhibited slight steatosis change. Feeding MCD diet, the liver sections showed pronounced steatosis and necroinflammatory foci changes. However, treatment with *P. urinaria* attenuated MCD-induced NASH as indicated by improved liver histology (Figure 5.4). Histological grading of liver sections further supported that *P. urinaria* significantly improved hepatic steatosis and necroinflammation (Table 5.2)

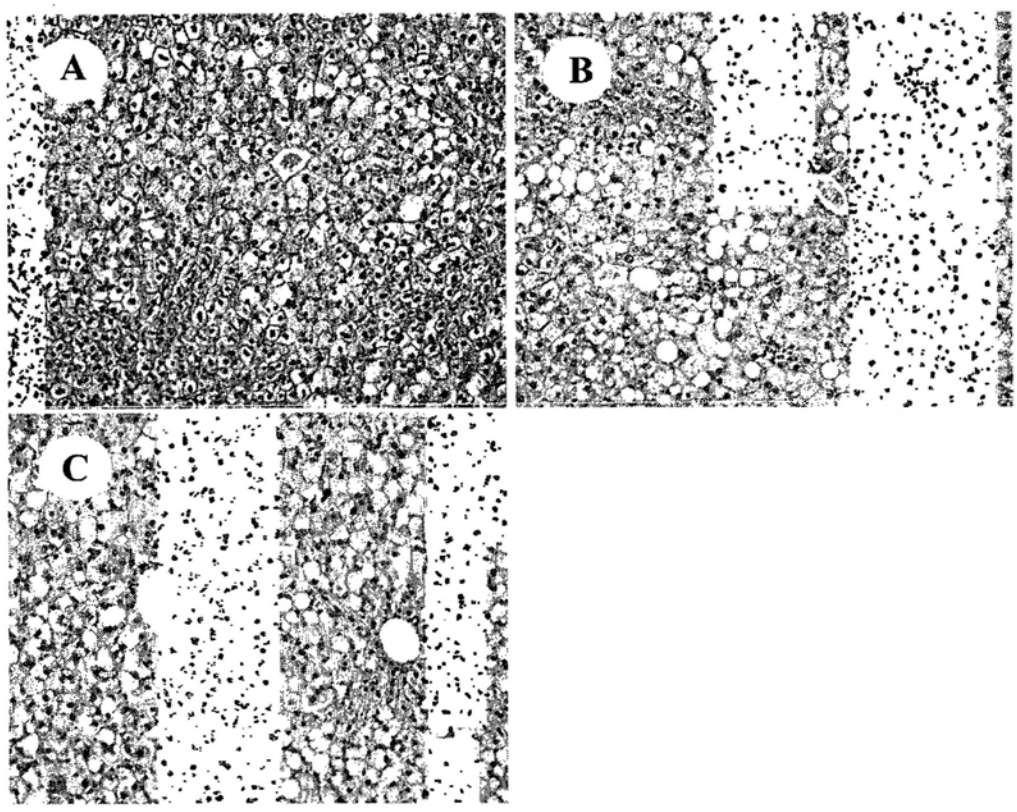

Figure 5.4 **Effect of *P. urinaria* on MCD diet-induced NASH in db/db mice.** Hematoxylin and eosin-stained liver sections from db/db mice fed: (**A**) the control diet -appearances are normal. (**B**) MCD diet. Liver shows foci of necroinflammation and macrovesicular fat droplets. (**C**) In mice fed the MCD diet, treatment with *P. urinaria* (1000 ppm) largely ameliorated NASH. Experiment lasted for 10 days. Slides are representative of 5 separate experiments (original magnification x 200).

Table 5.2 **Effect of *P. urinaria*on Scores for Hepatic Steatosis and Necroinflammation**

Diet	Control	MCD	MCD+PU 1000 ppm
Steatosis	0.6 ± 0.49	2.8 ± 0.50*	2.0 ± 1.00
Necroinflammation	0.0 ± 0.00	2.8 ± 0.45*	1.8 ± 0.84#

PU, *P. urinaria*. The severity of hepatic steatosis and necroinflammation was scored as described in Materials and Methods.

Duration of this experiment is 10 days.

Values of hepatic steatosis and necroinflammation are mean ± SD (n = 5/group).

*P<0.0001 control vs MCD diet, # P<0.05, ##P<0.01 MCD vs MCD+PU-treated mice.

5.3.2.2 Hepatic lipid peroxidation

The hepatic TBARS level in MCD diet-fed db/db mice was 23-fold increased compared with that of the control diet-fed db/db mice (9.81±3.23 versus 0.42±0.15 nmol/mg liver, $P<0.01$). In contrast, feeding db/db mice mice with *P. urinaria* at 1000 ppm significantly reduced hepatic TBARs level compared to the MCD group (5.16±2.49 nmol/mg liver, $P<0.05$) (Figure 5.5). Thus, the effect of Phyllanthus on MCD diet-induced steatohepatitis in db/db mice was consistent with the results obtained from C57BL/6.

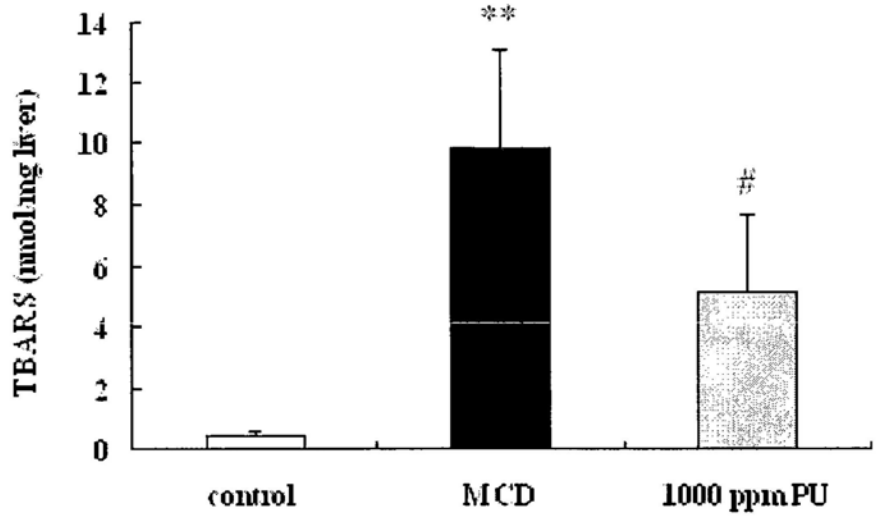

Figure 5.5 **Effects of the MCD diet and treatment with *P. urinaria* (PU) on hepatic lipoperoxide content in db/db mice.** Lipoperoxide content was assessed in db/db mice fed the control, MCD diet or MCD diet treated with 1000 ppm *P. urinaria* for 10 days. Data are mean ± SD with 5 animals in each group. $^{**}P<0.01$, MCD compared with control diet-fed mice. $\#P<0.05$, *P. urinaria*-treated MCD-fed compared with the mice fed the MCD diet.

5.3.3 Molecular mechanism of protective effects of *P. urinaria* on NASH

To understand the potential mechanisms by which *P. urinaria* ameliorated NASH, we further investigated the effect of *P. urinaria* on the expression of genes involved in inflammatory responses, antioxidative activities, oxidation and fatty acid regulations.

5.3.3.1 Effect of *P. urinaria* on the expression of proinflammatory factors

It is known that the hepatic expression of genes encoding several proinflammatory mediators can be induced by MCD diet feeding in mice.[Dela Pena, A., 2005] To evaluate the mechanisms of the effect of *P. urinaria* on NASH, we investigated expression levels of proinflammatory factors including TNF-α, IL-6, IL-1β, TGF-β1, ICAM-1 by real-time PCR . Mice fed with MCD diet had a marked elevation of hepatic mRNA expression of TNF-α, IL-6 and IL-1β as compared to mice fed with control diet. Conversely, treatment with *P. urinaria* at 1000 ppm or 2000 ppm in mice fed with MCD diet significantly lowered the mRNA expression of TNF-α and IL-6. This effect was not seen on mRNA expression of IL-1β, TGF-β1 and ICAM-1 (Figure 5.6).

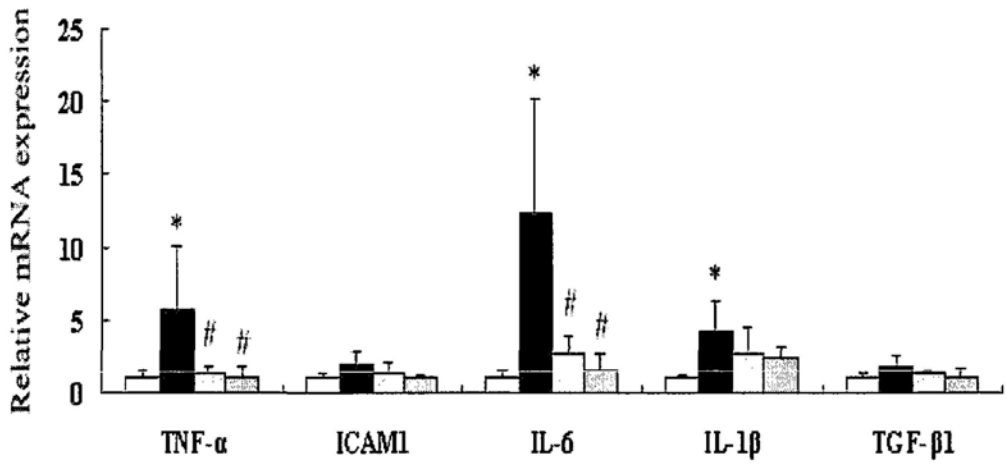

Figure 5.6 **Effect of the MCD diet and treatment with *P. urinaria* on hepatic mRNA expression of inflammatory mediators.** Expression of proinflammatory genes were measured in mice fed the control diet, MCD diet, or MCD diet with *P. urinaria* 1000 or 2000 ppm for 10 days. Hepatic mRNA was assessed by real time RT-PCR, normalized to GAPDH, and is expressed as fold-induction. Data are mean ± SD with 5 animals in each group. *P<0.05, MCD compared with control diet-fed mice. #P<0.05, *P. urinaria*-treated MCD-fed compared with the mice fed the MCD diet.

5.3.3.2 Effect of *P. urinaria* on the activation of inflammatory pathways

JNK has been suggested to be a critical modulator in liver injury mediated by TNF-α in the development of steatosis and insulin resistance. [Hotamisligil GS, 2005] Mice fed a MCD diet displayed activated JNK as determined by western blot showing upregulated phosphorylated JNK. In mice fed MCD diet with *P. urinaria*, protein expression of P-JNK was reduced (Figure 5.7E). In addition, increased NF-κB activity is known associated with induction of proinflammatory gene expression and involved in the progress of NASH induced by MCD diet. In this study, hepatic nuclear protein expression of p65, a key component of NF-κB, was significantly increased after 10 days of MCD dietary feeding, and this induction was blunted by *P. urinaria* as determined by immunohistochemistry assay (Figure 5.7A-D).

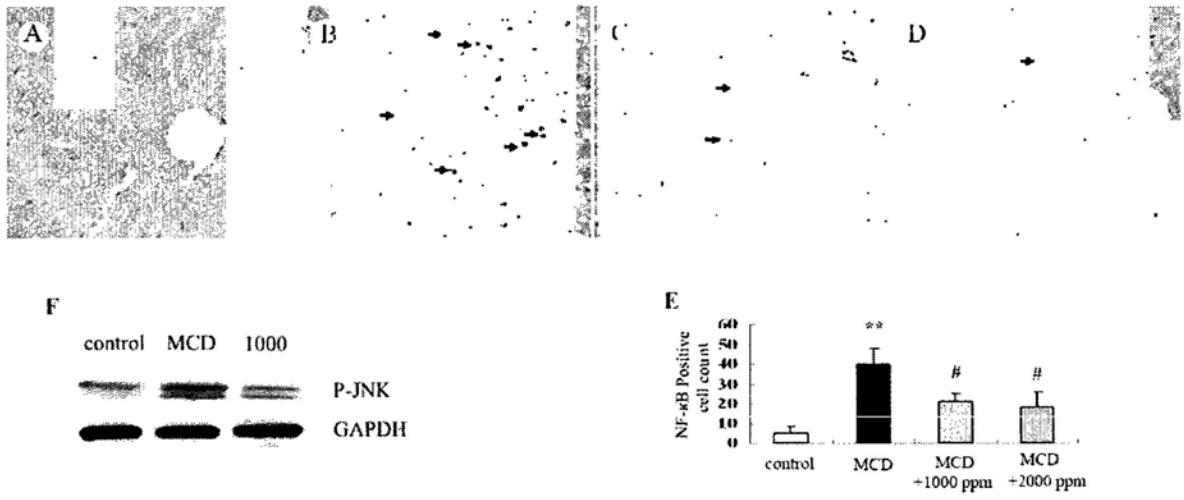

Figure 5.7 **Effects of *P. urinaria* (PU) on hepatic P-JNK protein expression and NF-κB activation in C57BL/6 mice.** Immunohistochemistry for NF-κB subunit p65 in sections from mice fed with: (**A**) the control diet, little nuclear accumulation of p65 is present; (**B**) MCD diet, frequent p65 nuclear immunoreactivity was observed (arrows); (**C**) MCD diet supplement with 1000 ppm PU and (**D**) MCD with 2000 ppm PU, the positive nuclear p65 was reduced. (**E**) A decrease in the number of nuclear p65-positive cells is evident in PU-treated mice. Representative slides obtained from 5 animals per group. (original magnification x 400). **P*<0.05, MCD compared with control diet-fed mice. #*P*<0.05, Phyllanthus-treated MCD-fed mice compared with the mice fed the MCD diet. Data are mean ± SD. (**F**) Protein expression of P-JNK were determinedby Western blotting. GAPDH was used as the internal control.

5.3.3.3 Effect of *P. urinaria* on the expression of genes related to fatty acid regulation

To seek an explanation for the hepatic triglyceride-lowering effects of *P. urinaria*, we assessed the hepatic expression levels of the lipogenic genes PPARγ, FAS, LXRα, LXRβ, SCD1 and C/EBPβ, and lipolytic genes involved in the β-oxidation of fatty acid, such as ACO, LCAD, PPARα and PPARα downstream target molecules Cyp4a10 and Cyp4a14 (Figure 5.8B-D). Compared with mice fed with MCD diet alone, administration of *P. urinaria* with the MCD diet greatly reduced expression of C/EBPβ which was highly induced by MCD diet (Figure 5.8A), but had no effect on other lipogenic genes (Figure 5.8C and D). On the other hand, *P. urinaria* significantly induced mRNA levels for the lipolytic gene Cyp4A10 (4-fold) (Figure 5.8B). Expression of ACO and LCAD involved in lipid β-oxidation were repressed significantly by MCD diet. However, *P. urinaria* supplementation did not restore their expression (Figure 5.8B and C).

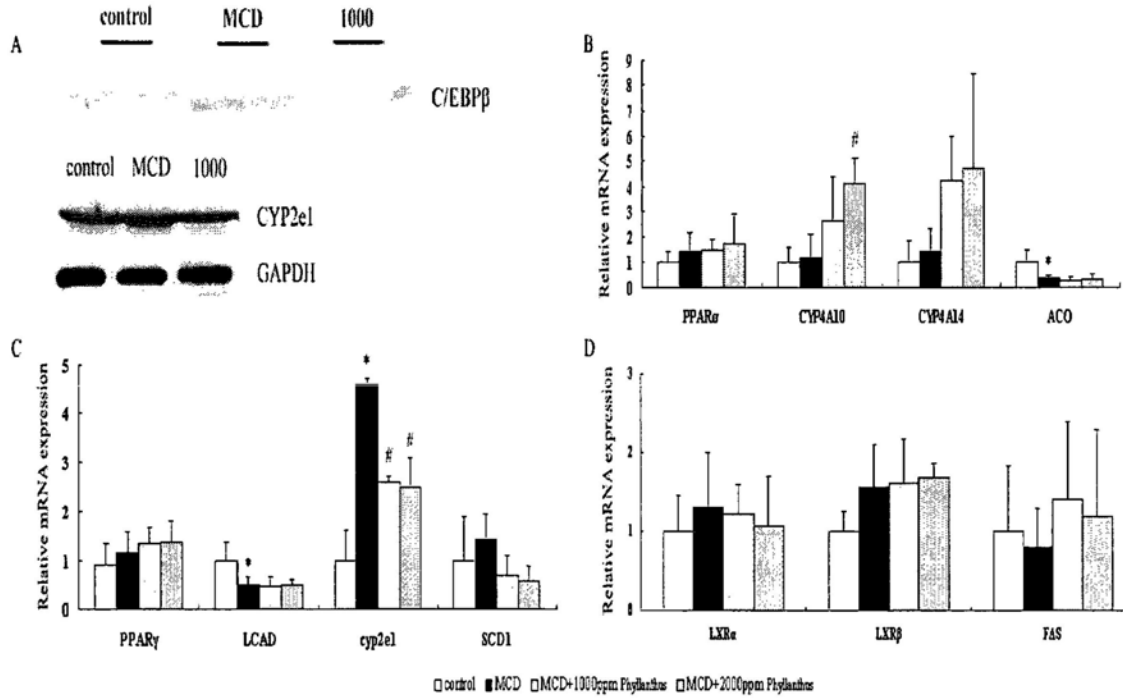

Figure 5.8 **Effects of the MCD diet and treatment with *P. urinaria* on hepatic mRNA and protein expression of fat metabolic genes.** **(A)** Protein expression of C/EBPβ and CYP2e1, and **(B, C, D)** mRNA expression of fatty acid regulation genes were measured in mice fed the control diet, MCD diet, or MCD diet with *P. urinaria* 1000 or 2000 ppm for 10 days. Proteins were determined by Western blotting, GAPDH used as the internal control. Hepatic mRNA was assessed by real time RT-PCR, normalized to GAPDH, and is expressed as fold-induction. Data are mean ± SD with 5 animals in each group. *P<0.05, MCD compared with control diet-fed mice. #P<0.05, *P. urinaria*-treated MCD-fed compared with the mice fed the MCD diet.

5.3.3.4 Effect of *P. urinaria* on the expression of CYP2e1

CYP2e1, a major mediator of lipid peroxidation, has been proven to play an important role in generation of oxidative stress in a MCD dietary NASH murin model. [Leclercq IA, 2000] In the present study, the mRNA and protein expression of CYP2e1 was induced by MCD diet. Administration of *P. urinaria* at 1000 ppm and 2000 ppm prevented the induction of CYP2e1 (Figure 5.8A and C).

CHAPTER 6. D ISCUSSION

As the increasing prevalence of NAFLD/NASH around the world, there has been mounting evidence from recent studies that NASH has become the main cause of cirrhosis and liver failure among the chronic liver diseases. [Matteoni CA, 1999] A number of different stratagies were proposed to reduce or reverse NASH based on increasing knowledge on the pathogenesis of this disorder. These include lifestyle modifications related to obesity and metabolic syndrome, agents against insulin resistance, vitamin E for antioxidant therapy, and interference with cytokine signaling. Unfortunately, up to now, there is no medication proven to be an effective treatment for NASH. Currently, it is generally believed that excessive accumulation of hepatic fat and oxidative stress play a critical role in the pathogenesis of NASH. [Day CP, 1998; Farrell GC, 2006] Therefore, inhibition of fatty acid cytotoxicity and liver inflammatory change is an important goal in the treatment of NASH.

Many clinical and experimental studies have shown that phyllanthus species are hepatoprotective through their anti-oxidative, anti-viral and anti-inflammatory in chronic liver diseases. Whether phyllanthus species could benefit NASH has not been assessed. *P. urinaria* is one of the phyllanthus species that has been proven to be safe in human and animal research. [www.Hepaguard.com] In the present study, we process a series of experiments to evaluate the effect of *P. urinaria* on

the prevention of NASH. The underlying mechanisms of the prevention of nutritionally induced NASH by *P. urinaria* were also explored.

6.1 The *in vitro* effects of *P. urinaria* on NASH

Our results demonstrated that incubating cultured AML-12 hepatocytes with MCD medium increased cellular steatosis and exposed to oxidative insult. MCD medium caused damages to cells attributing to increased production of ROS in hepatocytes as evidenced from release of ALT and cellular TBARS. Treatment with *P. urinaria* led to a dose-dependent decrease in steatosis and ALT as well as a concomitant suppression of cellular TBARS. To avoid any misleading results secondary to the immortalization of AML-12 by the stably transfected TGF-α, we also determined the effects of *P. urinaria* on cultured primary rat hepatocytes with MCD medium. Administration of *P. urinaria* appreciably protected cells from steatosis and ROS specifically probed by DCF.

The effects of *P. urinaria* on protection hepatocytes from MCD medium may result from biologically active lignans, ellagitannins, flavonoids and glycosides which responsible for the antioxidant and anti-inflammatory properties. [Xu M, 2007] In a recent study, Fang et al. isolated 9 compounds from *Phyllanthus urinaria Linnea*. Most of the compounds were investigated to exhibit antioxidant and anti-inflammatory activities *in vitro*. Moreover, some active ingredients, including trimethyl-3,4-dehydrochebulate and methyl brevifolincarboxylate, supressed the production of NO, TNF-α and IL-6. [Fang SH, 2008]

6.2 The *in vivo* effects of *P. urinaria* on NASH

Having observed substantial suppression of steatohepatitis by *P. urinaria* treatment *in vitro*, we conducted experiments designed to test the potential for *P. urinaria* to exert preventive effects against steatohepatitis in *vivo*.

6.2.1 Animal models of NASH

Animal models are invaluable for understanding of the pathogenic mechanism of steatosis and steatohepatitis. Moreover, animal models are particularly useful for testing potential therapeutic interventions on NASH. However, no existing model can provide the entire NASH phenotype as encountered in clinical practice, with identical causative factors and pathobiological processes. In this project, we established two types of animal models, including diabetic db/db and wild-type C57BL/6 murine model fed with MCD diet (Figure. 6.1).

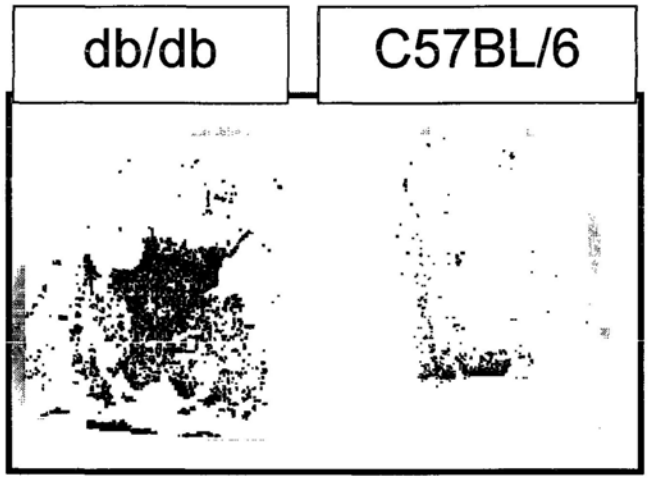

Figure 6.1 **Db/db and C57BL/6 mouse models for NASH**

The principle dietary model used in the study was C57BL/6 mice with NASH induced by MCD diet. The MCD diet is a lipid-rich diet (10% fat, versus 4% in normal chow) lack of two essential components, methionine and choline. Previous studies showed that mice fed the MCD diet might experience weight loss up to 40% of starting body weight with apparently normal physiological function. In mice fed MCD diet, researchers found lipid peroxidates accumulated from day 2 and reached massive levels by day 10. Lipid peroxidation persisted and oxidative stress occurred throughout the course of MCD diet chowing, which accelerated the progression from steatosis to steatohepatitis. In our study, C57BL/6 mice fed MCD diet experienced about 20% weight loss, but remaining physically active with good coat color. By day 10, the livers of MCD diet group exhibited macrovesicular and microvesicular fatty change accompanied by diffuse inflammation as compared to the mice fed on the control diet with methionine and choline.

The db/db mouse has mutation in the diabetes (db) gene which encodes the leptin receptor, resulting in dysfunction of the leptin receptor and consequently leptin resistance. [Chen H, 1996, Anstee QM, 2006] The db/db mouse appears uncontrolled food intake with obesity, insulin resistance, hyperglycemia and diabetes. The mechanism of steatosis is related to an increased delivery of fatty acid to the liver with high serum triglycerides and FFA and enhanced hepatic lipogenesis. This model will not develop steatohepatitis spontaneously without MCD diet induction. In present study, db/db mice fed MCD diet developed

NASH with macrovesicular fatty change and inflammation infusion by day 10. Hepatic oxidative stress was also significantly increased compared to the control.

6.2.2 Prevention of NASH

C57BL/6 mice fed with MCD diet for 10 days developed steatohepatitis. The liver histology exhibited steatosis, cellular inflammatory infiltrate and hepatocellular necrosis. Administering high dose *P. urinaria* (1000 ppm or 2000 ppm) in MCD-fed mice resulted in attenuation of the steatohepatitis, as evident by diminished histologic evidence of steatosis and inflammation. In addition, mice receiving *P. urinaria* had lower hepatic triglyceride content than in MCD diet-alone fed mice. *P. urinaria* inhibited the progress of the steatohepatitis induced by MCD diet in mice and significantly reduced the formation of TBARs and ROS.

Moreover, in the genetic NASH model, db/db mice fed MCD diet, virtually all hepatocytes show steatosis with infusion of necroinflammatory changes. The db/db mice fed MCD diet supplemented with *P. urinaria* (1000 ppm) improved liver histology with reduced cellular TBARS, compared with those fed on MCD diet alone.

It has been reported that Phyllanthus species contain more than 30 kinds of active chemical ingredients (Leslie Taylor, 1996; Yao QQ, 1993; Kassuya CA, 2005). In this study, the whole herbal extract of *P. urinaria* has been tested to

include no methionine and choline components. Some researches showed Phyllanthus played a hepatoprotective role via its antioxidant properties. [Bhattacharjee R, 2007; Chatterjee M, 2006] The results of current study revealed that *P. urinaria* was associated with a significant reduction of intrahepatic oxidative stress, and its antioxidant effect appears to play an important role in the attenuation of steatohepatitis. The mechanisms for prevention of steatohepatitis by *P. urinaria* were investigated thereafter.

6.3 Underlying mechanism of *P. urinaria* inhibition of NASH induced by MCD diet in mice

The pathogenesis of progression of NASH is complex. A growing body of evidence strongly suggests that increased FFA esterification and lipid storage, oxidative stress-induced lipid peroxidation and cytokine-mediated injury play a role in the liver injury occurring in NASH. Our study demonstrated administration of *P. urinaria* significantly improved the steatosis and oxidative stress in the lives compared to the MCD group. To investigate the hepatoprotective mechanisms of *P. urinaria* on NASH, we gained access to the expression of mRNA and proteins related to oxidative stress, fat metabolism regulation and inflammation.

6.3.1 Antioxidant effect of *P. urinaria* via suppressing CYP2e1

CYP2e1 is a major microsomal source of hydrogen peroxide and NADPH-dependent lipid peroxidation contributing to oxdative stress damage.

[Leclercq IA, 2000; Ekstrom G, 1989] Up-regulation of CYP2e1 was reported both in human NASH and in experimental steatohepatitis in mice fed MCD. [Sanyal AJ, 2001; Weltman MD, 1996; Weltman MD, 1998] Hepatocytes isolated from pyrazole-treated rats with induction of CYP2e1 showed greater sensitivity to the toxicities of ethanol and polyunsaturated fatty acid. [Wu D, 2000] MCD model causes steatohepatitis most likely through the CYP2e1-induced oxidative stress. [Quentin M. Anstee, 2006] In the present work, we showed that administration of *P. urinaria* led to a dose-dependent reduction of CYP2e1 expression from about 2-fold to 5-fold level compared with controls, thus alleviating lipid peroxidation in the liver. The effect of *P. urinaria* on preventing steatohepatitis and reducing lipid peroxidation is therefore likely to be related to the down-regulation of CYP2e1 expression. The regulation of CYP2e1 is complex and mediated through both transcriptional and posttranscriptional enzyme activation. The promoter of CYP2e1 contained many transcription factor binding sites including activator protein-1 (AP-1), hepatocyte nuclear factor and C/EBP. [Abdel-Razzak Z, 2004] One possible pathway of down-regulation of CYP2e1 by *P. urinaria* was through reducing nuclear accumulation of C/EBPβ. MCD diet-induced steatohepatitis is associated with elevation of both C/EBPβ and CYP2e1, whereas levels of CYP2e1 protein were lower in C/EBPβ$^{-/-}$ mice on MCD diet than in wild type MCD diet-fed mice. [Rahman SM, 2007] C/EBPβ binding mediated the induction of CYP2e1 by lipopolysaccharide in astrocytes. [Kelicen P, 2004] Thus, C/EBPβ played an important role in control of CYP2e1 in MCD model and *P. urinaria*

down-regulated CYP2e1 probably by effectively lowering C/EBPβ nuclear accumulation. On the other hand, CYP2e1 over-expression in NASH models impaired hepatic insulin signaling that was partially dependent on JNK activation. This phenomenon has been found to play an important role in the promotion of insulin resistance. [Schattenberg JM, 2005] In our study, decreased JNK activation was correlated with low expression of CYP2e1 in mice administered *P. urinaria*.

6.3.2 Inhibition of fatty acid synthesis and induction of fatty acid oxidation by *P. urinaria*

An excessive accumulation of triglycerides in the hepatocytes is mostly attributable to enhanced uptake and synthesis of fatty acids and reduced fatty acid oxidation. *P. urinaria* significantly improved the steatosis both *in vitro* and *in vivo*.

6.3.2.1 *P. urinaria* suppressed lipogenic regulator C/EBPβ

Administration of *P. urinaria* clearly lowered the nuclear accumulation of C/EBPβ, a newly established hepatic lipogenic regulator. Rahman SM et al. found that C/EBPβ over-expression in the liver of mice fed MCD diet led to elevated TG levels, endoplasmic reticulum (ER) stress, increased PPAR γ expression and NF-κB activation. [Rahman SM, 2007] A recent study showed that C/EBP-/- mice fed on a high-fat diet was protected against the development of obesity and the hepatic fat accumulation through increasing energy

expenditure and regulating critical genes related with lipid metabolism, including SREBP1c, PPARγ and SCD-1. [Millward CA, 2007] Moreover, genetic deletion of C/EBP dramatically reduced hepatic triglyceride accumulation and dampened steatohepatitis in C/EBP-/- mice fed with MCD [Rahman SM, 2007] or in the cross strain of C/EBP-/- and db/db mice. [Schroeder-Gloeckler JM, 2007] Thus, *P. urinaria* may have an effect on the control of fat input pathway through down-regulation of C/EBPβ.

6.3.2.2 *P. urinaria* promoted CYP4a10-catalyzed lipid ω-oxidation

Hepatic fatty acid oxidation occurs via mitochondrial β-oxidation, peroxisomal β-oxidation and CYP4A-catalyzed ω-oxidation. ACO and LCAD are the key enzymes of these three fatty acid oxidation systems in liver and are regulated by the lipolytic transcription factor PPARα. [Rao MS, 2004; Reddy JK, 2001] In our study, supplementation of the MCD diet with *P. urinaria* increased the expression of Cyp4a10, a PPARα downstream molecule. It has been shown that PPARα activation through its ligand (Wy-14,643) is effective against steatohepatitis through enhancing the CYP4A expression in MCD-fed mice. [Ip E, 2003 & 2004] Moreover, Cyp4a10 is involved in the degradation of proinflammatory lipid mediators such as prostaglandins, leukotrienes and the end products of lipid peroxidation. [Ip E, 2004] Collectively, the dramatically enhanced expression of lipolytic CYP4A10 by *P. urinaria* contributed to increased CYP4a-catalyzed fatty acid oxidation. In a situation of steatosis, stimulation of lipid combustion depletes the substrates for lipid peroxidation and

thereby decreases oxidative stress and prevents the development of steatohepatitis.

6.3.3 Anti-inflammatory effect of *P. urinaria* on NASH

Phyllanthus is a traditional herb used for anti-hepatitis mainly via its function on inhibition of oxidant and inflammation. We have demonstrated that *P. urinaria* protected against steatohepatitis through suppressing JNK and NF-κB related inflammatory signaling pathways and inhibiting pro-inflammatory cytokines such as TNF-α and IL-6.

6.3.3.1 *P. urinaria* blocked JNK and NF-κB pathways

In this study, we demonstrated that NF-κB and JNK, two major mediators in inflammatory response pathway, were highly active in MCD diet-fed mice but were substantially suppressed by *P. urinaria* administration. Both NF-κB and JNK, activated by a variety of stress signals such as TNF-α and ROS, mediate the interaction among obesity, inflammation, and insulin resistance. In addition, FFA increases the activities of various protein kinase C isoforms, which can in turn activate JNK. [Hirosumi J, 2002; Poli G, 2005, Hotamisligil GS, 2005] It has been well established that the JNK/c-Jun pathway mediates hepatocyte lipoapoptosis and liver injury from TNF- α, free fatty acids and oxidative stress. [Schwabe RF, 2004; Czaja MJ, 2003] Therefore, NF-κB and JNK are functional as both the cause and the consequence of increased inflammation and become self-reinforcing cycle factors. [Koteish A, 2002; Hotamisligil GS, 2005] In this

regard, one of the potential mechanisms for the anti-inflammation of *P. urinaria* is its ability to ameliorate the cellular ROS production and blunt the origin of this vicious cycle, thereby suppressing NF-κB and JNK pathways. This was supported by the evidence that hepatic triglyceride, inflammation and liver injury were reduced by JNK1 [Schattenberg JM, 2006] or NF-κB blockade [Dela Pena A, 2005] in mice from MCD diet-induced steatohepatitis.

6.3.3.2 *P. urinaria* downregulated cytokines TNF-α and IL-6

TNF-α and IL-6 are key inflammatory factors involved in the development of human and experimental steatohepatitis. [Abiru S, 2006; Koteish A, 2002] These pro-inflammatory regulators are mediated, at least in part, through oxidative stress. [Farrell GC.2003] Clinical trials found patients with NASH had an increased expression of TNF-α mRNA in both their liver and adipose tissue compared with obese non-NASH controls. Furthermore, the level of TNF-α expression paralleled the severity of histological appearance. [Crespo J, 2001] As a key inflammatory mediator, TNF-α plays a critical role in the evolution of steatohepatitis. TNF-α aggravates oxidative stress and is involved in the pathogenesis of mitochondrial dysfunction, a predominant source of oxidative stress in NASH. Treating cells with TNF-α was found to dramatically increase ROS, decrease the expression of adenosine triphosphatase and cytochrome c oxidase [Sanchez-Alcazar JA, 2003] and impair the electron flow of the mitochondrial respiratory chain. [Sanchez-Alcazar JA, 2000] Moreover, the pathogenic role of TNF-α has been proved to be related to the induction of

insulin resistance and further increase of lipolysis. Increased delivery of TNF-α to the liver will exaggerate the storage and oxidation of FFA, which generate ROS and induce oxidative stress. [Qin B, 2008]

IL-6 is another important mediator of inflammation in liver diseases, including fatty liver of obesity, type 2 diabetes mellitus and liver cirrhosis. IL-6 has been linked to impairment of hepatic insulin receptor signaling in hepatocyte culture and animal model. [Senn JJ, 2003; Klover PJ, 2003] Long-term exposure of IL-6 sensitized the liver to injury and impaired liver regeneration because of activation of the IL-6/signal transducer and activator of transcription protein-3 pathway. [Jin X., 2006; Torbenson M., 2002]

In this study, *P. urinaria* administration significantly suppressed oxidative stress and blunted TNF-α and IL-6 gene expression. The anti-inflammatory effects of *P. urinaria* may be partly related to inhibition of hepatic lipoperoxide and reduction in the mRNA expression levels of these regulators. TNF-α and IL-6 are NF-κB response elements. Upregulation of TNF-α and IL-6 may therefore be a result of the activation of NF-κB and c-Jun N-terminal kinase/activator protein-1 following MCD dietary feeding.

CHAPTER 7. SUMM ARY

In summary, *P. urinaria* clearly attenuated the liver injury of steatohepatitis in cultured hepatocytes *in vitro* and in MCD diet–fed mice *in vivo*. The mechanisms by which *P. urinaria* ameliorated steatohepatitis could be attributed to its antioxidant properties via suppression of CYP2e1, anti-inflammatory effects by suppressing inflammatory JNK and NF-κB pathways and downregulating critical inflammatory mediators TNF-α and IL-6, and induction of fatty acid oxidation through upregulation of CYP4a10 and suppression of lipogenic transcription factor C/EBPβ (Figure 7.1). Our findings supported a potential beneficial role of *P. urinaria* for the prevention and treatment of NASH in humans, and future clinical studies are warranted for validation.

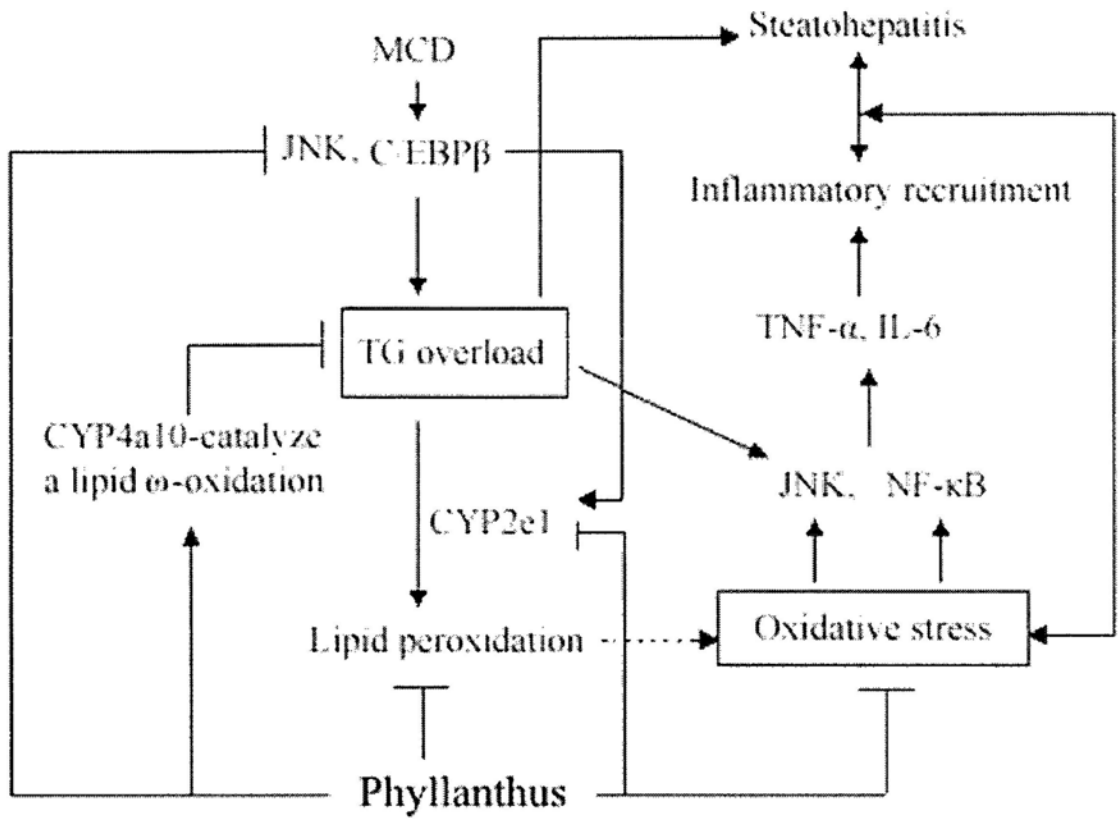

Figure 7.1 **Model for the mechanisms of protective effects of *P. urinaria* on MCD diet-induced steatohepatitis.** MCD diet-induced hepatic triglyceride (TG) overload triggers steatohepatitis via activating inflammatory JNK and NF-κB directly and by initiating CYP2e1-mediated oxidative stress, resulting in increased inflammatory recruitment. Phyllanthus relieves TG overload by promoting CYP4a10-catalyzed lipid ω-oxidation and suppressing lipogenic regulator C/EBPβ. On the other hand, Phyllanthus can lower oxidative stress directly and via blocking CYP2e1-mediated lipid peroxidation, thereby dampening the inflammatory cycle along with reduced activation of JNK and NF-κB pathways and expression of TNF-α and IL-6.

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