ผลของเจนิสทีนต่อภาวะไขมันลงตับที่ถูกเหนี่ยวนำโดยอาหารไขมันและฟรุกโตสสูงในหนูที่ถูกตัดรังไข่ทั้งสองข้าง

นางสาวสุดาพร พุ่มเมือง

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Effects of genistein on non-alcoholic steatohepatitis (NASH) induced by high-fat high-fructose diet in bilateral ovariectomized rats.

Miss Sudaporn Pummoung

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Science
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สุดาพร พุ่มเมือง : ผลของเจนิสทีนต่อภาวะไขมันลงตับที่ถูกเหนี่ยวนำโดยอาหารไขมันและฟรุกโตสสูงในหนูที่ถูกตัดรังไข่ทั้งสองข้าง (Effects of genistein on non-alcoholic steatohepatitis (NASH) induced by high-fat high-fructose diet in bilateral ovariectomized rats.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดวงพร วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ. ประสงค์ ศิริวิริยะกุล, 125 หน้า.

เพื่อศึกษาผลของเจนิสทีนต่อภาวะตับอักเสบที่ถูกเหนี่ยวนำด้วยอาหารไขมันและฟรุกโตสสูงในหนูที่ขาดฮอร์โมนเอสโตรเจน แบ่งหนูแรทเพศเมีย (n = 48) เป็นกลุ่มที่ถูกตัดรังไข่ทั้งสองข้างและกลุ่มที่ไม่ถูกตัดรังไข่ จากนั้นหนูทั้งสองกลุ่มจะถูกแบ่งเป็นอีก 3 กลุ่มย่อย คือ หนูกลุ่มที่ได้รับอาหารสูตรปกติ (กลุ่มควบคุม) กลุ่มที่ได้รับอาหารสูตรไขมันและฟรุกโตสสูง (กลุ่มเป็นโรค) และกลุ่มที่ได้รับอาหารสูตรไขมันและฟรุกโตสสูงร่วมกับเจนิสทีน ความเข้มข้น 16 มล./กก. วันละครั้ง ทุกวัน เป็นเวลา 8 สัปดาห์ (กลุ่มเจนิสทีน) เมื่อสิ้นสุดการทดลอง ทำการเก็บตัวอย่างเลือดเพื่อวิเคราะห์หาระดับเอนไซม์ตับเอเอสที เอแอลที และไซโตไคน์ทีเอ็นเอฟแอลฟา และเก็บเนื้อตับย้อมสีทางพยาธิวิทยาวิเคราะห์พยาธิสภาพของโรค การสะสมของไขมันในตับด้วยการย้อมสีออยล์เรดโอ วิเคราะห์ระดับเอ็นเอฟแคปปาบี ระดับไขมันไตรกลีเซอไรด์และกรดไขมันอิสระ ระดับเอ็มดีเอ การตายของเซลล์ตับด้วยวิธีทูเนล วิเคราะห์หาระดับพีพาร์แกมมา อะดิโปเนกทิน และตัวรับเอสโตรเจน ผลการศึกษาพบว่าหนูที่ได้รับอาหารสูตรไขมันและฟรุกโตสสูง มีพยาธิสภาพของตับอักเสบ โดยคะแนนพยาธิสภาพของตับที่ให้ตามเกณฑ์ของบรุนท์เพิ่มขึ้นอย่างมีนัยสำคัญ โดยมีคะแนนสูงที่สุดในหนูกลุ่มที่ถูกตัดรังไข่และเหนี่ยวนำให้เป็นโรค และพบการสะสมของไขมันในตับที่ย้อมด้วยสีออยล์เรดโอ ระดับไตรกลีเซอไรด์และกรดไขมันอิสระเพิ่มสูงขึ้นในหนูที่เป็นโรค ซึ่งพบว่าหนูกลุ่มเป็นโรคร่วมกับถูกตัดรังไข่มีการสะสมของไขมันในตับมากกว่าหนูที่ไม่ถูกตัดรังไข่ พบระดับทีเอ็นเอฟแอลฟาในซีรั่ม ระดับเอ็มดีเอ เอ็นเอฟแคปปาบี พีพาร์แกมมาในตับ และการตายของเซลล์ตับ เพิ่มสูงขึ้นอย่างมีนัยสำคัญในหนูที่เป็นโรค นอกจากนี้ยังพบว่าหนูที่ถูกตัดรังไข่มีระดับเอ็มดีเอ กรดไขมันอิสระ และพีพาร์แกมมาในตับไม่แตกต่างกันระหว่างกลุ่มควบคุมและกลุ่มเป็นโรค แสดงให้เห็นถึงบทบาทของเอสโตรเจนต่อกระบวนการเกิดตับอักเสบในหนู พบการแสดงออกของตัวรับเอสโตรเจนชนิดแอลฟาเพิ่มสูงขึ้นในตับของหนูกลุ่มเป็นโรค ขณะที่ตัวรับเอสโตรเจนชนิดเบตาในตับหนูกลุ่มเป็นโรคลดลง หนูกลุ่มเป็นโรคทั้งที่ไม่ได้ตัดและตัดรังไข่ มีคะแนนพยาธิสภาพของตับที่ให้ตามเกณฑ์ของบรุนท์ลดลงอย่างมีนัยสำคัญหลังจากได้รับเจนิสทีน ความเข้มข้น 16 มล./กก. วันละครั้ง ทุกวัน เป็นเวลา 8 สัปดาห์ นอกจากนี้ยังพบว่าเจนิสทีนลดการอักเสบ การสะสมของไขมัน และการตายของเซลล์ตับในหนูเป็นโรคที่ไม่ได้ตัดและตัดรังไข่อย่างมีนัยสำคัญ เจนนิสทีนลดการแสดงออกของพีพาร์แกมมา และเพิ่มการแสดงออกของอะดิโพเนกตินในตับหนูที่เป็นโรค ขณะเดียวกันเจนนิสทีนมีผลลดการแสดงออกของตัวรับเอสโตรเจนชนิดแอลฟาในตับ แต่ไม่มีผลเปลี่ยนแปลงการแสดงออกของตัวรับเอสโตรเจนชนิดเบตาในหนูกลุ่มเป็นโรคทั้งที่ไม่ได้ตัดและตัดรังไข่ การศึกษานี้แสดงให้เห็นว่าอาหารสูตรไขมันและฟรุกโตสสูงสามารถกระตุ้นให้เกิดภาวะตับอักเสบได้ ซึ่งภาวะตับอักเสบจะแย่ลงเมื่อเกิดร่วมกับการขาดฮอร์โมนเอสโตรเจน เอสโตรเจนจึงมีบทบาทในการป้องกันการเกิดภาวะตับอักเสบจากไขมันลงตับ การได้รับเจนิสทีนจะช่วยป้องกันการเกิดภาวะตับอักเสบได้ในหนูทั้งที่ไม่ได้ตัดและตัดรังไข่

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต ....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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KEYWORDS: BILATERAL OVARIECTOMIZED RATS, NON-ALCOHOLIC STEATOHEPATITIS, GENISTEIN, HIGH-FAT HIGH-FRUCTOSE DIET

SUDAPORN PUMMOUNG: Effects of genistein on non-alcoholic steatohepatitis (NASH) induced by high-fat high-fructose diet in bilateral ovariectomized rats.. ADVISOR: PROF. DUANGPORN WERAWATGANON, M.D., CO-ADVISOR: ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D., 125 pp.

To investigate the effects of genistein on estrogen deficiency with high-fat high-fructose (HFHF) diet-induced NASH rats. Female Sprague-Dawley rats (n=48) were randomly divided into ovariectomized (OVX) and non-ovariectomized (non-OVX) group. Both rat groups, further allocated into 3 subgroups; controls, rats fed with HFHF diet (NASH), and rats fed with HFHF diet plus daily 16 mg/kg BW of genistein (NASH+Gen) for 8 weeks. Serum samples were collected for liver enzymes (AST and ALT) and TNF-α level analysis. Liver tissue samples were harvested for histopathology, hepatic lipid accumulation (oil red o stained, FFA and TG level), hepatic lipid peroxidation (MDA level), hepatic inflammation (TNF-α and NF-kB) and hepatocytes apoptosis (TUNELs) examination. Protein expression of PPARγ, adiponectin, and estrogen receptor subtypes were analyzed by western blot. Rats fed with HFHF showed typical histopathology of NASH in both non-OVX and OVX groups as demonstrated by significantly increased of all histological feature scores of Brunt's criteria when compared with controls. The most severe histopathological damage was observed in OVX rats with NASH group. Hepatic lipid accumulations (ORO, TG, and FFA) were augmented in both non-OVX and OVX rats fed with HFHF. These augmentation were significantly higher in NASH rats with OVX than non-OVX. Additionally, inflammatory markers; serum TNF-α, hepatic MDA, and NF-kB, were enhanced in diet-induced NASH with both non-OVX and OVX groups. Moreover, hepatocytes apoptosis, and hepatic PPARγ expression were increased in both non-OVX and OVX rats with NASH. Interestingly, similarly levels of hepatic MDA, FFA, PPARγ, and adiponectin were observed between control and NASH with OVX groups. These might be the influence of estrogen deficiency and suggested the impact of estrogen deficiency in NASH pathogenesis. Estrogen receptors (ERα and ERβ) expression in liver displayed the opposite pattern after induced to NASH, since, ERα increased and ERβ decreased in NASH rats of both non-OVX and OVX. NASH rats that received daily 16 mg/kg BW of genistein improved the histopathological damage in both non-OVX and OVX as exhibited significantly lowered of all histological features scores. Furthermore, genistein significantly attenuated the inflammation, lipid accumulation, oxidative stress, and hepatocyte apoptosis in both non-OVX and OVX rats with NASH. Genistein displayed the properties as PPARγ antagonist and adiponectin agonist in NASH rats. In both non-OVX and OVX rats, the expression of ERα was declined with genistein administration as compared to the NASH groups, whereas, ERβ expression was not altered. In conclusion, HFHF diet is able to develop NASH pathogenesis and could be intensified by estrogen deficiency, suggesting the protective effect of estrogen on NASH. Genistein administration may protect NASH in both non-OVX and OVX rats.

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Chulalongkorn University

Field of Study: Medical Science
Academic Year: 2017

Student's Signature ....
Advisor's Signature ....
Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

αERKO	=	Estrogen receptor alpha knockout
ALT	=	Alanine aminotransferase
AMPK	=	5' adenosine monophosphate-activated protein kinase
ANOVA	=	Analysis of variance
ApoB	=	Apolipoprotein B
ApoE	=	Apolipoprotein E
ArKO	=	Aromatase knockout
AST	=	Aspartate aminotransferase
ATP	=	Adenosine triphosphate
BCA	=	Bicinchoninic acid
βERKO	=	Estrogen receptor beta knockout
CD-1	=	Cluster of differentiation-1
CD-36	=	Cluster of differentiation-36
CoA	=	Acetyl-coenzyme A
CYP2E1	=	Cytochrome P450 2E1
DAB	=	Diaminobezidine
DDW	=	Deuterium-depleted water
DMSO	=	Dimethyl sulfoxide
DNL	=	De novo lipogenesis
DW	=	Distill water
E2	=	Estradiol
ECL	=	Enhanced chemiluminescent
eNOS	=	Endothelial nitric oxide synthase
ERα	=	Estrogen receptor alpha
ERβ	=	Estrogen receptor beta
FADD	=	Fas-associated protein with death domain
FAS	=	Fatty acid synthase
FFA	=	Free fatty acid

HDL	=	High density lipoprotein
HFHF	=	High-fat high-fructose
IHC	=	Immunohistochemistry
IL-6	=	Interleukin-6
JNK	=	c-Jun N-terminal kinase
LDL	=	Low density lipoprotein
LPL	=	Lipoprotein lipase
LPS	=	Lipopolysaccharide
LSD	=	Least significant difference
MAPK	=	Mitogen-activated protein kinase
MCD	=	Methionine choline-deficient
MCF-7	=	Michigan Cancer Foundation-7
MDA	=	Malondialdehyde
MI	=	Myocardial infarction
NAFLD	=	Non-alcoholic fatty liver disease
NaOH	=	Sodium hydroxide
NASH	=	Non-alcoholic steatohepatitis
NF-kB	=	Nuclear factor-kappa beta
NON-OVX	=	Non-ovariectomized
NSS	=	Normal saline solution
ORO	=	Oil red o
OVX	=	Ovariectomized
PBST	=	Phosphate buffered saline with Tween 20
PNPLA3	=	Patatin-like phospholipase domain-containing protein 3
PPARα	=	Peroxisome proliferator-activated receptor alpha
PPARγ	=	Peroxisome proliferator-activated receptor gamma
PVDF	=	Polyvinylidene fluoride
RIPA	=	Radioimmunoprecipitation assay
ROS	=	Reactive oxygen species
SCD-1	=	Stearoyl-coA desaturase-1
SD	=	Standard deviation

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SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SREBP-1C = Sterol regulatory element-binding protein-1c

TBARS = Thiobarbituric acid reactive substances

TBST = Tris buffered saline with Tween 20

TG = triglyceride

TMA = Tissue microarray

TNF-α = Tumor necrosis factor alpha

TUNELS = Terminal deoxynucleotidyl transferase dUTP nick end labeling

VLDL = Very low density lipoprotein

WHO = World health organization

CHAPTER I
INTRODUCTION

Background and rationale

Nonalcoholic steatohepatitis (NASH) is the chronic progressive form of nonalcoholic fatty liver disease (NAFLD). The characteristics are demonstrated as necroinflammation, hepatocellular injury (1) and also cirrhosis at the end stage. Further progression of NASH may lead from hepatic steatosis and cirrhosis to hepatocellular carcinoma (2). The recent studied in a large middle-aged population showed that the prevalence of NAFLD and NASH is 20-30% (1) and 12.2% (3), respectively. Moreover, the development of NAFLD or NASH is strongly related with metabolic syndrome and obesity (4). Since, obesity is increasing worldwide, NASH become the most concern of liver diseases.

The mechanism of NASH pathogenesis is complex and involve with many processes of hepatic lipid metabolism at the "first hit" and resulting to inflammation and hepatocellular damage at the "second hit" (5). However, the lipotoxicity from lipid accumulation is not the only one process that accounting for the inflammation in the second hit. There are many molecular and metabolic alteration responsible for more progressive of fatty liver such as insulin resistance, metabolic syndrome, gut-derived endotoxin, adipose tissue signals, and genetic factors. Therefore, the "multiple hits" hypothesis was proposed for more precisely explanation of NASH pathogenesis (6).

One of the factors that influence the pathogenesis of NASH is dietary intake, especially fat and carbohydrate. However, the role of dietary fat in NASH pathogenesis is still controversial. Previous studied showed that *ad libitum* high-fat feeding induced the accumulation of lipid in liver of rats (7). Whereas, dietary fat ingestion responsible for only 15% of intrahepatic lipid (8). Interestingly, Pasquet and colleague (9) reported that fructose, which is the carbohydrate, is more steatogenic than fat because fructose generates reactive oxygen species (ROS) more than 100 times as compared with

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glucose (10). These can promote the damage of hepatocytes, however, the role of both dietaries fat and fructose in pathogenesis of NASH has not been fully elucidated.

Adipose tissue has many roles in NASH pathogenesis. It promotes the low grade of inflammatory stage with pro-inflammatory cytokines; IL-6 and TNF-α, production in obese individuals (11). Recently, adipose tissue is well accepted as the endocrine organ which not only secretes the cytokines, but also hormones (adipokines) such as adiponectin, leptin, resistin, and visfatin. Obesity, metabolic syndrome, and insulin resistance are the health conditions resulting an imbalance of adipokines that may greatly effect on liver functions (12).

The persistent of NF-kB activation was shown in animal models and patients with NASH (13). Tumor necrosis factor (TNF-α) shown to increase in patients with NASH (14) and positively associated with histological severity of liver damage (15). In contrary, interleukin-10, the anti-inflammatory cytokine reported significantly lower in NAFLD patients (14). The imbalance between pro-inflammatory and anti-inflammatory cytokines in NASH leads to hepatocyte necrosis and apoptosis, which finally turn simple steatosis and NASH to more progressive forms.

Estrogen has an important role in lipid and glucose metabolism which might be linked with NASH. Mice with estrogen deficiency showed hypercholesterolemia and increased NASH progression (16). In human studied, postmenopausal women markedly increased visceral fat accumulation and high incidence of metabolic syndrome (17, 18). Moreover, before 50 years of aged, men had 1.8-fold increased risk of having greater NASH than women. However, this different disappeared after 50 years of aged or postmenopausal status (19). Therefore, estrogen could be another factors that involved and should be more concerned in pathogenesis of NASH.

Although NASH is recently the most common liver disease, the effective treatment for NASH has not been identified (20). Genistein is a major isoflavone in soybean that mimic the estrogen effect. It has been investigated that not only estrogenic effect but genistein also has the hypolipidemic, antioxidant, and anti-inflammatory effect in both intact and deficient estrogen rats (21, 22). Previous studies demonstrated that genistein decreased the levels of TNF-α, IL-6, and TBARS in both serum and liver of high fat diet-induced NASH rats (23). Ovariectomized rats treated

with estrogen (E2) and estrogen receptor alpha (ERα) agonists has been shown to decrease body weight and total cholesterol level (24). Moreover, genistein reduced the activity of hepatic fatty acid synthetase (25) and increased the expression of PPARγ which is the hepatic transcription factor that regulates fat metabolism and inflammation (26) in NASH. However, it has been reported that the expression of PPARγ increased in high-fat diet-induced NASH rats (27) and genistein attenuated it expression level in liver of NASH ApoE knockout mice (28). Therefore, genistein might be useful to improve NASH pathogenesis, however, these effects of genistein need more study to clarify in diet-induced NASH with estrogen deficiency rats.

Research Questions

1. Does estrogen deficiency enhance the severity of high-fat high-fructose diet-induced non-alcoholic steatohepatitis?
2. Does genistein attenuate the pathogenesis of non-alcoholic steatohepatitis in estrogen deficiency rats?

Research Objectives

1. To examine the effect of genistein on estrogen deficiency rats fed with high-fat high-fructose diet to induce non-alcoholic steatohepatitis.
2. To investigate the severity of non-alcoholic steatohepatitis pathology in rats with estrogen deficiency.

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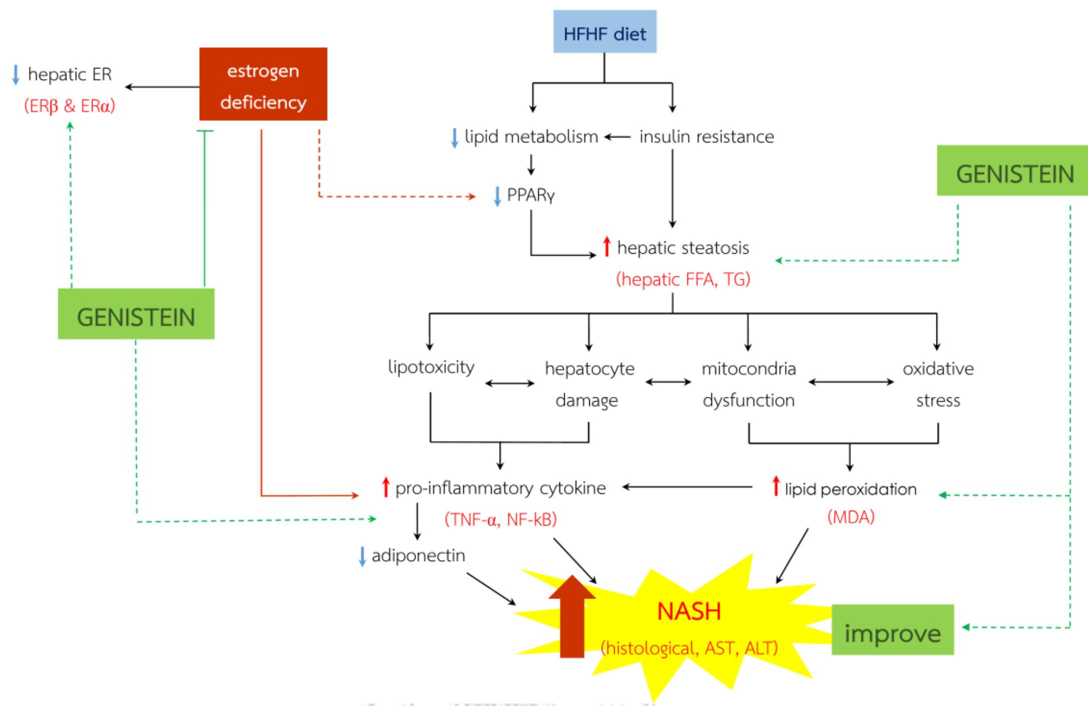
Hypothesis

Genistein diminish the severity of non-alcoholic steatohepatitis which induced by high-fat high-fructose diet in estrogen deficiency rats.

Conceptual Framework



Expected Benefit and Application

The results of this study will confer the information about the predisposing effect of estrogen deficiency on the pathogenesis of non-alcoholic steatohepatitis. In addition, they will provide an understanding of the genistein intake outcome on high-fat high-fructose diet-induced non-alcoholic steatohepatitis in estrogen deficiency rats. These might be useful for the applications of non-alcoholic steatohepatitis treatments in estrogen deficiency individuals.

Keywords

bilateral ovariectomized rats, non-alcoholic steatohepatitis, genistein, high-fat high-fructose diet

CHAPTER II
LITERATURE REVIEW

Non-alcoholic steatohepatitis (NASH)

Pathophysiology of NASH

NASH is the progressive form of simple steatosis or fatty liver which considered as a benign condition with low risk of progression. The hepatic fat accumulation exceeding 5-10% by weight (29) without a history of significant alcohol use is the crucial feature of NASH. The hallmarks of NASH are hepatocellular injury, inflammation, and fibrosis. Obesity and metabolic syndrome are the most common conditions that associate with NASH, however, there are some conditions that rarely relate with NASH such as polycystic ovary syndrome, sleep apnea, and pancreatoduodenal resection (30). Nevertheless, the exactly mechanisms that responsible for the progression of NAFLD to NASH are still largely unknown.

In 1998, the two-hit hypothesis was proposed to explain the pathogenesis of NASH (6). According to this hypothesis, “first hit” is responsible for the fat accumulation in liver resulting to inflammation, mitochondria dysfunction, and oxidative stress production in “second hit”. At the first hit, the intrahepatic lipid accumulation occurs when the rate of hepatic influx is over than hepatic lipid clearance. The dietary fat ingestion, obesity, and insulin resistance increase free fatty acids from adipocytes, *de novo* lipogenesis, and impairment of hepatic β-oxidation. The exportation of triglycerides are decreased from aberrant alteration of ApoB and VLDL synthesis and secretion (31). These are the important factors to increase the availability of free fatty acids resulting to fat accumulation as triglycerides in the liver (32). The lipotoxicity from hepatic fat accumulation might be the factor that responsible for second hit initiation. The damage of hepatocytes which induced by lipotoxicity leads to the expression of several inflammatory cytokines and oxidative stress. Moreover, reactive oxygen species formation from exceed mitochondria β-oxidation can further stimulate inflammatory

cytokines release. These mechanisms finally increase hepatocyte inflammation and predispose fatty liver to greater injury and then turn simple steatosis to NASH. Persistently stimulation of pro-inflammatory and fibrogenic cytokines releasing subsequence the activation of hepatic stellate cells resulting in extra-cellular matrix generation. Finally, progressive fibrogenesis consequences to cirrhosis and might be developed hepatocellular carcinoma (33) (Figure 2.1).

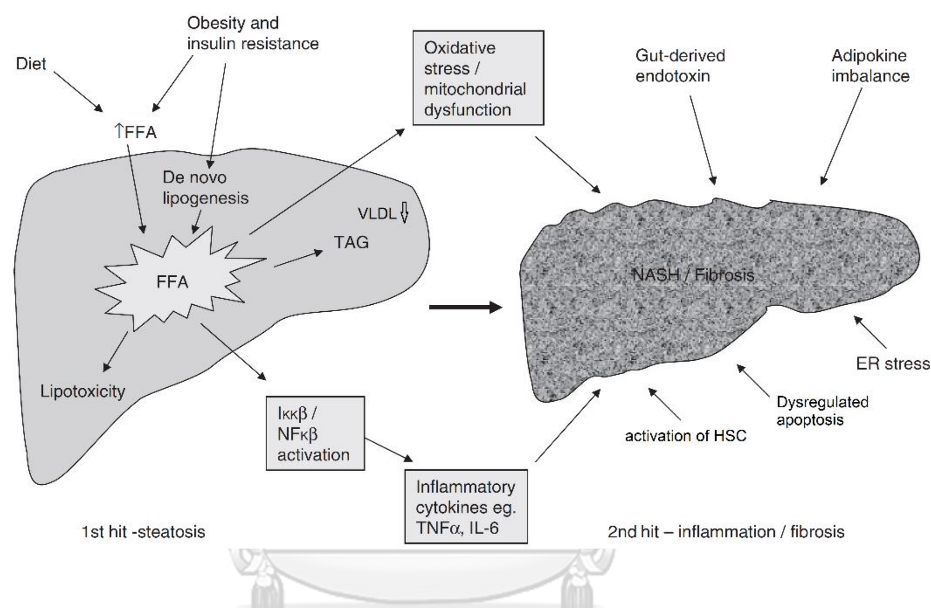


Figure 2.1 The two-hit hypothesis of NASH pathogenesis.
(modified from JK Dowman) (34)

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However, it is truly unknown which factors could be responsible for inflammation, fibrosis, and cirrhosis in NASH pathology. In addition, the pathogenesis of NASH seem to be the multifactorial disease, therefore, the two-hit hypothesis may not provide the accurate information for NASH pathogenesis. In multiple-hit hypothesis, dietary habits and genetic factors result to insulin resistance, obesity, metabolic syndrome, and alteration in the intestinal microbiome. Obesity reported to increase microbiota in the intestine and presentation of lipopolysaccharide (LPS) in those microbiota resulting to initiate innate immune response and may also accounting

for “second hit” in NASH model (6). The enlargement of LPS in circulating levels lead to systemic inflammation and hepatic insulin resistance (35). Moreover, NAFLD patients exhibit the relationship between the increasing of small bowel permeability and degree of liver steatosis (36). The disturbing microbiota system by probiotics showed improved systemic inflammation (37). This is suggesting the effect of gut-derived signals in pathogenesis of NASH.

Adipose tissue has also pivotal role in NASH pathology, since, adipokines; the hormones that secret from adipose tissue, may effect on liver function (12) when the imbalance of these adipokines occur. Additionally, genetic factors influence on hepatocyte fat content and inflammatory condition in liver. The polymorphisms in *PNPLA3*; gene that encoding a protein which homology to lipid acyl hydrolases, strongly associated with hepatic fat content in NAFLD (38). However, how PNPLA3 mutation increase risk of NASH is remains unclear. Therefore, the pathogenesis of NASH is a complex process which unable to explain by two-hit hypothesis and need more study to understand the mechanism of the disease.

Diagnosis of NASH

There are several biochemical markers and imaging modalities to use for NASH diagnosis. Currently, non-invasive imaging tecniques are commonly applied for hepatic steatosis assessment such as abdominal ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT). However, these modalities has several limitations; poor sensitivity in mild steatosis, skill-operated requirement, and expensive. Up-to-date research tried to discover the remarkable surrogate markers for NASH, unfortunately, they were still missing. Therefore, liver biopsy remains definitive gold standard for NASH diagnosis.

Factors influence on NASH

1.) Obesity and metabolic syndrome

In 2014, World Health Organization or WHO reported that the prevalence of obesity is 11% in men and 15% in women, worldwide (39). Thus, more than half of billion adults are classified as obese. Since, NAFLD is found in over two thirds of obese individuals (40), this condition may exist more than two hundred millions of the adult population. This might be led to NASH which is the most common cause of liver disease. However, there are evidences found that hepatic lipid accumulations were independently with obesity in men (41). Therefore, obesity alone might not be explained all hepatic lipid accumulation.

NASH is the hepatic manifestation of metabolic syndrome which closely related to obesity, insulin resistance, dyslipidemia, and hypertension (42). Patients with fatty liver are more likely to have dyslipidemia and high blood pressure than without fatty liver individuals (43). Insulin resistance in obesity and metabolic syndrome plays a key role on hepatic lipid accumulation in NASH, since, insulin influence several intracellular metabolic pathway; especially glucose and lipid metabolism (44). The impairment of insulin sensitivity leads to increase free fatty acid released from adipose tissue (45). Patients with NASH showed higher level of serum insulin (46). Peripheral insulin resistance increased lipolysis, hepatic fatty acid β-oxidation, and hepatic oxidative stress resulting to imbalance between hepatic pro-oxidant and antioxidant molecules (47) that might cause more susceptibility of hepatocytes to injury. Moreover, it has data indicated that NASH with peripheral insulin resistance associated with the defection of mitochondrial structure (44). Therefore, insulin resistance which closely related to obesity and metabolic syndrome is may the essential factor for NASH.

Nowadays, it is well known that obesity is the state of chronic inflammation (48) because adipose tissue secreted the large amount of inflammatory cytokines; TNF-α, which induced insulin resistance. Type 2 diabetes mice which induced by high fat diet exhibited hepatic and systemic insulin resistance and showed to increase the hepatic production of downstream proinflammatory cytokines of NF-kB pathway,

including IL-6, IL-1β, and TNF-α (13). The resistant of insulin signaling may lead more aggravation of NASH via increase proinflammatory cytokines and *vice versa*.

2.) Dietary

Daily calorie intake enlargement is parallel with the prevalence of obesity and metabolic syndrome. Whether the excessive energy intake is the contributor of NASH epidemic, the specific components of the diet should be considered.

High fat dietary intake is well accepted for inducing obesity, insulin resistance, and metabolic syndrome which are the contributing factors on hepatic lipid accumulation in NASH pathogenesis. Long-term consumption of increased percentage of fat with low percentage of carbohydrate did not increase the risk of NAFLD (49) and seemed to ameliorate elevate liver enzyme levels (50). However, mice fed with 40% fat containing diet established the features of NASH (51) and rats *ad libitum* high-fat feeding induced the accumulation of lipid in liver of rats (7). Although the role of dietary fat consumption on pathogenesis of NASH has not been elucidated, the amount of fat composition in diet should be concerned.

In contrast to dietary fat intake, carbohydrate over feeding results to weight gain and hepatic steatosis in a short period of time. The predominant carbohydrate diet is the monosaccharide fructose, which consumed as sucrose. Because liver is the site of fructose metabolism, fructose consumption may implicate in pathogenesis of metabolic syndrome and NASH (52, 53). High fructose diet reported to increase fatty acids in liver through *de novo* lipogenesis stimulation and inhibit hepatic lipid oxidation. Moreover, high-fat with high-fructose diet consumption caused serious change in the liver. It induced NAFLD by increasing plasma total cholesterol and triglyceride, inducing hepatic lipogenesis mediated via SREBP-1c and PPARγ, and reducing hepatic β-oxidation mediated via PPARα (54). Moreover, dietary fructose enhanced cytotoxicity in methionine-choline-deficient diet-induced NASH mice which resulting to alter histological changes of liver and induce hepatocyte apoptosis (55). These assumed to the predominantly contributing effect of dietary fructose to NASH progression.

3.) Adipokines from adipose tissue

Adipokines, the endocrine properties proteins that produce from adipose tissue, have important role in pathogenesis of NASH. Leptin is anorexigenic adipokine that prevents the accumulation of lipid in the liver (56), however, it also acts as pro-inflammatory and prodiabetogenic adipokine (57). Leptin reported to increase progressively with raising severity of steatosis and fibrosis in NASH patients (58).

Adiponectin, another adipokine, showed the potential role in NASH. In circulation, adiponectin can be detected in variety isoforms; globular or full-length (low, medium, and high molecular weight) (Figure 2.2). Adiponectin binds to 2 distinct isoforms of receptor; AdipoR1 and AdipoR2, for cascade physiological stimulation. Both adiponectin receptors are expressed differently in tissues. AdipoR1 is generally expressed in skeletal muscle, while, AdipoR2 is prominently expressed in liver and involving in glucose and lipid metabolism, inflammation and oxidative stress (59).

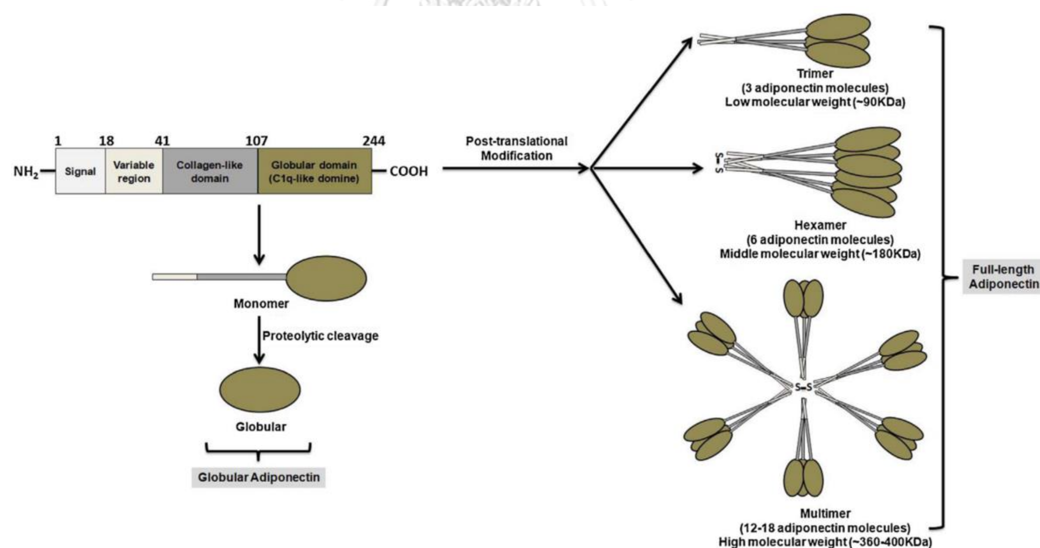

Figure 2.2 The different isoforms of adiponectin (59).

Adiponectin has been reported to hepato-protective properties by inhibiting the NF-kB activation and production of TNF-α and IL-6 (60). Additionally, high

adiponectin level decreased hepatic fat content and improved insulin resistance in leptin-deficient *ob/ob* mice (61). The reduction of adiponectin and augmentation of leptin might be involved in hepatic steatosis, inflammation, and fibrogenesis in obese individuals (62).

Resistin and visfatin are the recently discovered adipokines that may play a role in NASH patients. Rat fed with high fat diet resulted NASH and significant progressive increase in serum resistin and visfatin. In patients with NAFLD, serum resistin were associated with histological steatosis, portal inflammation, and NASH scores (63). On the contrary, in 114 NAFLD patients and 60 healthy individuals, visfatin levels showed no difference between two groups (64). These are conflicting results on the visfatin and NASH pathogenesis association. Therefore, the level of these adipokine and the effect on NASH are still undescribed.

4.) Peroxisome proliferaror-activated receptors (PPARs)

Structure and distribution of PPARs

PPARs are the ligand-activated transcription factors that are members of the nuclear receptor. In mammal, PPARs has been classified into 3 isoforms; PPARα, PPARβ (also known as PPARδ), and PPARγ. Each isoforms has a particular physiological functions due to different of biochemical properties, specific distributions in organs, and distinct responses to ligands (65). There are various exogenous and endogenous ligands that can be bonded with PPARs and activated the relating biological consequences such as fatty acids and their derivatives, eicosanoids, metabolites of linoleic acid, etc.

Basically, stimulated PPARs are heterodimerization with retinoid X receptor (RXR). Afterward, multi-protein complex binds to DNA on the specific element which named peroxisome proliferator response element (PPRE) and encodes several proteins involving in energy homeostasis, lipid metabolism, and cell proliferation (66).

Tissue distribution of PPAR isoforms are differed from each other and their functions are depended on specificity of tissue types. PPARα is widely expressed in skeletal muscle, brown adipose tissue, heart, and liver. Whereas, the expression of PPARγ is highly in adipocyte and lesser expressed in liver and spleen. PPARβ/δ is

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ubiquitous expressed in all tissues, nevertheless, it is abundant in liver, kidney, and skeletal muscle (67).

Function of PPARs on lipid metabolism

The experimental evidences are well-accepted the key role of PPARα on the regulation of fatty acid oxidation in liver. Additionally, PPARα is involved in pro-inflammatory response, since, PPARα agonists exhibited the inhibitory effect on NF-kB and AP-1 transcription activities (67). Moreover, PPARα also contributed to implicate in the development of embryo and glucocorticoids secretion (68).

The implications of PPARβ/δ are generally ranging from regulation of cell proliferation, thermogenesis to energy homeostasis. Otherwise, physiological functions of PPARβ/δ are still being clear up.

The rest isoform of PPARs; PPARγ, exists 2 isoforms which are PPARγ1 and PPARγ2. PPARγ1 is expressed in liver, while, PPARγ2 is expressed preferentially in adipose tissue (69). PPARγ exhibits the opposite action from PPARα because it serves as a regulator adipocytes differentiaton and lipid storage. Thus, PPARγ is potential target for insulin-sensitizing drug; Thiazolidinedione (TZD), that used for type 2 diabetes treatment. However, long-term TZD usage which means persistently activate PPARγ reported to gain weight (70) and promote hepatic steatosis (69).

The controversial actions of PPARγ in liver are reported. Diet-induced hepatic steatosis demonstrated up-regulation of PPARγ through stimulation of genes involving in lipid uptake and storage (54, 71, 72). On the contrary, PPARγ sequestered fatty acid to adipose tissue resulting to protect liver from fat accumulation (67, 73). This speculate that, the effects of PPARγ in liver remain unidentified.

5.) Estrogen deficiency

Estrogen is the primary female sex hormone that not only involved in reproductive regulation but also in many biological functions such as cell differentiation and inflammatory modulation (74). The most potent form of estrogen is estradiol-17β or E2 which synthesized mainly in ovary by testosterone aromatization. Physiological function of E2 occurs via the activation through plasma membrane-

associated and nucleus estrogen receptors (74). Binding with nuclear receptors, E2 acts as the transcription factors that leads to increase several genes numbers, including the low-density lipoprotein (LDL) receptor, endothelial nitric oxide synthase (eNOS), and cyclin D1 (75). While, the plasma membrane-associated estrogen receptors are responsible for the alteration and regulation of gene expression in cell type-specific manner. In liver, E2 signals are related to LDL receptor expression, which resulting to the reduction of serum LDL-cholesterol.

Sex hormones are required to integrate metabolic interaction between major organs, and distributions of fat are partially determined by concentrations of sex hormones (76). Estrogen deficiency is associated with the alteration of lipid metabolism and affected the markedly increasing of lipid in blood circulation (77). After menopause, estrogen deficiency leads to visceral obesity that accomplished by insulin sensitivity reduction (78). Nevertheless, the mechanisms of estrogen deficiency induced obesity are not clear.

The incidence of NASH in women is increased when they underwent to menopause (19) and hepatic nuclear estrogen receptors are dependent on age but not sex (79). Mice fed with high-fat high-cholesterol diet demonstrated that estrogen deficiency worsen histological features, lipid profiles, and inflammation in NASH mice (16).

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Genistein

Structure, bioactivation, and absorption of genistein

Genistein is one of isoflavones that mainly found in soybean, which has three principle isoflavones: genistein, daidzein, and glycetein in a percentage concentration of 50 : 40 : 10% (80). Isoflavone is the one of six major subclasses of flavonoids, which are the secondary metabolite of plant polyphenolic compound (Figure 2.3)

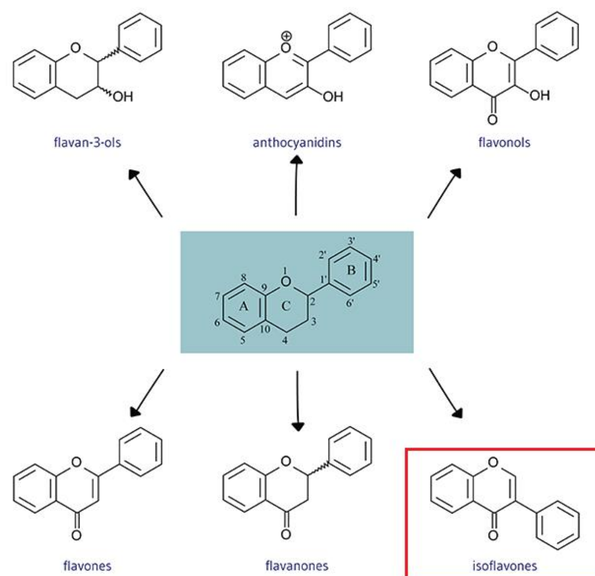

Figure 2.3 Basic structures of six flavonoids subclasses.
(modified from Hossain MK) (81)

Almost source of isoflavone in plants are belonging to the Fabacae family (82). For human dietary, the compatible nutritional isoflavones source is soy. However, there are other isoflavones sources such as red clover, but these are for cattle industry (83). The distribution of isoflavones in diet are primarily binding with at least one molecule of sugar and called glycosylated or glucoside isoflavones.

In soybean, genistein presents as inactive form by conjugated with the glycosides that called genistin (84). The glycoside-conjugated form is hardly absorb through the epithelial cells of intestine because of high molecular weight and hydrophilic property. Furthermore, glycoside-conjugated form of genistein has weakly biological activities. After ingested, genistin requires the hydrolyzation by acid in stomach and β-glucosidases enzyme from bacteria in the intestinal wall for converting glycone to aglycone form which is genistein (85) (Figure 2.4). The deconjugation of genistin needed for the estrogenic potencies of genistein (86). The metabolites of genistein are uptake by liver and excretion into the bile as β-glycuronides genistein and transport through blood circulation. Consequently, a second round of hydrolysis

occurs again in the intestine (87). The enterohepatic circulating allowed tissues and organs prolong exposed with genistein after soy consumption. The highest concentration of genistein in circulation occurs within 4-8 hours after intake (88) and excretes with more hydrophilic metabolites via urine within 24 hours.

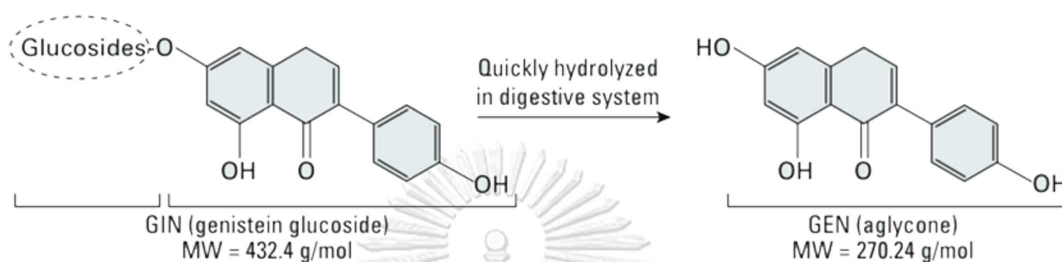

Figure 2.4 Glucosides and aglycones forms of isoflavones. (modified from Izumi T) (89)

Estrogenic effect of genistein

Genistein shares structural features with the estradiol-17β or E2 (90), therefore, genistein can bind with human estrogen receptor and exert estrogenic and anti-estrogenic activity (Figure 2.5).

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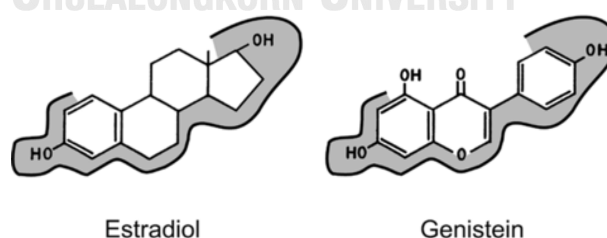

Figure 2.5 The resemblance of estrogen and genistein structure.

Genistein binds differentially to estrogen receptor subtypes; estrogen receptor alpha and beta (90), with higher affinities to ERβ than ERα (91, 92). Therefore, the

selective action of genistein may different between tissues which depend on the tissue distribution of estrogen receptor subtypes. Although, genistein has been reported that it binds to estrogen receptors with a weaker affinity than estradiol (93). Consumption of dietary containing high levels of genistein may result in biological response in both humans and animals.

There are many estrogenic effects of genistein, since, estrogen is involved in many biological processes. Genistein is useful to improve the symptoms that involved with the estrogen deficiency such as unpleasant symptoms of post-menopausal women (hot flushes and vaginal dryness), osteoporosis, cognitive function, obesity, and cardiovascular disease (94).

Genistein effected on the reduction of ovariectomized mice food intake resulting to decrease body weight (95). Moreover, it ameliorated insulin resistance and increased HDL cholesterol (96). Conversely, in high-fat diet-induced obesity in ovarectomized rat, administration of genistein had no effect on energy intake, body weight and lipid profile (24). Previous studies showed that plasma LDL level reduced up to 10% after isoflavone intake (97) and genistein attenuated the hyperlipidemia in male hamsters fed with high-fat diet by up-regulated both ERβ and ERα in liver (98). Female OVX Wistar rats treated with ERβ agonist, ERα agonist, or genistein and fed with high-fat diet demonstrated the reduction of lipogenesis and triglyceride accumulation in liver and muscle (99). Compared with daidzein, genistein showed stronger affecting on hepatic genes expression that involved in lipid and carbohydrate metabolism (100). In rat model of postmenopausal metabolic syndrome, genistein increased HDL cholesterol, and enhanced hepatic PPARα and PPARγ expression (96).

The peroxisome proliferator-activated receptors (PPARs) is a nuclear receptor that has an essential role in lipid and carbohydrate metabolism. These receptors are belong to the steroid/thyroid receptor superfamily, thus, genistein which is the ligand for estrogen receptor can bind and activate the biological action of PPARs (101). As an agonist of PPARα, genistein improved insulin sensitivity, glucose homeostasis, and adiposity through PPARα activation pathway. In addition, genistein down-regulated PPARγ during adipocyte differentiation via estrogen receptor-dependent mechanism (102). However, liver cells treated with genistein increased lipid metabolism genes in

an estrogen receptor-independent pathway but through activation of PPARα (103). In addition, increased hepatic PPARγ mRNA expression was observed in OVX treated with genistein Wistar rats (99). Therefore, the estrogenic action of genistein on lipid metabolism through PPARs pathway is still unclear.

Nonestrogenic effects of genistein

There are several non-estrogenic effects of genistein including anti-oxidative stress, inhibiting cell proliferation, inducing cell apoptosis, and anti-inflammation.

Recently epidemiological meta-analysis study reported that soy isoflavone intake reduced risk factor of breast cancer in both pre- and post-menopausal Asian women (104). In human breast cancer cell line (MCF-7), it was shown that genistein inhibit cells proliferation through cell cycle regulation (105).

The study of anti-inflammatory effect of genistein is very highlight nowadays. It has been found that genistein showed antioxidant effect and decreased renal inflammation in streptozotocin-induced diabetic mice (106). In addition, genistein decreased the plasma level of TNF-α and IL-6 in rats fed with fructose (107), and also inhibited IL-1β, IL-6, and TNF-α mRNA levels. Furthermore, macrophages and neutrophils which contributing to the inflammatory response are attenuated their activation by genistein (108).

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Effects of genistein on NASH

Since, the lipid metabolism and anti-inflammatory effect of genistein are involved with the estrogen, genistein may have the benefits on NASH progression in estrogen deficiency. The hepatic oxidative stress in NASH reduced by genistein via activation of enzymes and proteins that involved in antioxidative process such as superoxide dismutase and cytochrome P450 2E1 (109). Moreover, the induction of oxidative stress in ovariectomized rats can be attenuated by reversed the antioxidative enzymes level (22). The inflammatory cytokines; TNF-α, IL-1β, IL-6, and NF-kB in NASH are attenuated by genistein administration (23, 110). Besides, the activation of AMPK

and JNK pathway which are the inflammatory stimulated pathway can be inhibited by genistein (23, 110).

Genistein and soy protein supplementation improved NASH through sensitized insulin signaling which leads to decrease insulin resistance and improve lipid metabolism in both intact and ovariectomized rats (25, 109, 111). Moreover, genistein and soy protein lowered hepatic total cholesterol and triglyceride in a dose-dependent manner, resulting to the decreased of hepatic lipid accumulation in diet-induced NASH rats (26, 112). Inversely, mice fed with methionine-choline-deficient (MCD) diet-induced NASH did not show the significantly change in hepatic steatosis after genistein administration (110). These can be implied that effect of genistein on hepatic lipid accumulation may difference between NASH models. In addition, the effect of genistein on PPARγ is still controversy. Previous studies reported that genistein could increase hepatic PPARγ in NASH rats (26), however, it markedly decreased PPARγ in liver of weanling (112) and ApoE knockout (28) rats induced NASH models. Therefore, the further study of the effects of genistein on lipid metabolism and fat accumulation in NASH is still needed.

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CHAPTER III
MATERIALS AND METHODS

3.1 Study design

Animal experimental study

3.2 Ethic consideration

All animal procedures in this study were accomplished in agreement with recommendations and guidelines for experimental animals from the National Research Council of Thailand.

The study protocol was submitted and approved by Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University (MED-ACUC), the permission No. is 19/2559.

3.3 Sample size calculation

This study was calculated the number of rats per group per time point perform as Susutlertpanya W, et al. (26), the sample size was estimated by

$$n_{(/group/time\ point)} = \frac{(Z_{\alpha/2}+Z_{\beta})^2\sigma^2}{(\bar{x}_1-\bar{x}_2)^2}$$

This study chosen α value = 0.05; $Z_{\alpha/2}$ = 1.96; β value = 0.1; Z_β = 1.28, σ^2 = 6.27, average X_1 = 3.83, and average X_2 = 0.36. Therefore, the sample size in this study would be 8 in each group.

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3.4 Study protocol

Forty-eight female Sprague-Dawley rats, four weeks age, and 120-140 g weight were used in the study. Initially, all rats were group-caged, kept under standard conditions (temperature at 25°C and 12-hour dark-light cycle) and fed with standard diet. After one week for adaptation, rats were randomly allocated into six groups (8 rats per each group) as details in Table 3.1.

Table 3.1 The conditions of six experimental groups.

Experimental group	Ovariectomy (OVX)	HFHF diet	Genistein 16 mg/kg BW
control	-	-	-
NASH	-	✓	-
NASH + Gen	-	✓	✓
OVX	✓	-	-
OVX + NASH	✓	✓	-
OVX + NASH + Gen	✓	✓	✓

n = 8 in each group. HFHF = high-fat high-fructose, BW = body weight, NASH = non-acoholic steatohepatitis, Gen = genistein, OVX = ovariectomized.

The rats were assigned as following groups;

1) control: rats fed with standard diet.
2) NASH: rats fed with high-fat high-fructose (HFHF) diet.
3) NASH + Gen: rats fed with HFHF diet and administered of 16 mg/kg body weight of genistein via oral gavage once daily.
4) OVX: rats were performed bilateral ovariectomy and fed with standard diet.
5) OVX + NASH: rats were performed bilateral ovariectomy and fed with HFHF diet.
6) OVX + NASH + Gen: rats were performed bilateral ovariectomy, fed with HFHF diet and administered of 16 mg/kg body weight of genistein via oral gavage once daily.

All rats fed with diets and water for eight weeks *ad libitum*. Body weight was measured and recorded weekly. At the end of experimental period, rats were 6-8 hours overnight fasted and sacrificed with overdose thiopental sodium through intraperitoneal injection. Afterwards, the abdomen of rats were opened and taken out of the whole livers rapidly, washed with cold normal saline solution (NSS) and cut into small pieces. A few pieces of livers were fixed overnight at room temperature in 10% formalin for paraffin embed and stained with hematoxylin and eosin for histological analysis or immunohistochemistry (NF-kB, PPARγ, and apoptosis) examination. The remaining pieces of liver were immediately frozen in liquid nitrogen and stored at -80°c until analysis.

Liver tissues were extracted, thereafter, the supernatant was collected for hepatic lipids (free fatty acid and triglyceride) and hepatic malondialdehyde (MDA) levels. In addition, the supernatant also used for estrogen receptor subtypes (ERα and ERβ), peroxisome proliferator-activated receptor gamma (PPARγ), and adiponectin protein expression measurement.

Blood samples were collected by cardiac puncture and allowed to clot for 30 min at 25°c. Subsequently, clotted blood were centrifuged at 2,000g for 10 minutes at 4°c. Eventually, the serum was collected and stored at -80°c until analysis. Liver enzymatic tests (aspartate transaminase; AST and alanine transaminase; ALT) and inflammatory cytokine; TNF-α assessment, were performed. The study protocols is shown in Figure 3.1.

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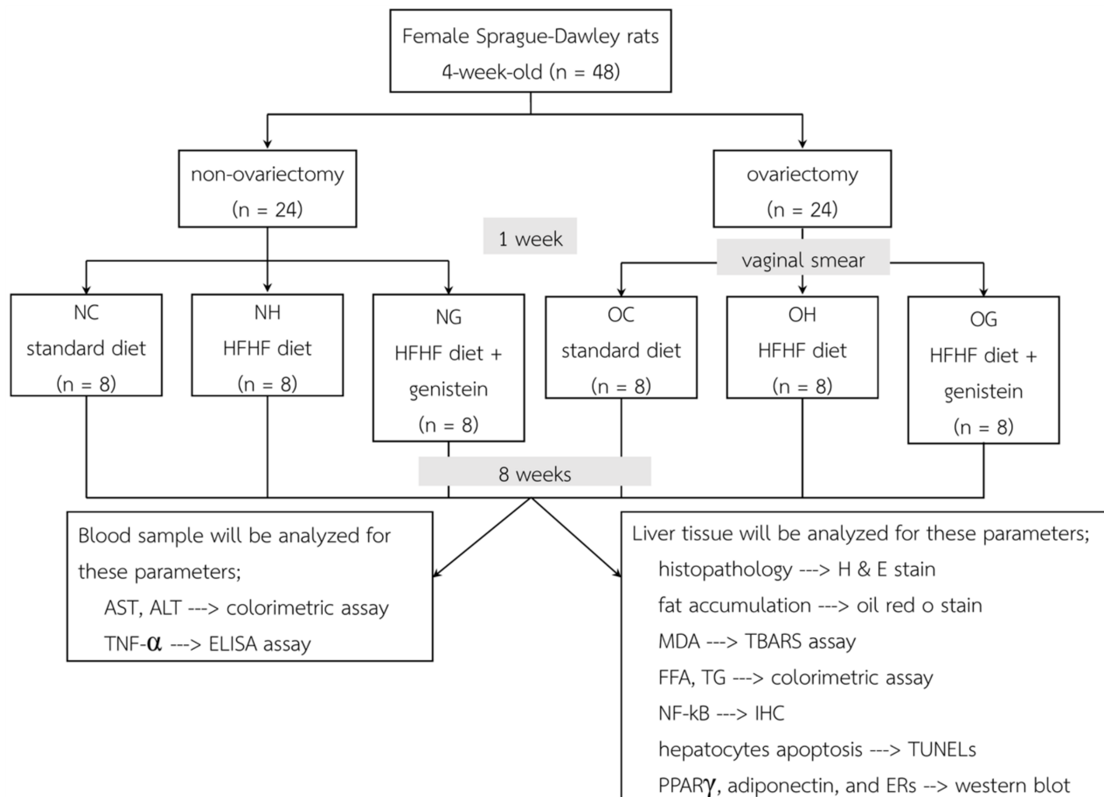

Figure 3.1 The schematic of study protocol.

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3.5 Bilateral ovariectomized protocol

Rats in ovariectomy groups were performed the bilateral ovariectomy with the bilateral dorsolateral approach (113, 114) under the anesthesia using thiopental sodium. Briefly, anesthetized rats laid on one side, skin incision was performed on the middle point between rib cage and proximal area of the thigh. Muscle layers were dissected through to the peritoneal cavity. The adipose tissue surrounding the ovary was cleared to identify the ovary and uterine tube and then performed the ligation at the distal uterine horn and the ovary completely removed. After that, muscle and skin were closed layer by layer (Figure 3.2).

Rats were caged individually for recovery after ovariectomized and performed vaginal smear at 8.00 A.M. for five days consecutively (115) to confirm the complete of bilateral ovariectomized.

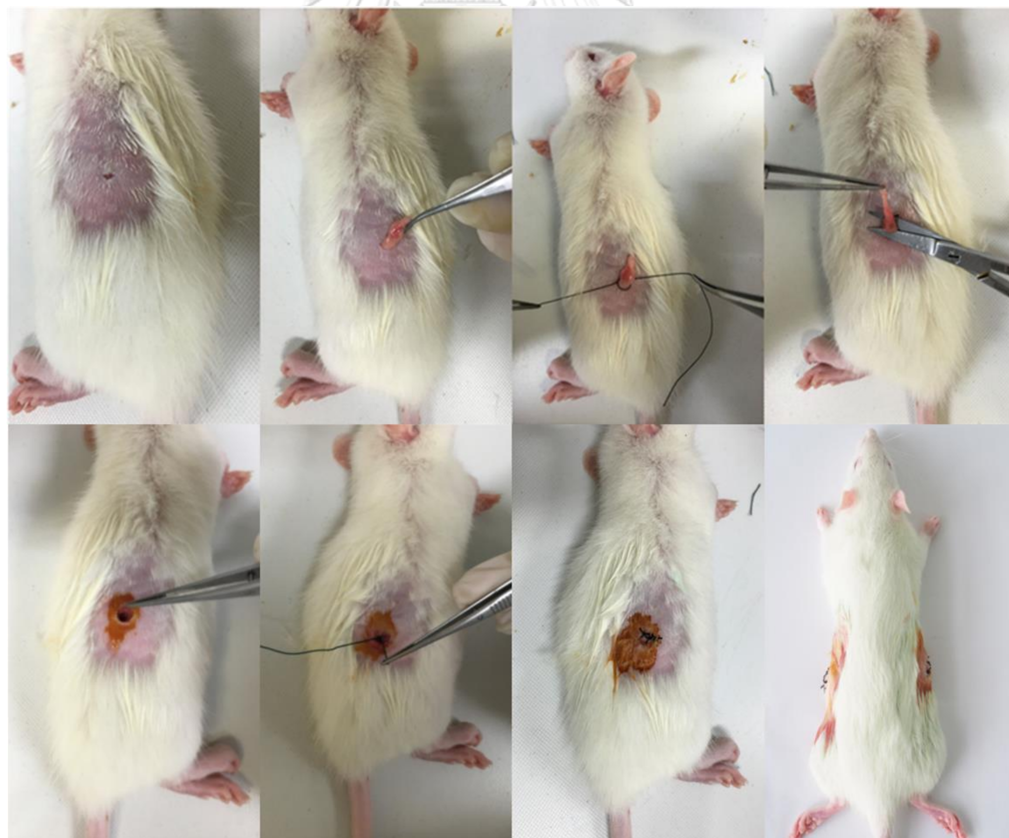

Figure 3.2 The bilateral dorsolateral approach procedure for bilateral ovariectomy.

3.6 Vaginal smear protocol

As shown in Figure 3.3, rats were performed vaginal smear with small glass dropper. Examiner gentle grasped the back and neck region of rat and pushed tail aside. The other hand of examiner passed in the tip of glass dropper that fill with small amount of normal saline solution into the vagina and flushed 2-3 times. In a while, the vaginal lavage samples were collected and placed on 2 glass slides equally. Samples were left to air-dried and stained with 2% methylene blue. The changes of vaginal epithelial cell types proportion were investigated under light microscope (116).

Figure 3.3 The procedure of vaginal smear.

The full estrous cycle in rodent occurs over 4 or 5 days and can be divided into 4 phases; proestrous, estrous, metestrous, and diestrous (117). There are three cell types that can be detected in vaginal smear samples; nucleated epithelial cells, cornified squamous epithelial cells, and leukocytes. The relative ratio of these cells was used to identify the phase of estrous cycle (Table 3.2). The complete

ovariectomized rats showed the relative ratio of epithelial cells in vaginal smear samples similar to cells that observe in the diestrous phase and called anestrous phase (Figure 3.4).

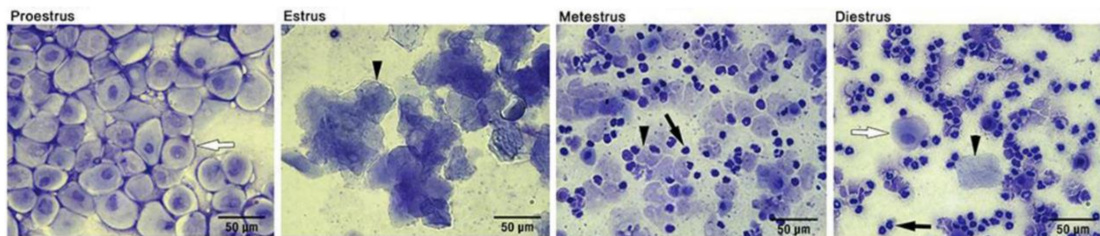

Figure 3.4 Cytology of vaginal smear sample in each stages (118).

Table 3.2 Cell types in each stage of estrous cycle.

Estrous cylcle	Cell types
Proestrous (12-14 hrs)	• predominance of nucleated epithelial cells (clusters or individually) • some cornified squamous epithelial cells
Estrous (25-38 hrs)	• predominance of cornified squamous epithelial cells (clusters)
Metestrous (6-8 hrs)	• mix of three cell types with a predominance of leukocytes • few nucleated epithelial and/or cornified squamous epithelial cells
Diestrous (48-53 hrs)	• predominance of leukocytes

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3.7 Experimental diets

Standard diet for NC and OC groups consist of 7% fat, 47% carbohydrate, and 27% protein which providing from Perfect companion group co., Ltd, Thailand.

High-fat high-fructose diet (Figure 3.5) was prepared from the modification of Pickens MK formula (55). The composition of HFHF diet in this study consists of 55% fat (vegetable oil), 35% carbohydrate (20% is fructose), and 10% protein (albumen) (Table 3.3).

Table 3.3 The ingredient of high-fat high-fructose (HFHF) diet.

Ingredients (%)	Standard	HFHF
protein	27	10
carbohydrate	47	35#
fat	7	55*

*vegetable oil, #consist of 20% fructose

Figure 3.5 High-fat high-fructose diet to induce NASH.

3.8 Genistein preparation

A crystalline solid of genistein (Cayman chemical, MI, USA) was freshly dissolved in 1 mL of 0.1% dimethyl sulfoxide (DMSO) at the concentration of 16 mg/kg body weight of rats (23, 26). All rats were weighed and recorded weekly for monitoring and genistein dose calculation.

Genistein administered to NASH with non-OVX and OVX rats via oral gavage which is the procedure that can deliver liquid compound directly to the stomach of rats. Prior to perform oral gavage, the stainless steel gavage needle would be measured for rat sizes and lengths. The distance from mouth to the last rib of rats was equal to the esophagus length. The other side of gavage needle; bulb tip, is connected to a syringe that containing the genistein solution.

Rats were gently restrained in upright position to immobilize the head without signs of distress. The gavage needle passed along the side of mouth, following through the roof of the mouth, toward the stomach. Subsequently, genistein was push at the corrected length and withdraw the needle. The unpleasant signs such as coughs or chokes were monitored (Figure 3.6).

Figure 3.6 The characteristics of genistein and oral gavage technique.

3.9 Hepatic histopathology

Principle of hematoxylin-eosin (H & E) stains

The hematoxylin and eosin stain is used widely for visualizing and studying the morphological changes of variety tissue types in their natural environment. Hematoxylin stains nucleic acid yielding the deep blue-purple color, while, eosin stains nonspecific; cytoplasm and extracellular matrix, proteins which resulting to several shades and intensities of pink, orange, and red colors (119). In the staining solution, hematoxylin is oxidized to heamatin which more soluble in aqueous acidic solution and presenting red color. On the contrary, heamatin is less soluble in basic condition and displaying blue color. The heamatin exhibits cell nuclei due to bind with lysine residues of nuclear histones in acidic conditions. Counterstaining with eosin obtains full details of cell.

Hematoxylin-eosin stain procedures

Reagents preparation

1. Hematoxylin

aluminium ammonium sulphate	200	g
hematoxylin	20	g
ethanol	40	mL
sodium iodate	4	g
acetic acid	80	mL
glycerol	1,200	mL
DW	2,800	mL

2. Eosin

1% eosin	400	mL
1% aqueous phloxine	40	mL
95% alcohol	3,100	mL
acetic acid	16	mL

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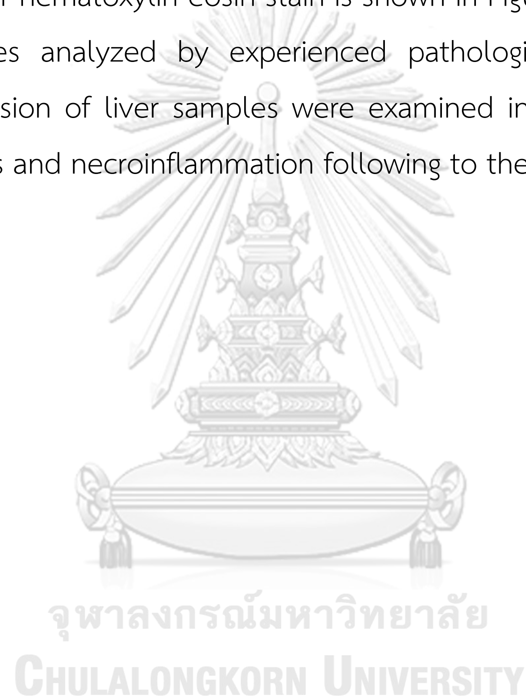
Tissue samples were fixed in 10% formaldehyde for 24-48 hours duration before embedding. Embedded liver sample blocks were sectioned into 3 μm thick (Thermo electron corporation, USA) and prepared with hot air oven, overnight. In the beginning of the stain, the sections were deparaffinized in order with xylene and rehydrated with alcohol for penetration of staining dyes into tissue section. Subsequently, sections were stained with hematoxylin for nuclear staining, rinsed 5 minutes with running tap water, and dipped in 95% alcohol. Latterly, sections were counterstained with eosin, dehydrated, cleared with xylene, and covered by coverslip. The procedures for hematoxylin-eosin stain is shown in Figure 3.7.

All samples analyzed by experienced pathologist with blinded manner. Histopathologic lesion of liver samples were examined in all fields for grading and staging of steatosis and necroinflammation following to the criteria of Brunt (120).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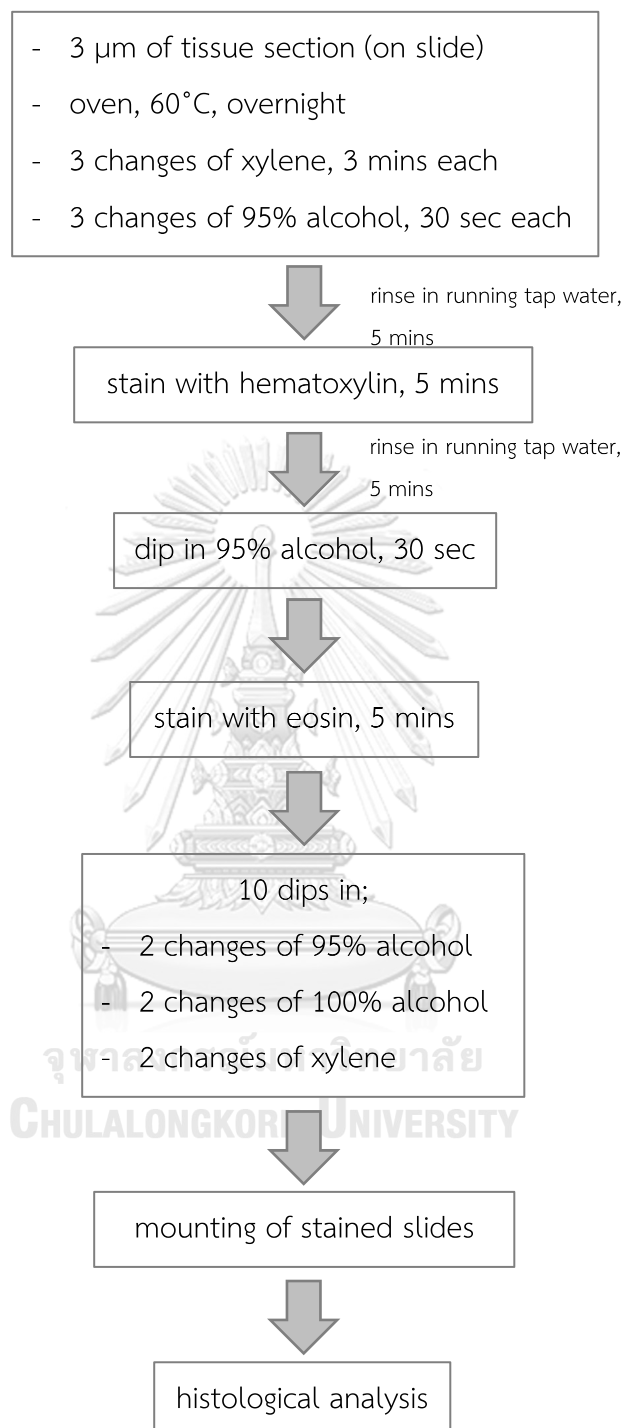

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Figure 3.7 The procedure of hematoxylin-eosin staining.

The histological variables; macrovesicular steatosis, lobular inflammation, and hepatocellular ballooning were graded as following; (Table 3.4).

Table 3.4 The histopathological grading using Brunt's criteria.

Histological variables	Grade	Description
Steatosis	0	no hepatocytes containing fat
	1	< 33% of the hepatocytes containing fat
	2	33% - 66% of the hepatocytes containing fat
	3	> 66% of the hepatocytes containing fat
Lobular inflammation	0	no inflammation and necrosis
	1	< 2 foci per 20x field
	2	2 – 4 foci per 20x field
	3	> 4 foci per 20x field
Hepatocellular ballooning	0	no ballooning degeneration
	1	few ballooning cells
	2	many cells or prominent ballooning

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3.10 Oil red o stain

Oil red o (ORO) is the staining technique that use for neutral lipid and cholesteryl esters detection but not biological membranes (121). This diazol dye has the maximally soluble in fat, while, minimally soluble in solvent. The dilution before use of ORO with water resulting to subside the dye solubility, accordingly, shift this hydrophobic dye from the solvent to lipids in tissue sections. Thereupon, ORO can quantify the amount of lipid accumulation in tissues.

Oil red o stain procedures

Reagents preparation

Oil red o solution

ORO dye	0.1 g
acetone	50 mL
70% alcohol	50 mL

Freshly prepare and filter before use.

Liver tissues were processed in cryostat microtome at -20°C. The -80°C frozen livers were thawed on ice, then, 0.5 cm of samples were embedded with Tissue-Tek® OCT compound (Sakura Finetek, Torrance, CA) on cryomolds. Liver tissue was placed on adequate amount of OCT in corrected orientation for sectioning. OCT was poured on top of tissues and avoided bubbles until samples completely wrapped. After consolidation, frozen-embedded liver tissue was further cutting into 5 μm thick and pasted on adhesive microscope slides (Matsunami Platinum PRO, Tarjan Scientific, Australia). Immediately, liver sections were stained 3 minutes with ORO solution and rinsed with running tap water. Subsequently, section were counterstained with 10 seconds of hematoxylin, rinsed with running tap water, air dried, and mounted with fluorescence mounting medium (Dako, CA, USA). Photographics analysis with regular light microscope was performed within 24 hours. Twenty fields of each liver section at 40x magnificent were analyzed the area that occupied by lipid droplets (122) with Image-Pro plus software (Media Cybernetics, MD, USA) and expressed as percentage of ORO positive area. The protocol of ORO stain is demonstrated in Figure 3.8.

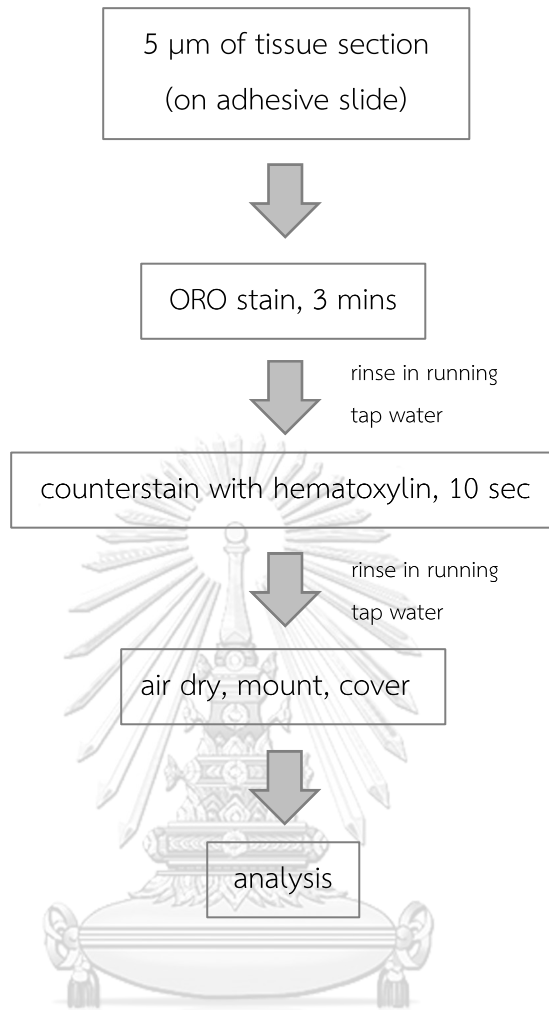

Figure 3.8 The procedure of oil red o staining.

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3.11 Hepatic immunohistochemistry

Tissue microarray (TMA) construction

Tissue microarray is a histopathologic method that proposed and published by Battifora H in 1986. This technique is useful for organizing tiny amount of biological samples, less laboratory personnel, short duration, and cost effective (123). Additionally, each tissue sample in TMA is treated in an identical of experimental manner with a wide range of techniques such as histochemical and immunological stains, etc. The cores sampled of these TMAs reported to represent the whole tissue sections (124-126).

TMA construction was initiated with paraffin-embedded blocks (donor blocks) which retrieved, sectioned, and stained with hematoxylin and eosin. The areas of NASH pathological features were identified and marked on hematoxylin and eosin stained slides by experienced pathologist. The areas on donor block that corresponding to marked areas on slide were cored 10 times. A while later, cores were placed in an empty paraffin block for making recipient block (127) (Figure 3.9). These TMA blocks were used for further immunohistochemistry and TUNELs analyses.

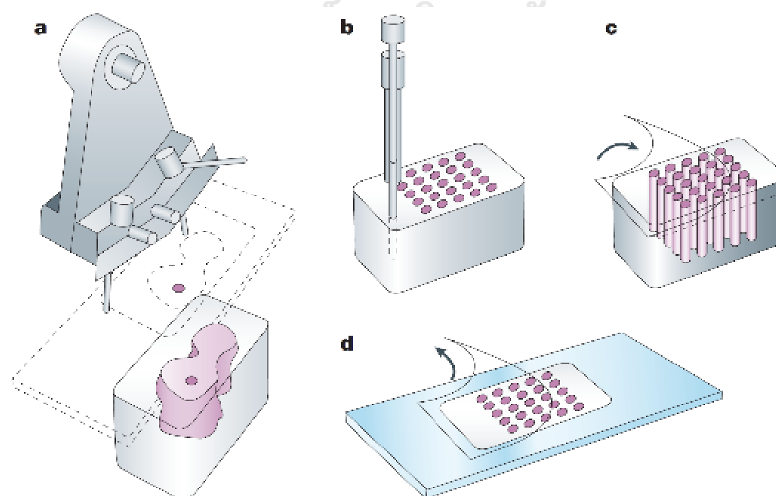

Figure 3.9 Tissue microarray construction (127).

Principle of immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a laboratory technique that use to expose the antigen which characterize abnormal cells or localize proteins of interest in tissue samples. According to the antigen-antibody complex, IHC widely accepted as excellent tool for specific molecules detection. Antibodies these use to detect antigens are conjugated with a marker; enzyme, fluorescent dye, gold particles, or radioactive, for signal amplification. Thereafter, signals are determination by colorimetric reaction or fluorescent signal depending on the conjugation substrate.

Immunohistochemistry protocol for nuclear factor kappa B (NF-kB) and PPARγ measurement

The 3 μm liver sections thick from TMA blocks were 10 minutes deparaffinized with xylene and ethanol. After washing with water, sections were undergone to antigen retrieval process via automate retrieved (Dako, CA, USA). The endogenous peroxidase activity and nonspecific binding were blocked with 5 minutes of 3% H_2O_2 and 20 minutes of 3% normal horse serum, respectively. The primary antibodies of NF-kB p65 (abcam, MA, USA; 1:800) or PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) was applied for 30 minutes at room temperature, subsequently, incubated with specific secondary antibodies for 30 minutes. The immunoreactivity was visualized with diaminobenzidine (DAB) incubation. The slides were counterstained with hematoxylin (128). Under light microscopy, the positive stained cells presented the dark brown in nucleus. The digital images were taken in high magnification field from each sample. The numbers of positive stained cells were counted by the Aperio ImageScope software (Leica Biosystems Imaging, Inc., MD, USA) and expressed as the percentage of immunoreactive cells. The IHC protocol of NF-kB and PPARγ are shown in Figure 3.10.

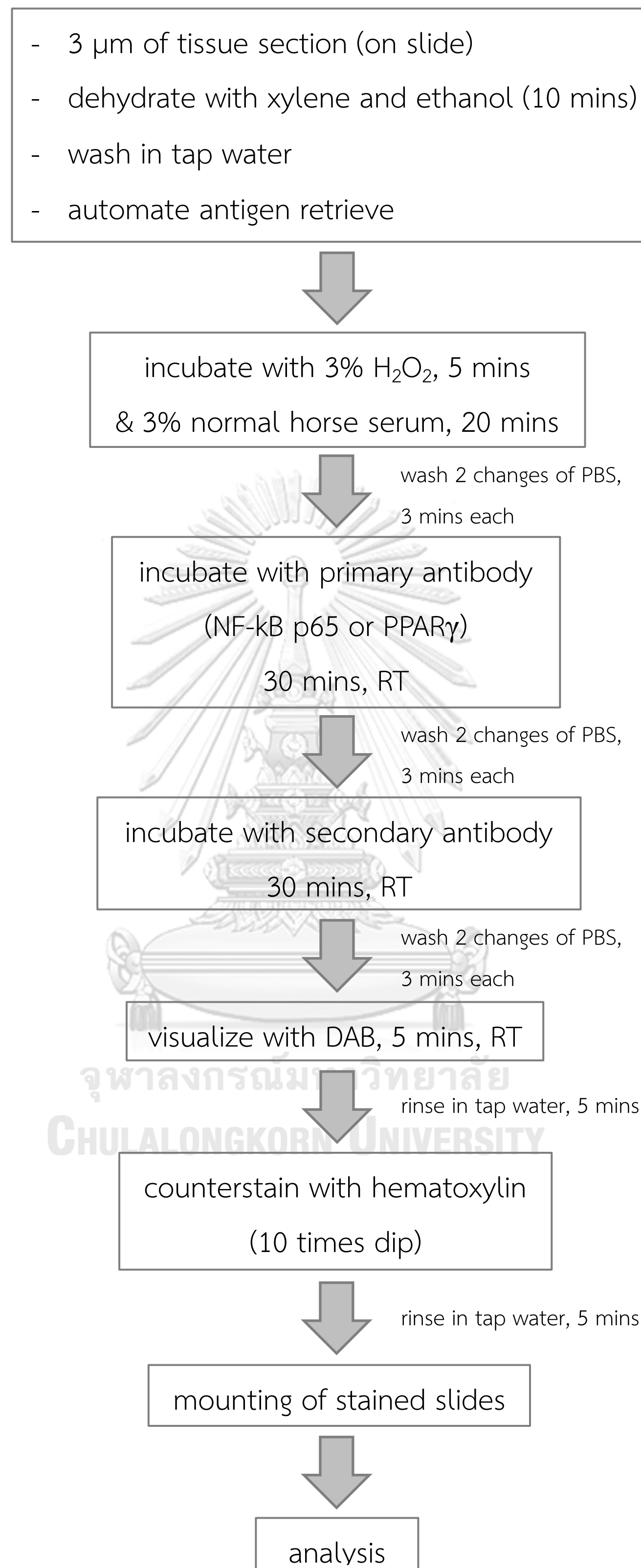

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Figure 3.10 The IHC procedure of NF-kB and PPARγ.

3.12 Hepatocyte apoptosis

Principle of TUNELS method

Apoptosis is the physiological process of program cell-death that appears under normal and abnormal conditions. The hallmark of apoptosis is internucleosomal DNA fragmentation via activity of endonuclease (129). Based on template-independent identification of double-stranded DNA end breaks by TdT, enzyme that catalyzes the additional dUTPs labeled to free 3’-hydroxyl termini of DNA end is used for DNA damage detection with immunohistochemistry techniques. For this reason, Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling assay has been established to monitor DNA fragmentation at the last phases of apoptosis.

TUNELS protocol for hepatocyte apoptosis measurement

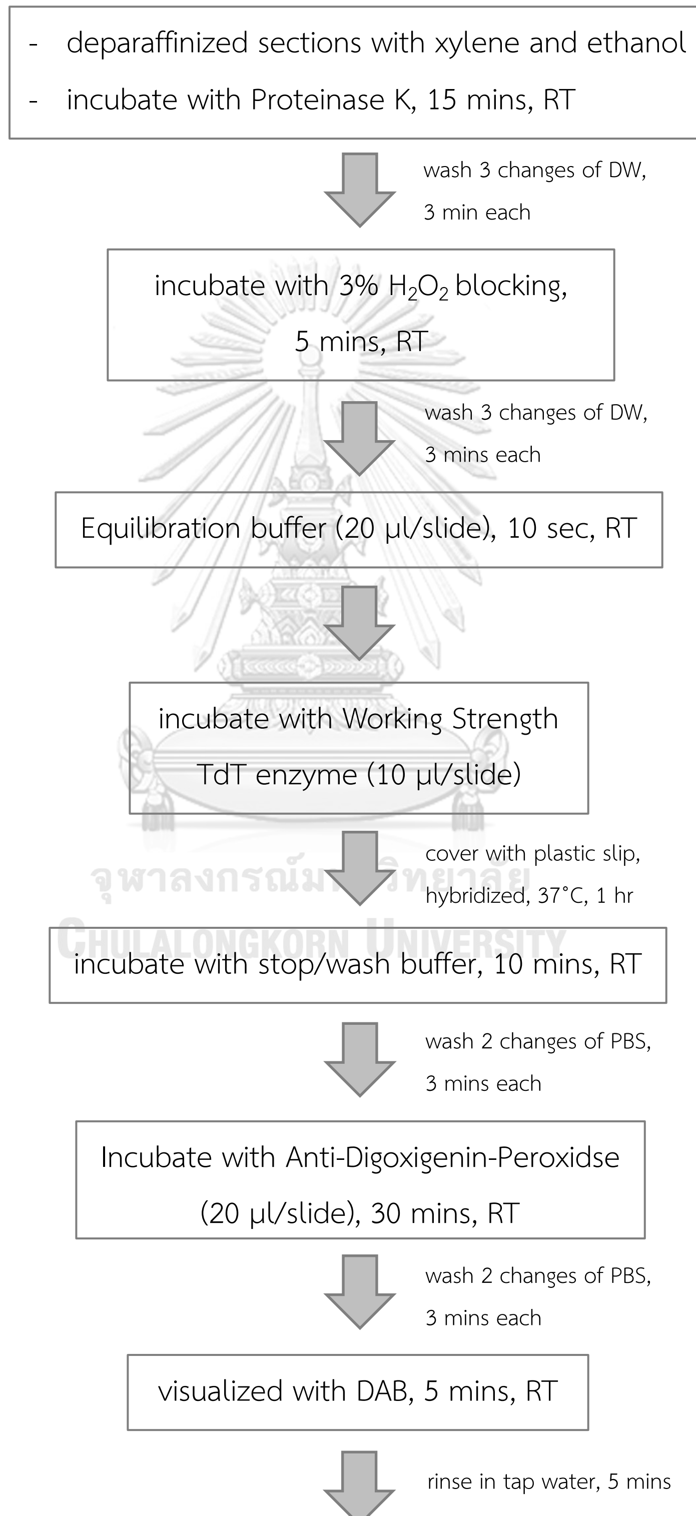
TUNELs assay was performed by ApopTag® Peroxidase In Situ Apoptosis Detection kit (Millipore, CA, USA).

Reagent preparation

1. Equilibration buffer: ready to use.
2. Reaction buffer: ready to use.
3. Working Strength TdT: dilute with Reaction buffer in a ratio of 70% Reaction buffer to 30% TdT enzyme.
4. Anti-Digoxigenin-Peroxidase: ready to use.
5. DAB peroxidase substrate: prepare 0.05% DAB in 0.05M Tris.
6. Proteinase K: dilute 200 µg/mL to 20 µg/mL by PBS.
7. Working Strength H_2O_2: dilute 10-fold of 30% H_2O_2 to 3% H_2O_2 by PBS.
8. Working Strength stop/wash buffer: prepare with 1 mL of stop/wash buffer and 34 mL of DW.

The procedure of TUNELs assay was achieved following to manufacturer’s instruction and showed in Figure 3.11. Deep dark-brown color at nucleus was detected as TUNELs positive cells and counted by the Aperio ImageScope software (Leica

Biosystems Imaging, Inc., MD, USA). The result was expressed as the percentage of immunoreactive cells.



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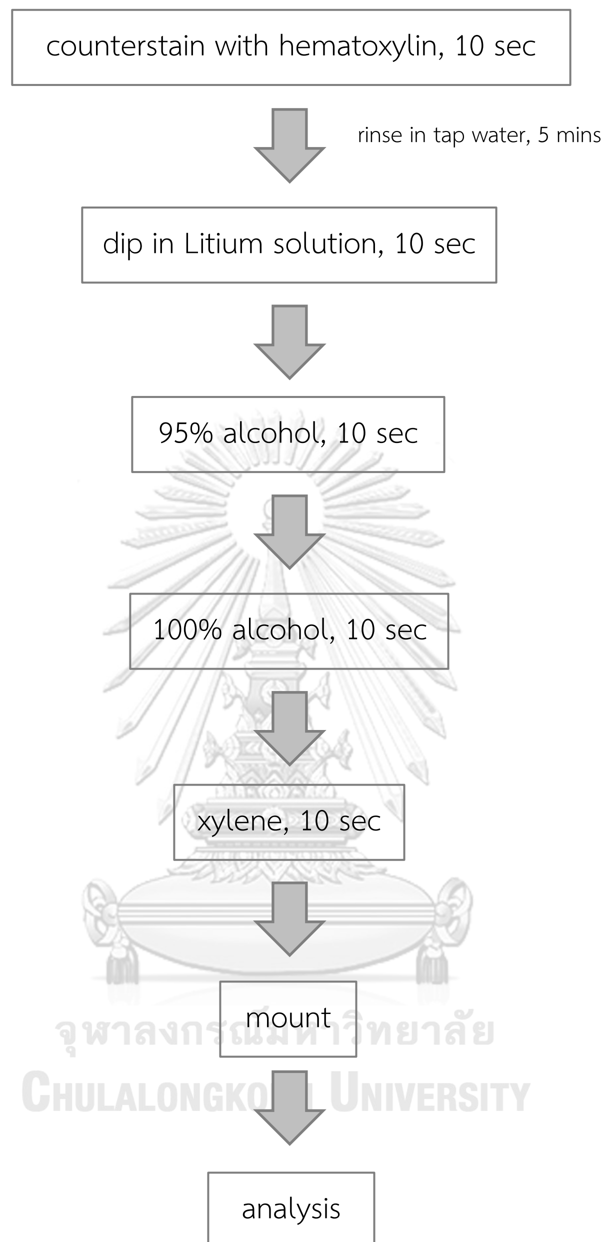

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Figure 3.11 The procedure of TUNELs assay.

3.13 Hepatic biochemical analysis

Free fatty acids and triglyceride (FFA and TG) analysis

Hepatic lipid extraction

The lipid was extracted from the liver tissue with lipid extraction kit (BioVision, Inc., CA, USA). According to the manufacturer's protocol, 20 mg of liver tissues were minced into small pieces in 500 µl of lipid extract buffer, homogenized on ice and centrifuged at 10,000g, 4°C for 5 minutes. The supernatant was collected and allowed to dry in incubator at 37°C overnight (or until dry). A thin film of lipid extracted was seen at the bottom of collecting tube and resuspended in 50 µl of lipid suspension buffer and sonicated 15-20 minutes at 37°C before used to quantify the amount of free fatty acids and triglyceride by colorimetric assays.

The procedure of lipid extract was performed following to manufacturer's instruction and showed in Figure 3.12.

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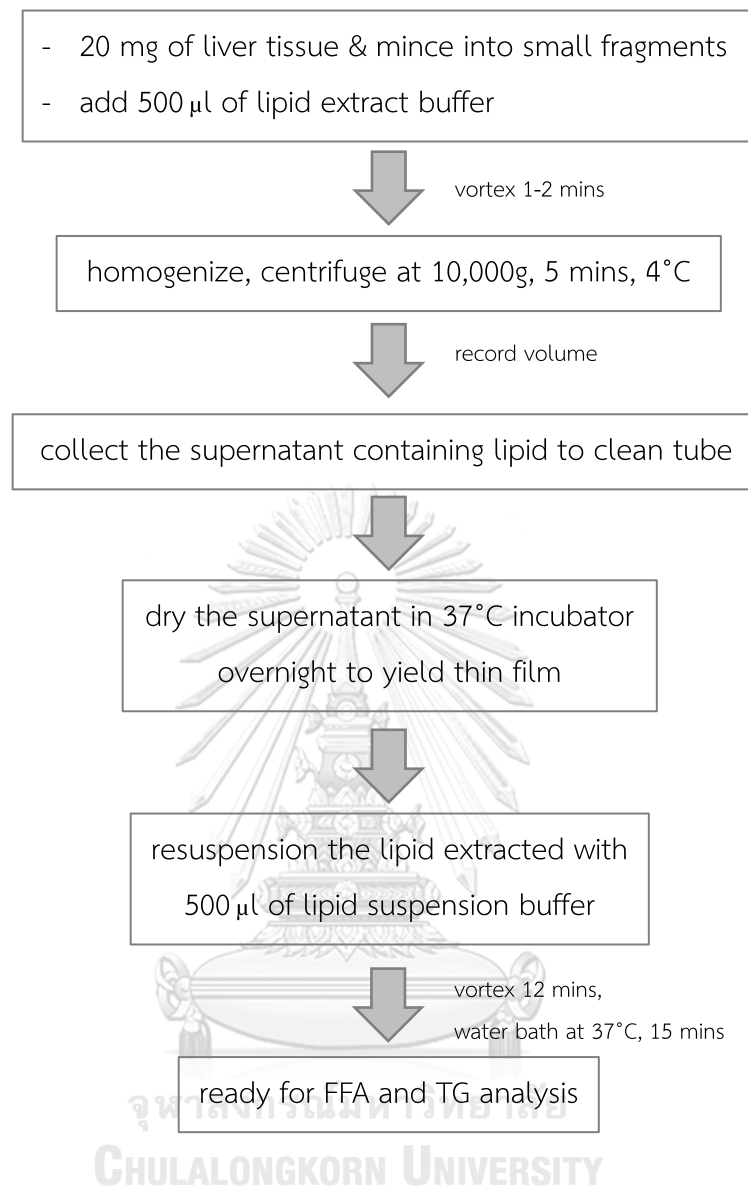

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Figure 3.12 Lipid extract assay.

Principle of colorimetry

Colorimetry is the technique that generally used in biochemical investigations. It can be used to measure any substances that is itself colored or reacted to produce a color. The absorbance of a solution at a particular frequency of visual light will be determined with colorimeter and verified the concentration from the intensity of color which must be proportional to the absorbance.

Protocol for hepatic FFA measurement

In assay (FFA quantification colorimetric kit, BioVision Inc., CA, USA), FFAs are converted to CoA derivatives, which latterly oxidized with the concomitant generation of color and can be detected at 570 nm wavelength.

Reagent preparation

1. Palmitic acid Standard: frozen storage placed in water bath at 80-100°C, for 1 min or until cloudy, vortex 30 sec. Repeat and vortex one more time for clearly standard.
2. Fatty acid assay buffer: ready to use.
3. Acyl-CoA synthetase (ACS) reagent: dissolve in 220 µl of assay buffer, mix well with pipetting.
4. Enzyme mix: dissolve in 220 µl of assay buffer, mix well with pipetting.
5. Fatty acid probe: ready to use.
6. Enhancer: ready to use.
7. Standard preparation: add 0, 2, 4, 6, 8, 10 µl standard individually into microplate well and adjust volume to 50 µl with assay buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of standard.

The protocol of FFA analysis is shown in Figure 3.13.

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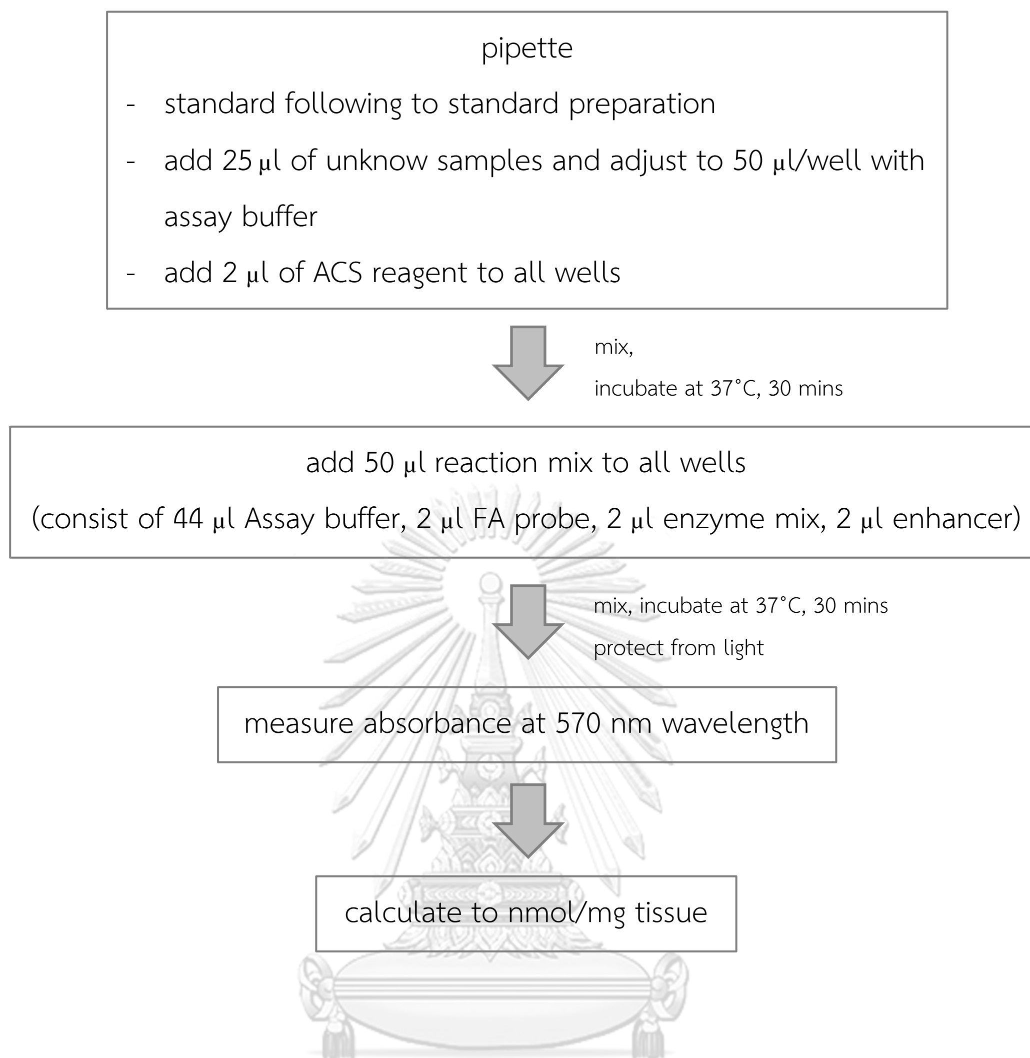

Figure 3.13 Free fatty acid colorimetric assay.

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Protocol for hepatic TG measurement

This assay (Triglyceride quantification colorimetric kit, BioVision Inc., CA, USA), triglyceride are converted to FFA and glycerol, subsequently, glycerol is oxidized to a product that reacts with the probe to generate color at 570 nm wavelength. The sensitivity of the kit is 2-10,000 μM.

Reagent preparation

1. Triglyceride Standard: frozen storage placed in water bath at 80-100°C, for 1 min or until cloudy, 30 sec., vortex. Repeat heat and vortex one more time for clearly standard.
2. Triglyceride probe: ready to use.
3. Triglyceride assay buffer: ready to use.
4. Triglyceride enzyme mix: dissolve with 220 μl of triglyceride assay buffer.
5. Lipase: dissolve in 220 μl triglyceride assay buffer.
6. Standard preparation: dilute 40 μl of standard into 160 μl triglyceride assay buffer and add 0, 10, 20, 30, 40, 50 μl of standard into a series of microplate well. Triglyceride assay buffer used for adjusted volume to 50 μl/well to generate 0, 2, 4, 6, 8, 10 nmol/well of triglyceride standard.

The protocol of FFA analysis is shown in Figure 3.14.

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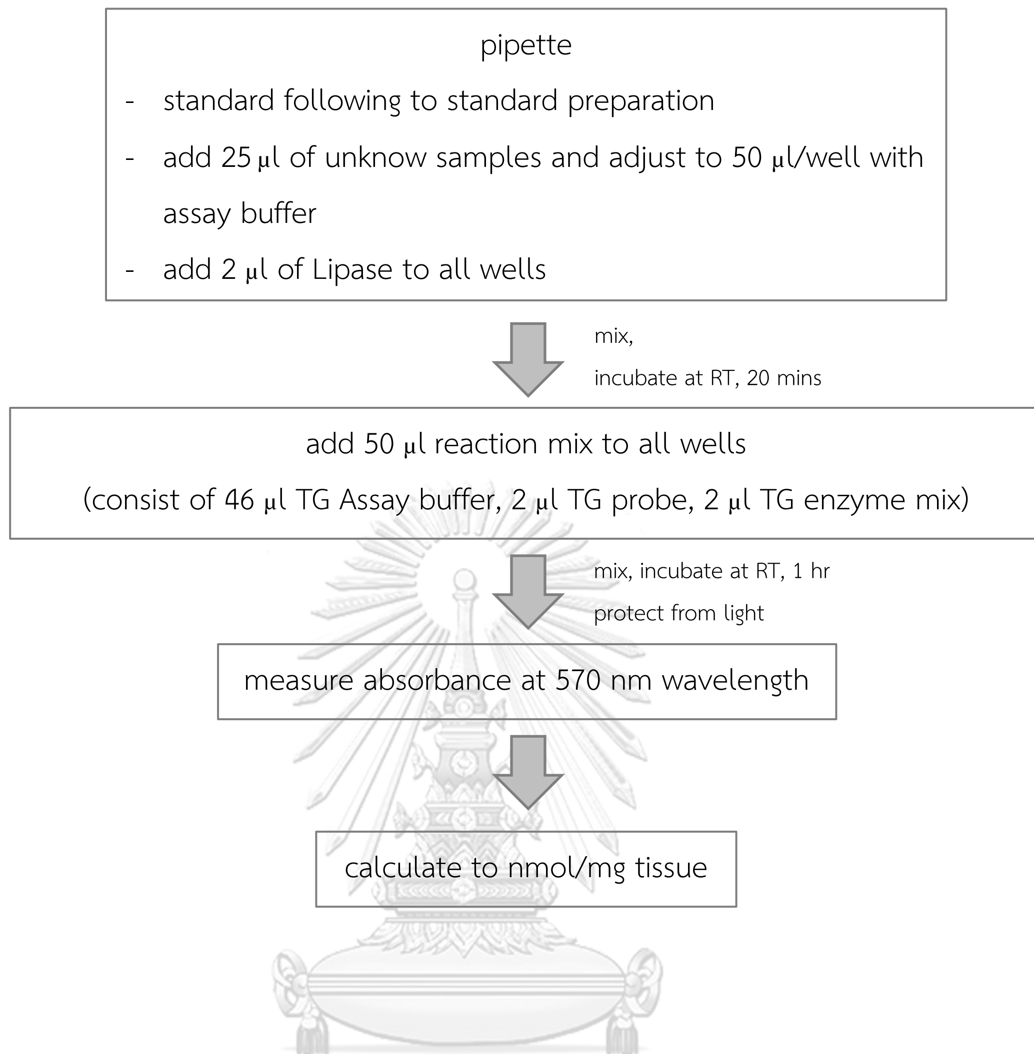

Figure 3.14 Triglyceride colorimetric assay.

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Malondialdehyde (MDA) analysis by TBARS assay

Tissue homogenate

Liver tissue (0.1 g) was homogenized on ice for 30 minutes in 1 ml of RIPA buffer (Cell Signaling Technology®, Inc., MA, USA) with protease inhibitor cocktails (Sigma-Aldrich, DS, Germany) in the ratio of 100:1 and cleared by centrifugation at 16,000g for 5 minutes at 4°C. Protein concentration in supernatant was collected and assayed to determine protein concentration by BCA method.

Total protein measurement with bicinchoninic acid (BCA) assay

The BCA principle based on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of Cu^{2+} to Cu^{+} which providing a basis to monitor the reduction of alkaline Cu^{2+} by protein. The amount of reduction is proportional to the amount of protein in the solution. The total protein concentration is exhibited by color change of sample solution (green to purple-blue), which can be absorbed light at a wavelength of 562 nm. (Figure 3.15)

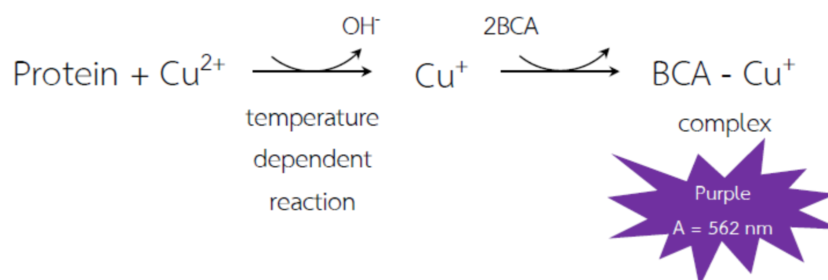


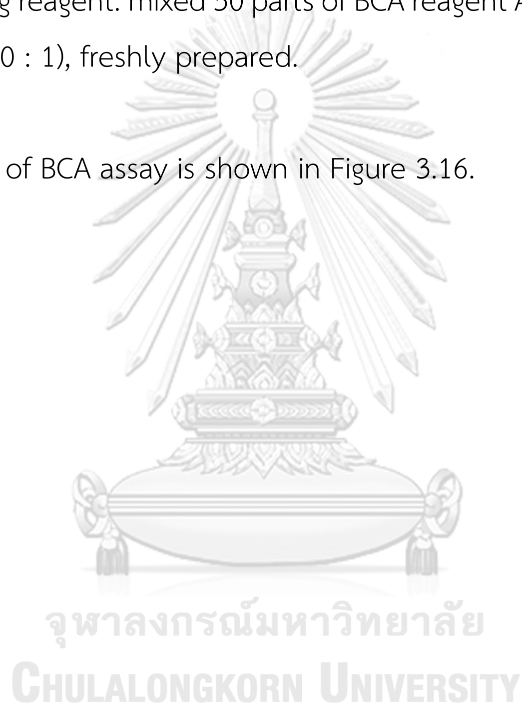
Figure 3.15 The reaction of protein and bicinchoninic acid in BCA assay.

The procedure of BCA assay was performed following to BCA protein assay kit (Pierce®, Thermo scientific, Inc., IL, USA).

Reagent preparation

1. Standard protein: Albumin standard (provided from kit) was used for standard preparation. Serial dilution of standard was performed into 125 – 2,000 μg/mL of assay range.
2. Working reconstitution buffer: ready to use.
3. Compatibility reagent solution: add 100 μl of working reconstitution buffer to dissolve compatibility reagent. Stirring solution with pipette 15-20 times and protection from light.
4. BCA working reagent: mixed 50 parts of BCA reagent A with 1 part of BCA reagent B (A : B = 50 : 1), freshly prepared.

The flow chart of BCA assay is shown in Figure 3.16.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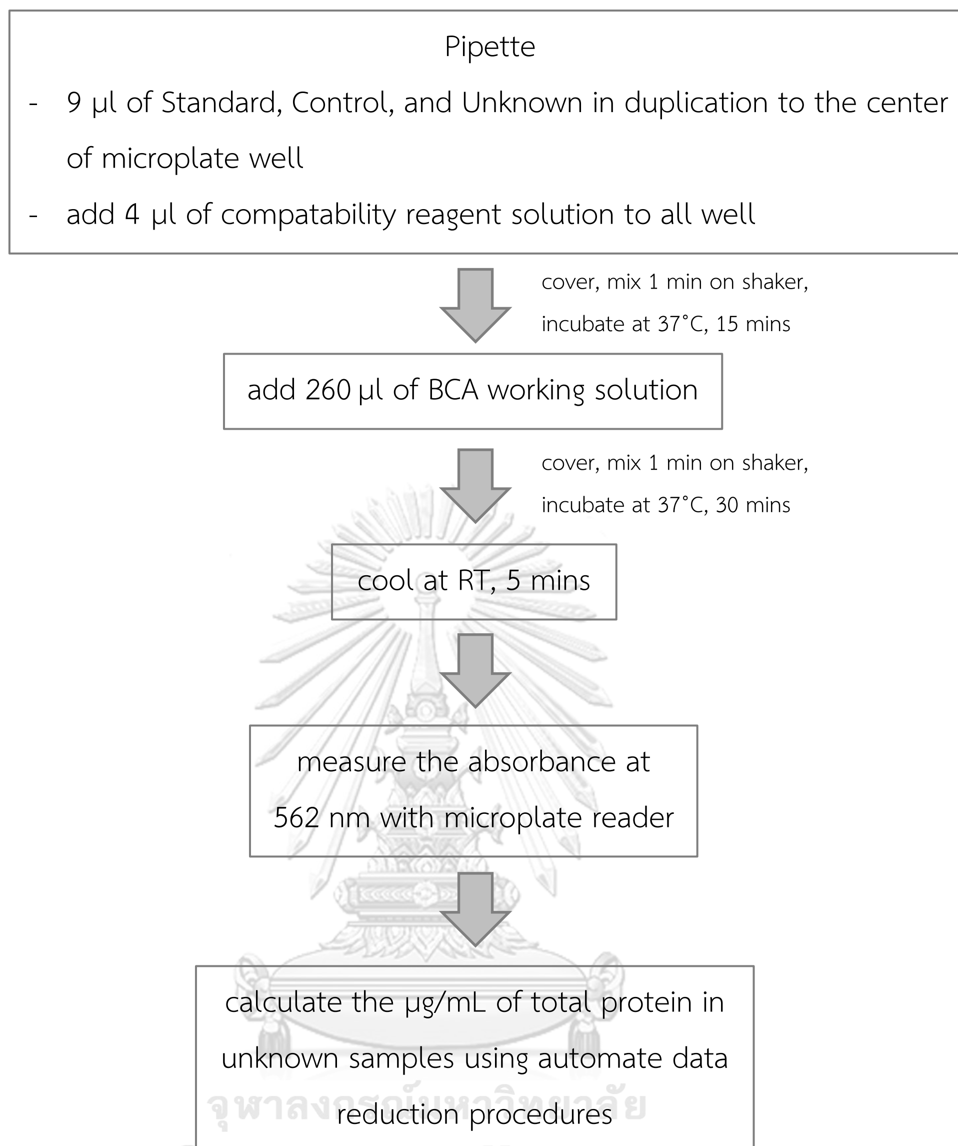

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Figure 3.16 BCA assay for total protein.

Principle of TBARS assay

Thiobarbituric acid substances (TBARS) is a well-establish method for monitoring lipid peroxidation, which used as the indicator of oxidative stress in cell and tissues. The principle of this method is measured the lipid peroxidation by product; malondialdehyde (MDA), and thiobarbituric acid (TBA) complex in samples. In the conditions of high temperature and acidic, MDA-TBA complex is measured colorimetrically at 530-540 nm wavelength. The level of hepatic MAD was performed by using TBARS assay kit (Cayman Chemical, MI, USA).

Reagent preparation

All reagents were equilibrated at least 30 mins, RT, before assay.

1. Thiobarbituric acid: ready to use.
2. TBA acetic acid: dilute 40 mL of TBA acetic acid with 160 mL DDW for color reagent preparation.
3. TBA NaOH: dilute 20 mL of TBA NaOH with 180 mL DDW for color reagent preparation.
4. TBA malondialdehyde Standard: ready to use.
5. TBA SDS solution: ready to use.
6. Color reagent: weigh 530 mg of TBA and dissolve in 50 mL of diluted TBA NaOH and 50 mL of diluted TBA acetic acid. Thereafter, mix until completely dissolve.
7. Standard preparation: dilute 250 µl of MDA standard with 750 µl of DDW. Add the volume of MDA stock solution and DDW to each tube as describe below.

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tube	MDA (µl)	DDW (µl)	MDA concentration (µM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

The flow chart of TBARS assay is shown in Figure 3.17.

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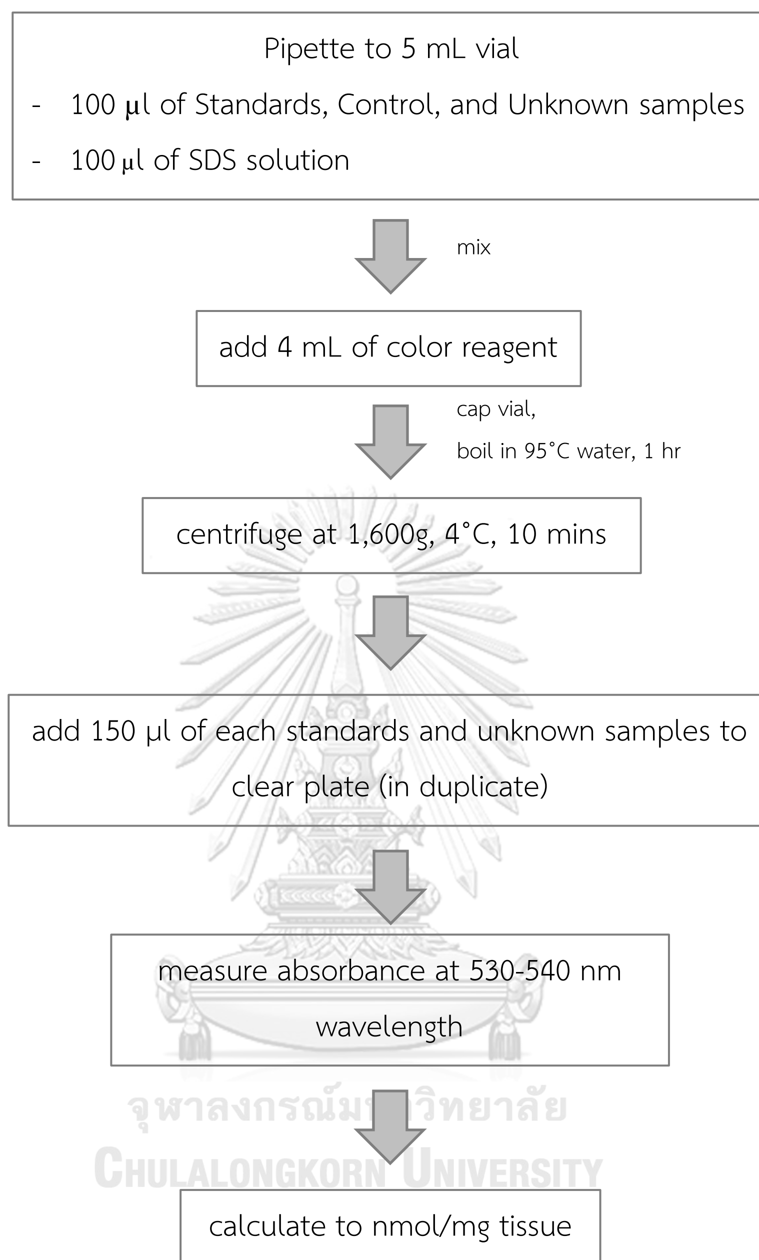

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Figure 3.17 Thiobarbituric acid substances (TBARS) assay.

Protocol for MDA measurement (TBARS assay)

Malondialdehyde (MDA) level, which is a marker of hepatic lipid peroxidation (28), was evaluated by determining the production rate of thiobarbituric acid reactive substances (TBARS) with commercial assay kits (Cayman chemical, MI, USA). Approximately 25 mg of liver tissue were homogenized on ice in 250 µl of radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor and centrifuged at 1,600g for 10 minutes at 4°C. Protein concentration in supernatant was assayed by BCA method.

Supernatant was mixed with the solution containing 20% acetic acid, 0.8% thiobarbituric acid, and 8.1% sodium dodecyl sulfate. The solution was boiled for 1 hour in 95°C water bath and, then, centrifuged for 10 minutes at 1,600g. The absorbance of supernatant fraction was measured at 532 nm wavelength and expressed in nmol/mg protein.

3.14 Western blot analysis

Principle of Western blot

Western blot is the common technique in biological research to separate and identify the specific proteins in a mixture that extracted from cells, by reason of, it is rapid accomplished, using simple equipment, easy to interpret and unambiguous result. In theoretical, there are three essential features to achieve this technique: protein separation based on molecular weight by gel electrophoresis; transfer separated protein to membrane; and detection a target protein by specific matched antibodies. Thereafter, target protein will be visualized by imaging system. The thickness of visual band appertain to target protein amount.

Protocol for Western blot analysis of PPARγ, adiponectin, and estrogen receptors

The 60 µg of denatured protein were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then, connected to power supply and ran in a buffer tank (Bio-Rad, USA); initially voltage was 80V for 30 minutes, immediately

increased voltage to 120V for 60 minutes. The negatively charged of protein fraction would migrated in an electric field through the gel towards the positive electrode. Eventually, separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane by Semi-dry transfer method. The gel and PVDF membrane were assembled into a sandwich with filter papers so that proteins migrate from the gel onto the membrane. The transferred PVDF membrane was overnight blocked at 4°C with 5% non-fat dry milk in phosphate buffer saline with Tween 20 (PBST). Subsequently, PVDF membrane was 2 hours incubated at room temperature with PPARγ (Santa Cruz Biotechnology, Inc., USA; 1:400), adiponectin (R&D system, MN, USA; 1:500), ERα (Santa Cruz Biotechnology, Inc., USA; 1:1,000) or ERβ (Thermo Scientific, IN, USA). After that, membrane was washed by Tris-buffer saline with Tween 20 (TBST) 3 times; 5 minutes each, and incubated with secondary antibody conjugated horseradish peroxidase (HRP) (1:10,000) (goat anti-mouse and anti-rabbit, Santa Cruz Biotechnology, Inc., USA; donkey anti-goat, Abcam, MA, USA) for 1 hour at room temperature. Membrane was washed 3 times; 5 minutes each, with TBST before visualized bands of target protein with enhanced chemiluminescence (ECL) western blotting system (ChemiDoc™ Touch Imaging System, BioRad laboratories, CL, USA) and normalized by β-actin using Image Lab™ Software (BioRad laboratories, CL, USA).

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3.15 Serum analysis

Serum preparation

The whole blood that taken from cardiac puncture, allowed to clot for 30 minutes at room temperature. Ensuingly, clotted blood was centrifuged at 2,000g for 10 minutes in 4°C refrigerated centrifuge. The supernatant was immediately transfer into a clean polypropylene tube; 200 µl aliquots, and kept at -80°C until analysis.

Aspartate transaminase (AST) and Alanine transaminase (ALT) analysis

The enzyme markers of hepatocellular injury were evaluated as the activity of AST and ALT by biochemical analyzer Reflotron® (Woodley equipment company Ltd.,

UK). Based on the colorimetric principle, serum was applied onto reagent strips which designed specifically for quantitative of AST and ALT determination. The quantity of AST and ALT were reported as enzyme concentration in units per liter (U/L).

Tumor necrotic factor alpha (TNF-α) analysis

Principle of enzyme-linked immunosorbent (ELISA) assay

ELISA is widely used as diagnostic that applied the basic immunology concept of an antigen-antibody binding, which can be detected of very small quantities of antigens such as proteins, peptides, or hormones in fluid sample. The antigen in sample is mobilized and bond to a specific antibody. Subsequently, antigen-antibody complex is detected by secondary enzyme-coupled antibody. Visible color change or fluorescence, which assess by colorimetric reader, represent the amount of antigen in sample (130).

Protocol for TNF-α measurement

TNF-α was assayed with the enzyme-linked immunosorbent assay (ELISA) technique by a Quantikine ELISA commercial kit (R&D system, Inc., MN, USA) following with the instructions of manufacturer. The level of TNF-α was expressed as pg/mL.

Sample preparation

Serums require 2-fold dilution with Calibrator Diluent RD5-17 before assay. The instruction suggests to dilute 75 µl of serum with 75 µl of Calibrator Diluent RD5-17.

Reagent preparation

All reagents were equilibrated and reconstituted at least 30 mins, RT, before assay.

1. Rat TNF-α coated microplate: ready to use.
2. Rat TNF-α control: reconstitute with 1 mL DW.
3. Calibrator Diluent RD5-17: ready to use.
4. Assay Diluent RD1-41: ready to use.
5. Rat TNF-α conjugate: ready to use.
6. Wash buffer: dilute 20 mL of wash buffer with 480 mL DW.

7. Substrate solution: mix color reagent A and B in 1 : 1 within 15 mins before use and protect from light. Volume per well is 100 µl of mixture.
8. Stop solution: ready to use.
9. Rat TNF-α Standard: reconstitute with 2 mL of Calibration Diluent RD5-17 which produces a stock solution of 800 pg/mL.
10. Standard preparation: add Calibration Diluent RD5-17 and stock solution to produce a dilution series in polypropylene tubes as describe below.

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Standard No.	Volume of Calibrator Diluent (µl)	Volume of standard (µl)	Concentration (pg/ML)
S7	-	reconstituted standard	800
S6	200	200 of S7	400
S5	200	200 of S6	200
S4	200	200 of S5	100
S3	200	200 of S4	50
S2	200	200 of S3	25
S1	200	200 of S2	12.5
S0	200	-	0

The flow chart of TNF-α assay is shown in Figure 3.18.

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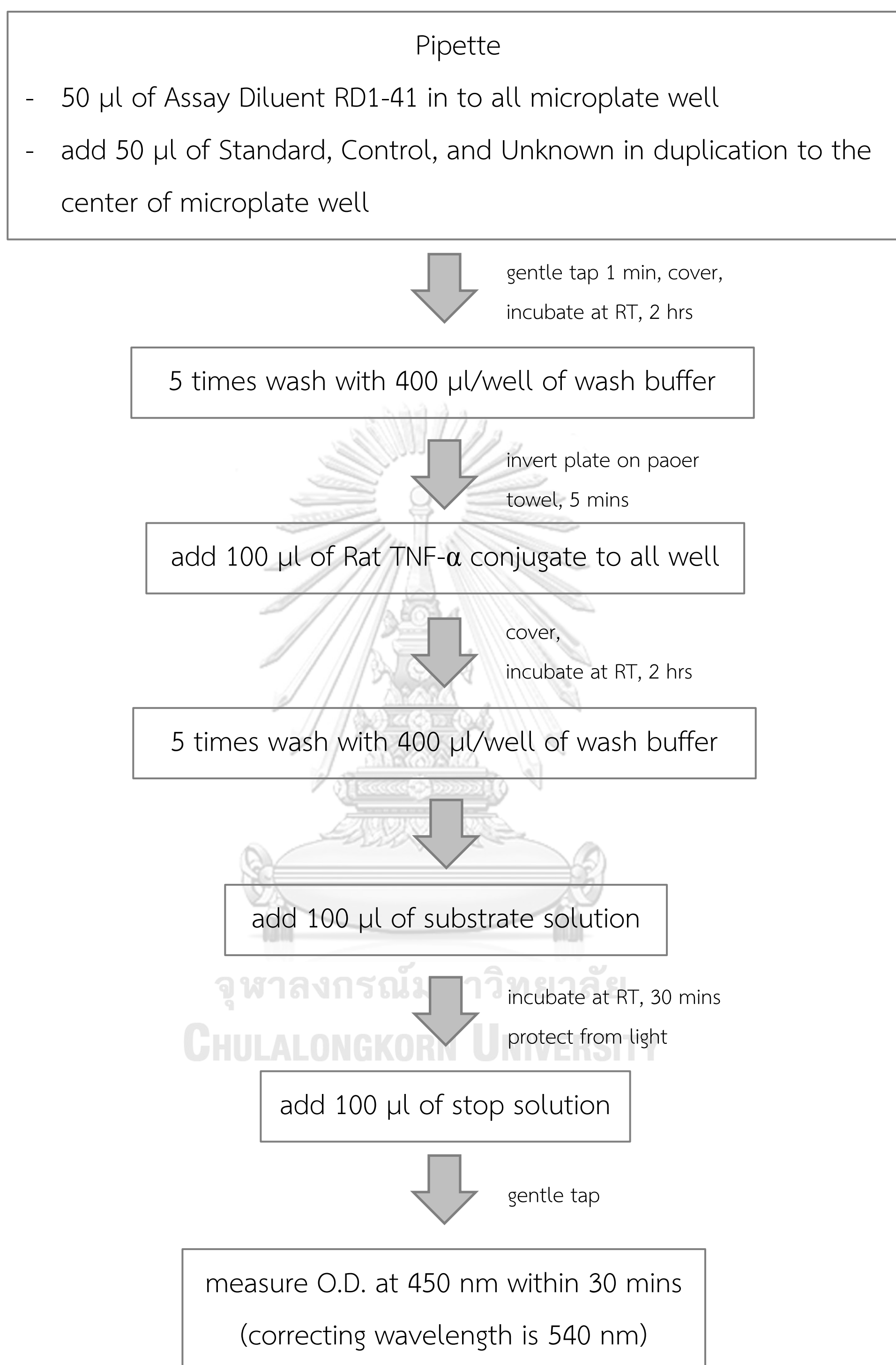

Figure 3.18 TNF-α assay by ELISA method.

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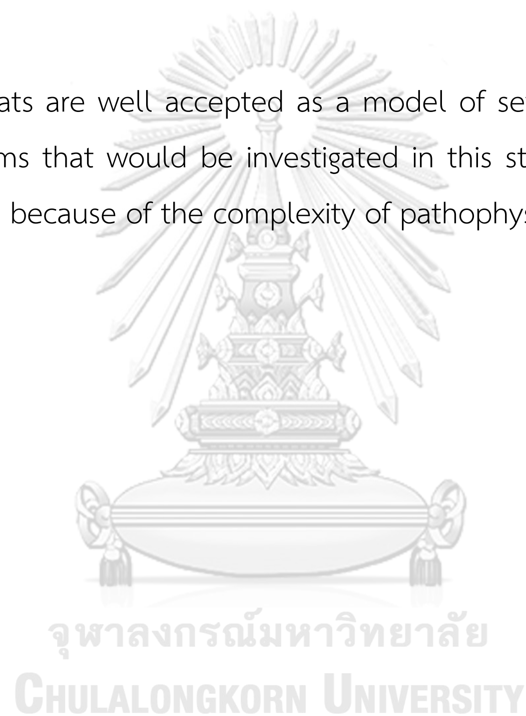
3.16 Statistical and data analysis

The data were expressed as mean ± standard deviation (SD).

One way analysis of variance (one-way ANOVA) and least significant difference (LSD) post-hoc test were used to compare the mean difference among experimental groups. Descriptive statistic was used for histological examination. A $P < 0.05$ considered as statistical significance.

3.17 Limitation

Although rats are well accepted as a model of several human diseases, the specific mechanisms that would be investigated in this study might not be directly referred to human because of the complexity of pathophysiology in human diseases.



CHAPTER IV
RESULTS

4.1 Change of rats' body weight

Female Sprague Dawley rats; aged 4 weeks, were allocated into 6 groups of experiment. At the beginning, the body weight of rats was not different among 6 groups ($p = 0.397$). The ovariectomy showed the affection on rats' body weight, since, ovariectomized (OVX) rats significantly increased body weight when compared with non-ovariectomized (non-OVX) rats even fed with normal diet (191.31 ± 12.99 vs 125.94 ± 14.52 g, $p < 0.01$). Both non-OVX and OVX rats fed with high-fat high-fructose (HFHF) diet decreased their weight. OVX rats with HFHF diet-induced NASH indicated more ∆BW than non-OVX that represent the effect of OVX on NASH severity. Although, these effects on weight change could not regain by genistein (Table 4.1), genistein could show to improve rats' general appearance in both non-OVX and OVX rats fed with HFHF diet (Figure 4.1).

Table 4.1 The body weight change of rats.

	Experimental groups					
	Non-OVX			OVX		
Body Weight (g)	control	NASH	NASH + Gen	control	NASH	NASH + Gen
Before experiment	123.44±7.77	121.87±8.44	126.81±5.92	122.19±13.35	127.69±11.18	130.44±8.18
At the end of experiment	249.38±15.79	90.94±6.51[a]	92.94±9.65	313.50±22.30[e]	94.62±14.35[c]	99.87±9.54
∆BW	125.94±14.52	-30.94±7.73[a]	-33.87±6.28	191.31±12.99[e]	-33.06±5.84[c]	-30.56±6.60

Data are shown as mean±SD. n = 8 in each groups.

[a]Comparison between control and NASH groups of non-OVX rats.

[b]Comparison between NASH and NASH with genistein groups of non-OVX rats.

[c]Comparison between control and NASH groups of OVX rats.

[d]Comparison between NASH and NASH with genistein groups of OVX rats.

[e]Comparison between control groups of non-OVX and OVX rats.

[f]Comparison between NASH groups of non-OVX and OVX rats.

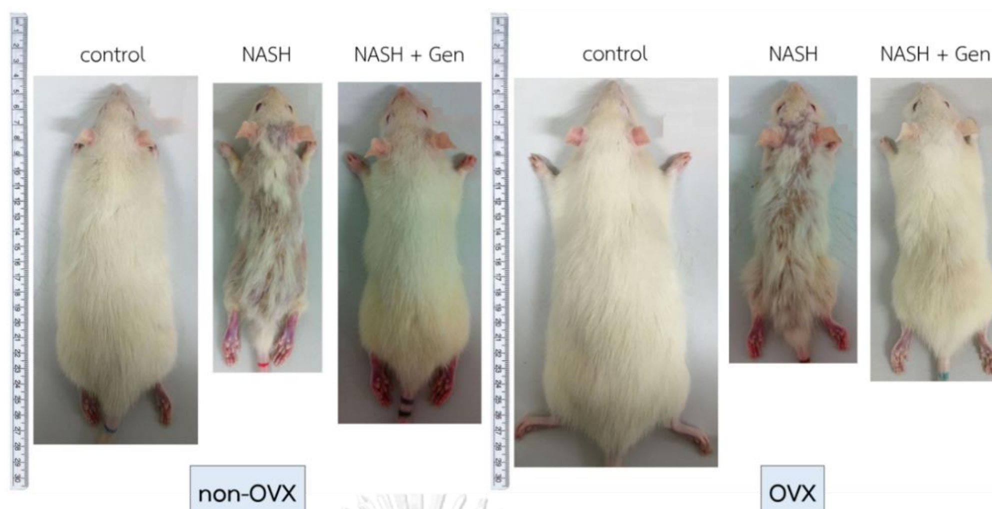


Figure 4.1 The general appearance of rats in each experimental groups. After OVX, rats markedly increased body weight and showed normal active. However, their body weight greatly decreased with feeding HFHF diet to induced NASH and slightly reversed by genistein. The general appearances such as size, hair were worsen in NASH with non-OVX and OVX groups. In addition, rats in NASH groups demonstrated drowsiness and inactive. Genistein administration improved the general appearances in both non-OVX and OVX with NASH rats and altered rats to more active.

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4.2 The confirmation of completely bilateral ovariectomy

All rats in OVX groups were operated to remove both ovaries with bilateral dorsolateral approach procedures as mentioned in methodology. The vaginal smear was used for confirmation of complete bilateral ovariectomy. In non-OVX rats, the variation of cellular types and proportion in vaginal lavage presented the estrous cycle of rat as identified to proestrous, estrous, metestrous, and diestrous phase (Figure 4.2-upper panel). OVX rats showed predominant of leukocytes; non-variable cellular types, which identified as anestrous cycle (Figure 4.2-lower panel).

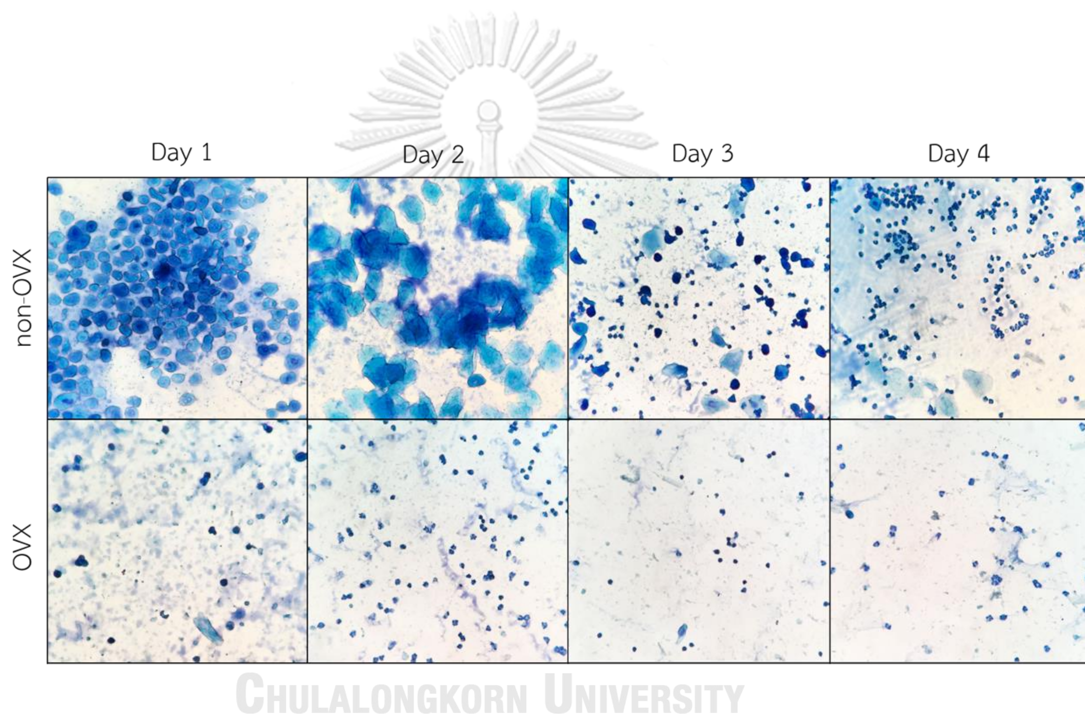

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Figure 4.2 Photomicrographs of vaginal smears from non-OVX and OVX rats for 4 consecutive days with methylene blue stained. Normal estrous cycle is described on the upper panel; proestrous in Day 1, estrous in Day 2, metestrous in Day 3, and diestrous in Day 4. Lower panel describe as anestrous. The original magnification is 400x.

4.3 Histopathology and gross liver appearance

The liver of both non-OVX and OVX rats with HFHF diet-induced NASH groups demonstrated dissimilar of liver tissue appearance from control groups. The soft and pale color were seen in NASH group. Moreover, the liver of OVX fed with normal (control) group slightly greasier than non-OVX control group. However, NASH rats with genistein revealed better liver tissue appearance in both non-OVX and OVX groups (Figure 4.3).

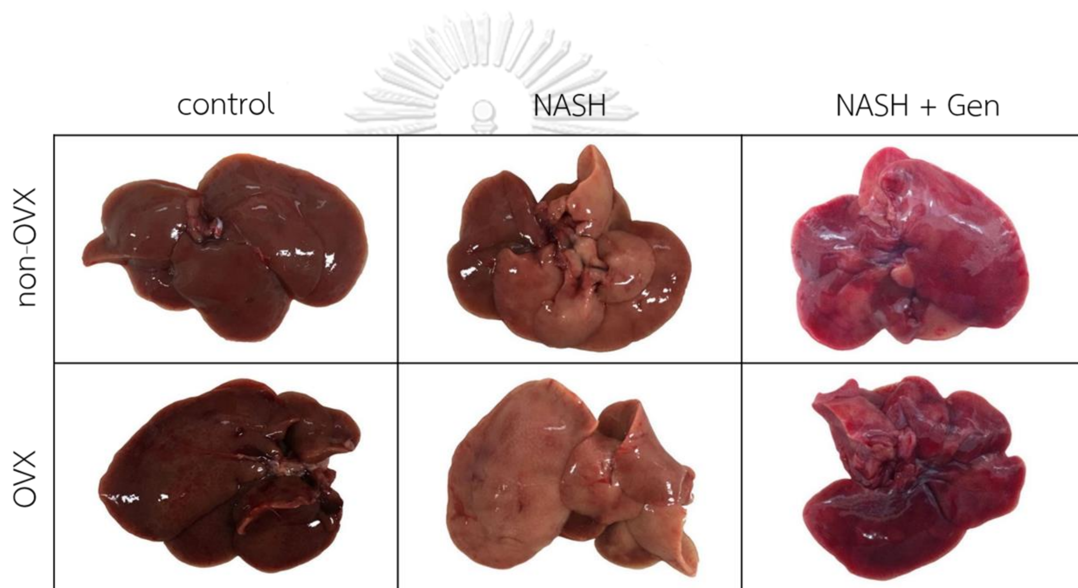

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Figure 4.3 The gross liver appearance of rats in each experimental groups. The color of liver is pale and greasy in non-OVX and OVX with NASH group. Genistein administration revealed pink-red color of liver.

Hematoxylin-eosin (H&E) stain of liver sections demonstrated that HFHF diet induced the typical features of NASH including; steatosis, lobular inflammation, and ballooning of hepatocytes in both non-OVX and OVX NASH groups with more exacerbated injuries in OVX than non-OVX rats. Moreover, there are interesting to observe that OVX rats fed with standard diet exhibited more lipid accumulation and lobular inflammation than the corresponding non-OVX rats. This may represent the influence of estrogen on NASH protection. Genistein administration showed to improve liver injuries via decreased steatosis, lobular inflammation, and hepatocellular ballooning in NASH rats as shown in histopathology of NASH + Gen group in both non-OVX and OVX rats (Figure 4.4).

Steatosis, lobular inflammation, and hepatocellular ballooning were graded with the Brunt's criteria (2). In normal control group, all rats were graded into score 0 of steatosis, lobular inflammation, and hepatocellular ballooning, however, the rats were increased in lobular inflammation and hepatocellular ballooning after ovariectomy (lobular inflammation; 4 rats for score 1 and 1 rat for score 2, hepatocellular ballooning; 3 rats for score 1 and 1 rat for score 2). HFHF were resulting to increase severity of steatosis, lobular inflammation, and hepatocellular ballooning of both non-OVX and OVX rats. Noticeably, the OVX with NASH rats were higher scores than non-OVX with NASH rats.

The number of rats that graded into highest score of all 3 histological features were diminished by daily genistein administration. Remarkably, genistein likely seem to reduce more number of rats got highest score in OVX than non-OVX rats with NASH (Table 4.2).

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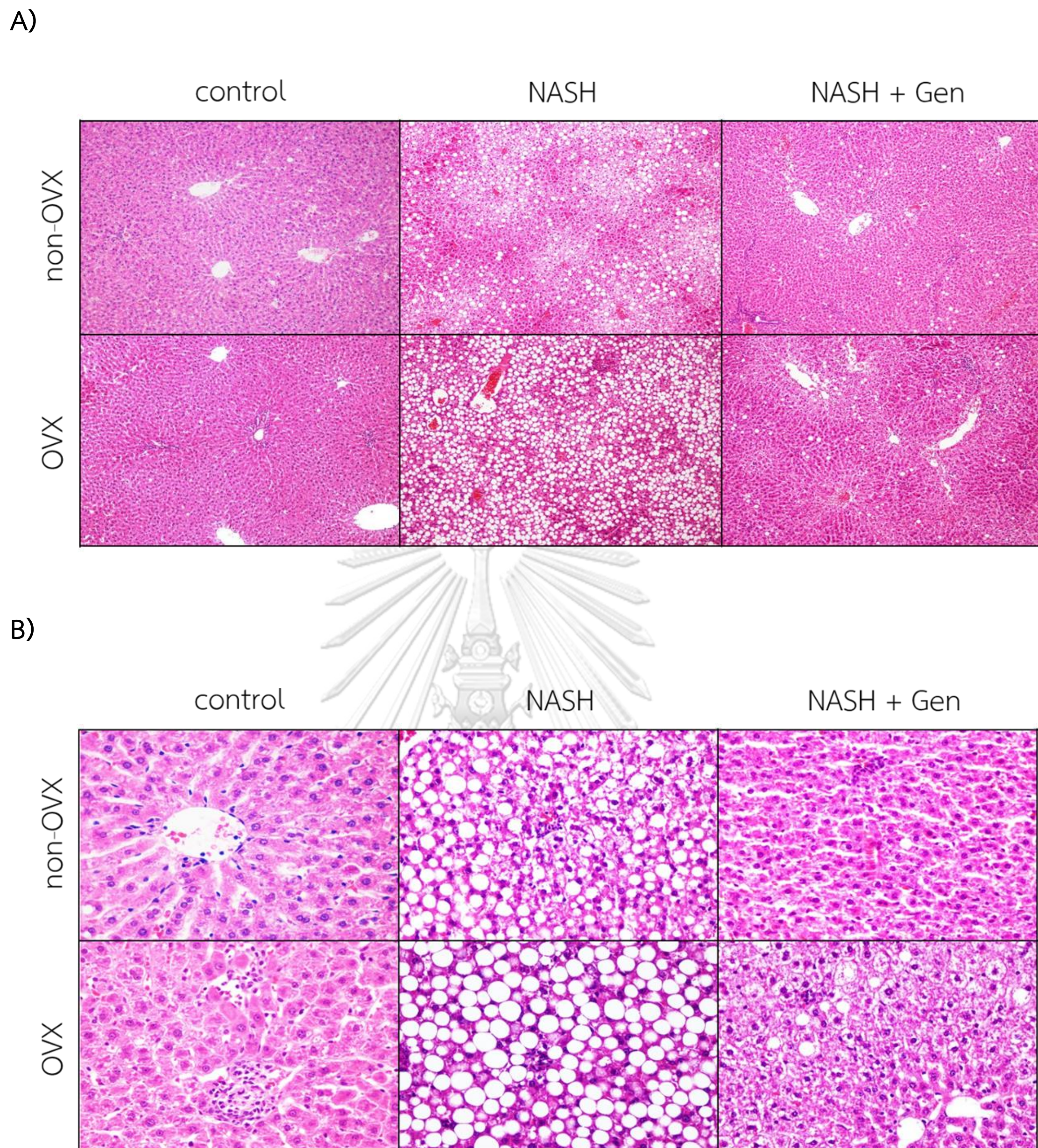

Figure 4.4 Hematoxylin-eosin (H&E) stain of liver sections. Steatosis, lobular inflammation, and hepatocellular ballooning showed the most severe in OVX rats feeding with HFHF diet. Genistein improved the steatosis, lobular inflammation, and hepatocellular ballooning in both non-OVX and OVX with NASH rats. The original magnification is 100x (A) and 400x (B).

Table 4.2 The frequency of rats in each histological variables grading of NASH.

Group	Steatosis				Lobular inflammation				Ballooning		
	0	1	2	3	0	1	2	3	0	1	2
control	8	-	-	-	8	-	-	-	8	-	-
NASH	-	3	3	2	-	6	2	-	-	1	7
NASH + Gen	1	4	2	1	1	6	1	-	-	6	2
OVX	8	-	-	-	3	4	1	-	4	3	1
OVX + NASH	-	-	-	8	-	4	4	-	-	2	6
OVX + NASH + Gen	1	6	1	-	4	4	-	-	-	5	3

Data are shown as number of rats. n = 8 in each groups.

Criteria of histopathological scores are (2);

Steatosis :

0 = no hepatocytes containing fat

1 = < 33% of the hepatocytes containing fat

2 = 33 - 66% of the hepatocytes containing fat

3 = > 66% of the hepatocytes containing fat

Ballooning :

0 = no ballooning

1 = few ballooning

2 = many ballooning

Lobular inflammation :

0 = no inflammation and necrosis

1 = mild zone 3 injury/inflammation

2 = noticeable zone 3 injury/inflammation

3 = severe zone 3 injury/inflammation

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According to the Brunt's criteria (2), the histological features of NASH including steatosis, lobular inflammation, and hepatocellular ballooning were scored into grades; 0-3 in steatosis, 0-3 in lobular inflammation, and 0-2 in hepatocellular ballooning by blinded experienced pathologist.

Steatosis score was significantly increased in non-OVX and OVX rats feeding with HFHF diet when compared with control groups (non-OVX; 1.88 ± 0.83 vs 0.00 ± 0.00, $p < 0.01$ and OVX; 3.00 ± 0.00 vs 0.00 ± 0.00, $p < 0.01$). Noticeably, HFHF diet led more fat accumulation in liver of OVX than non-OVX rats, since, OVX + NASH group presented higher score of steatosis than non-OVX NASH group (3.00 ± 0.00 vs 1.88 ± 0.83, $p < 0.01$). This may imply the role of estrogen deficiency on hepatic fat accumulation.

Normal diet remarkably enhanced the lobular inflammation score in OVX as compared to non-OVX group (0.75 ± 0.71 vs 0.00 ± 0.00, $p < 0.01$). HFHF diet also altered lobular inflammation score. When compared with control, lobular inflammation score was significantly increased in both non-OVX (0.00 ± 0.00 vs 1.25 ± 0.46, $p < 0.01$) and OVX (0.75 ± 0.71 vs 1.50 ± 0.53, $p < 0.01$) with NASH rats.

Estrogen deficiency due to ovariectomy demonstrated hepatocellular ballooning score increasing as compared to non-OVX control group (0.63 ± 0.74 vs 0.00 ± 0.00, $p < 0.05$). In the contrary, there was not different of hepatocellular ballooning score between non-OVX and OVX rats feeding with HFHF diet (1.88 ± 0.35 vs 1.75 ± 0.46, $p = 0.60$). HFHF diet markedly increased score of hepatocellular ballooning score in non-OVX (1.88 ± 0.35 vs 0.00 ± 0.00, $p < 0.01$) and OVX (1.75 ± 0.46 vs 0.63 ± 0.74, $p < 0.01$) when compared with control groups.

NASH activity score which summed from steatosis, lobular inflammation, and hepatocellular ballooning scores were expressed similarly with all 3 histological features score (Table 4.3). HFHF diet achieved the increasing of NASH score in both non-OVX and OVX rats compared with corresponding normal diet groups (5.00 ± 0.93 vs 0.00 ± 0.00, $p < 0.01$; 6.25 ± 0.71 vs 1.38 ± 1.30, $p < 0.01$, respectively). Estrogen deficiency was greater the augmentation of NASH score than intact-ovary rats in both rats groups that feeding with normal (1.38 ± 1.30 vs 0.00 ± 0.00, $p <0.01$) and HFHF

diet (6.25 ± 0.71 vs 5.00 ± 0.93, $p < 0.05$). The most severity of NASH exhibited in OVX rats feeding with HFHF diet.

Table 4.3 The NASH activity score.

Group	0 normal	1 - 3 mild NASH	4 - 5 moderate NASH	6 - 8 severe NASH	Mean ± SD
control	8	-	-	-	0.00 ± 0.00
NASH	-	-	5	3	5.00 ± 0.93
NASH + Gen	-	4	4	-	3.63 ± 1.30
OVX	3	5	-	-	1.38 ± 1.30
OVX + NASH	-	-	1	7	6.25 ± 0.71
OVX + NASH + Gen	-	7	1	-	2.88 ± 1.13

NASH = nonalcoholic steatohepatitis, OVX = ovariectomized, Gen = genistein

The administration of genistein to non-OVX rats with NASH could not lower steatosis (1.38 ± 0.92 vs 1.88 ± 0.83, $p = 0.07$) and lobular inflammation score (1.00 ± 0.53 vs 1.25 ± 0.46, $p = 0.33$), contrarily, hepatocellular ballooning score was significantly decreased (1.25 ± 0.46 vs 1.88 ± 0.35, $p < 0.05$) when compared with NASH group. In OVX groups, genistein was significantly diminished steatosis score (3.00 ± 0.00 vs 1.00 ± 0.53, $p < 0.01$) and lobular inflammation score (1.50 ± 0.53 vs 0.50 ± 0.53, $p < 0.01$) as compared to OVX + NASH groups. Unfortunately, hepatocellular ballooning score was not altered (1.38 ± 0.52 vs 1.75 ± 0.46, $p = 0.12$). However, genistein showed the ability to scale down the NASH score in both non-OVX and OVX rats with NASH when compared with corresponding NASH groups (non-OVX; 3.63 ± 1.30 vs 5.00 ± 0.93, $p < 0.01$ and OVX; 2.88 ± 1.13 vs 6.25 ± 0.71, $p < 0.01$) (Figure 4.5) (Table 4.3).

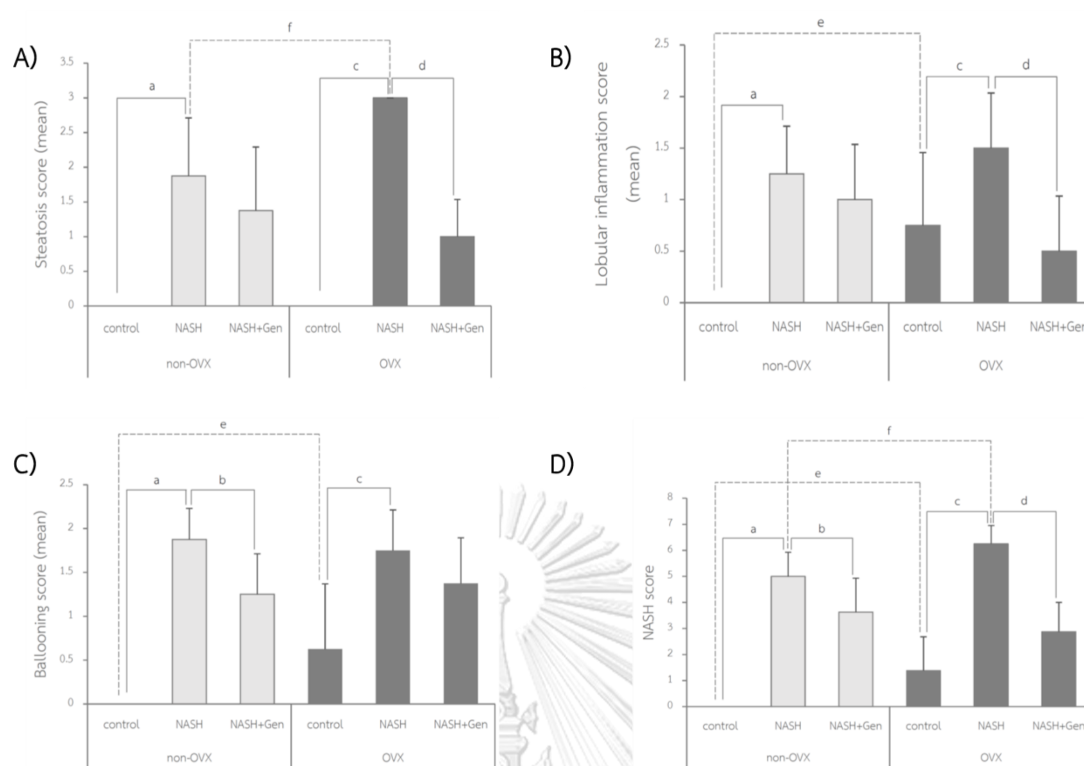

Figure 4.5 Histological changes in liver tissue were semi-quantitatively expressed as 0-3 in steatosis (A), 0-3 in lobular inflammation (B), 0-2 in hepatocellular ballooning (C), and summed 0-8 in NASH score (D). Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

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4.4 Serum biochemical markers of liver injury

Elevated of serum AST and ALT levels are the indicators of liver damage. Serum level of AST, as shown in Figure 4.6A, did not demonstrate the alteration among 6 experimental groups. In non-OVX rats, AST level of HFHF diet-induced NASH group was not differed from control group (573.31 ± 276.98 vs 507.94 ± 193.36 U/l, $p = 0.58$). The comparative level of AST was also found between OVX rats with NASH and control group (603.63 ± 262.26 vs 421.94 ± 169.78 U/l, $p = 0.13$). There were not statistical variation of AST level between non-OVX and OVX rats feeding with normal (507.94 ± 193.36 vs 421.94 ± 169.78 U/l, $p = 0.47$) and HFHF diet (573.31 ± 276.98 vs 603.63 ± 262.26 U/l, $p = 0.80$).

As shown in Figure 4.6B, serum ALT levels were not different between control and HFHF diet-induced NASH groups in non-OVX (56.48 ± 27.33 vs 59.22 ± 58.05 U/l, $p = 0.867$) and OVX rats (48.00 ± 12.68 vs 67.18 ± 41.18 U/l, $p = 0.248$), however, the increasing trend of ALT level in OVX + NASH group was observed. In addition, the different of ALT level between non-OVX and OVX rats feeding with normal (56.48 ± 27.33 vs 48.00 ± 12.68 U/l, $p = 0.61$) and HFHF diet (59.22 ± 58.05 vs 67.18 ± 41.18 U/l, $p = 0.63$) were not demonstrated.

Although, the significant reduction of serum ALT levels was only shown in NASH with OVX group after received genistein when compared with OVX + NASH group (26.58 ± 16.45 vs 67.18 ± 41.18 U/l, $p < 0.05$) decreasing trend was also found in non-OVX rats (28.37 ± 13.15 vs 59.22 ± 58.05 U/l, $p = 0.066$).

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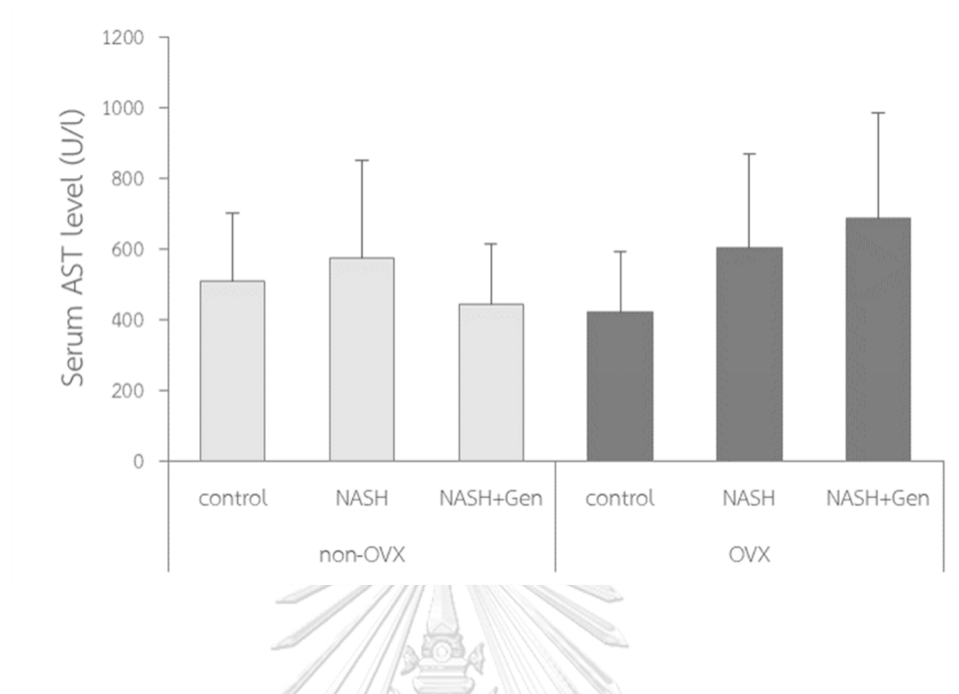
A)



B)

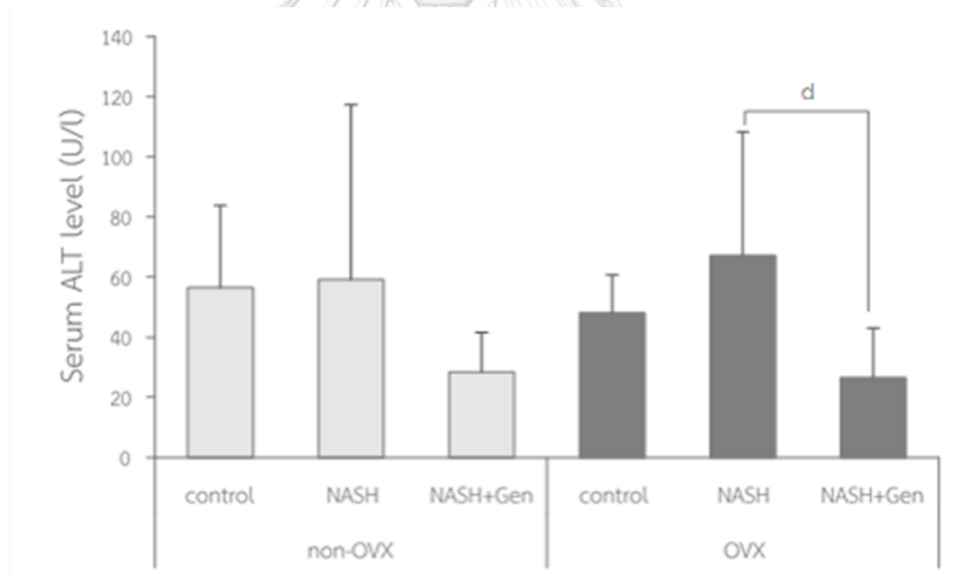


Figure 4.6 Serum level of biochemical markers of liver injury in rats. A) serum AST levels. B) serum ALT levels. Value represent mean ± SD.

d; $P < 0.05$ compared between NASH and NASH+Gen group of OVX rats

4.5 Serum inflammatory cytokine level

The inflammation was considered as up-regulation of inflammatory cytokines. HFHF diet-induced NASH group expressed higher serum TNF-α level in both non-OVX (124.13 ± 15.57 vs 62.63 ± 18.07 pg/mL, p < 0.01) and OVX rats (171.63 ± 22.34 vs 124.13 ± 16.04 pg/mL, p < 0.01) when compared with control groups.

Ovariectomy shown to intensify the liver inflammation in both diet conditions as expressed higher 1.9-fold serum TNF-α level than non-OVX in normal diet (124.13 ± 16.04 vs 62.63 ± 18.07 pg/mL, p < 0.01) and 1.3-fold in HFHF diets (171.63 ± 22.34 vs 124.13 ± 15.57 pg/mL, p < 0.01).

Nevertheless, TNF-α level in serum of NASH rats was significantly decreased by genistein in OVX rats (73.08 ± 19.31 vs 171.63 ± 22.34 pg/mL, p < 0.01) (Figure 4.7).

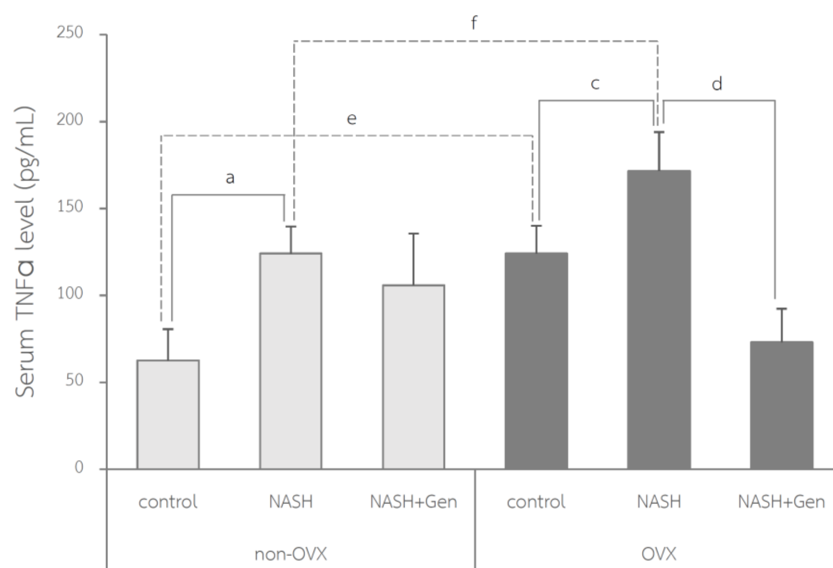


Figure 4.7 Serum TNF-α level of rats by ELISA analysis. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

4.6 Hepatic lipid peroxidation level

Lipid peroxidation is used as the indicator of cellular and tissue oxidative stress. The TBARS measurement is well-accepted method for determination of lipid peroxidation; malondialdehyde (MDA) detection. Eight weeks of HFHF diet-induced NASH significantly elevated TBARS levels in liver of non-OVX rats compared with normal diet (0.14 ± 0.03 vs 0.12 ± 0.01 nmol/mg protein, $p < 0.05$). Although, HFHF diet could not increase TBARS levels of OVX rats with NASH as compared to control group (0.14 ± 0.02 vs 0.12 ± 0.01 nmol/mg protein, $p = 0.051$), the raising trend was observed. Genistein was not reduced TBARS levels in non-OVX ($p = 0.055$). Conversely, it significant decreased TBARS levels in OVX rats when compared with NASH group (0.11 ± 0.01 vs 0.14 ± 0.02 nmol/mg protein, $p < 0.01$) (Figure 4.8).

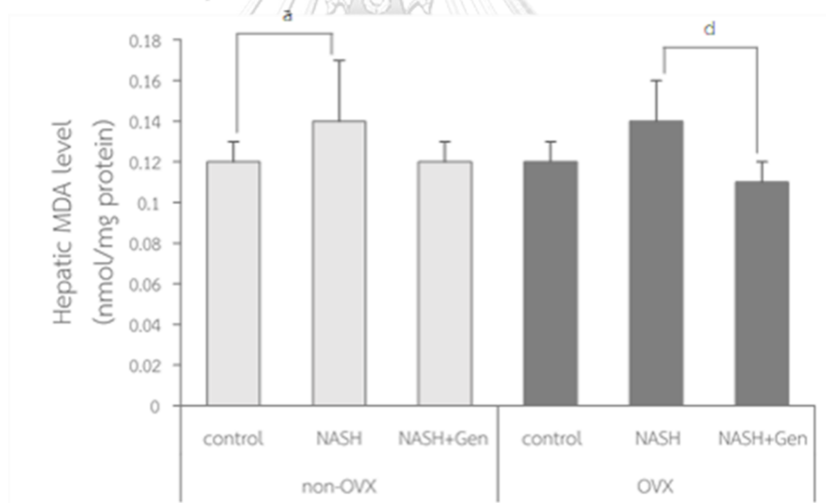


Figure 4.8 Hepatic malondialdehyde (MDA) level measured by TBARS assay. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

4.7 Hepatic lipid accumulation

Oil-red-O (ORO) stained in frozen section of liver tissue showed increased lipid accumulation in HFHF induced NASH groups with the most in OVX rats. These lipid were attenuated with genistein administration in both non-OVX and OVX rats (Figure 4.9A). Graph of ORO-positive area was displayed in percentage in Figure 4.9B.

In HFHF induced NASH groups, both non-OVX and OVX rats increased percentage of ORO positive area as compared with control groups (67.42 ± 4.33 vs 11.17 ± 1.86%, $p < 0.01$ and 81.07 ± 1.44 vs 23.24 ± 7.63%, $p < 0.01$; respectively).

Moreover, OVX rats showed higher hepatic lipid accumulation than non-OVX in both normal diet (23.24 ± 7.63 vs 11.17 ± 1.86%, $p < 0.01$) and after induced with HFHF diet (81.07 ± 1.44 vs 67.42 ± 4.33%, $p < 0.01$).

The administration of genistein decreased ORO positive area in both non-OVX (16.60 ± 7.07 vs 67.42 ± 4.33%, $p < 0.01$) and OVX rats (12.84 ± 3.96 vs 81.07 ± 1.44%, $p < 0.01$) as compared with NASH groups.

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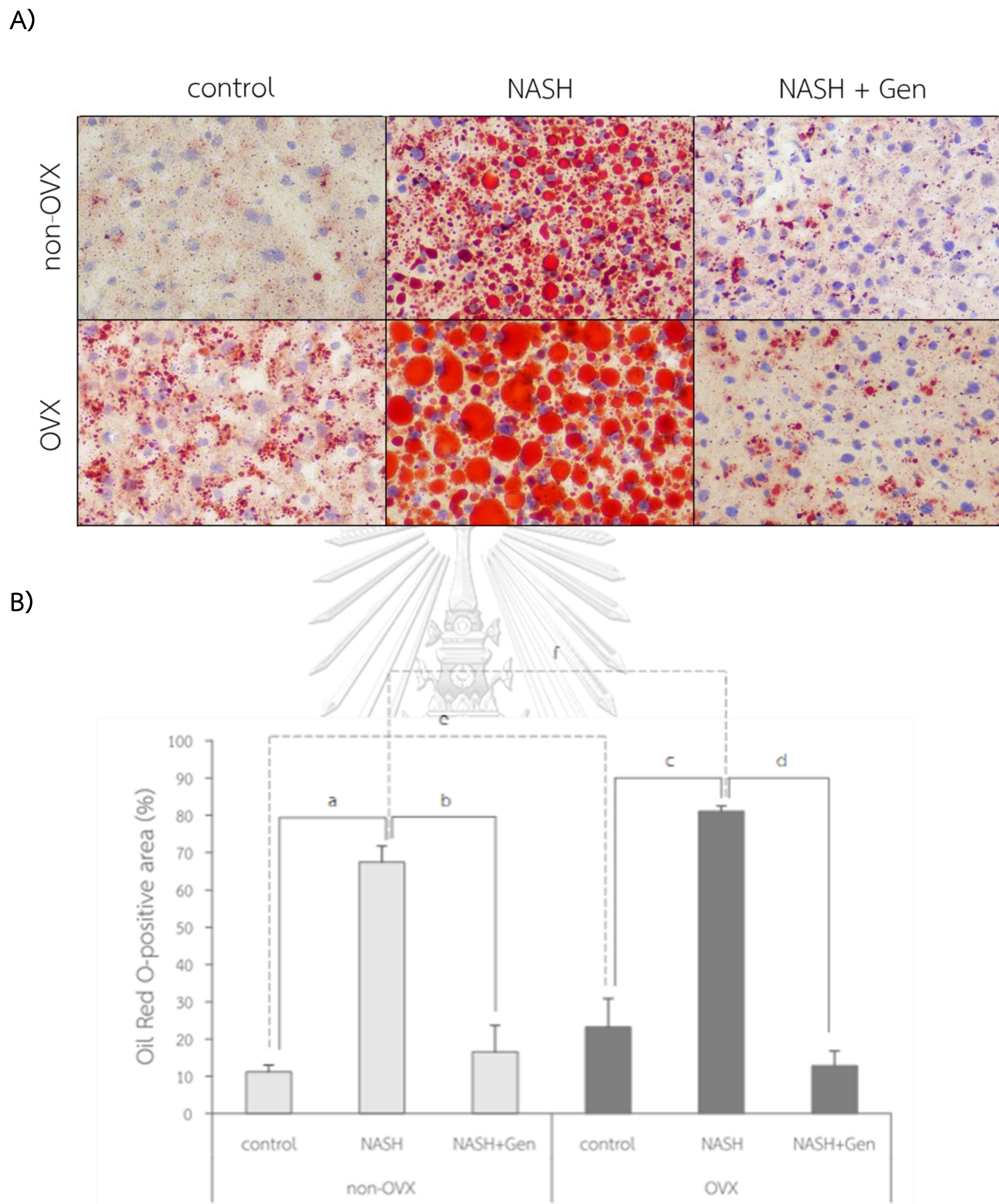


Figure 4.9 Lipid accumulation in liver tissue. A) photomicrographs of ORO staining in 6 experimental groups. The original magnification is 400x. B) the percentage of ORO positive area. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

Hepatic triglyceride (TG) level was significantly escalated in both HFHF induced NASH with non-OVX (115.28 ± 43.31 vs 37.30 ± 17.07 nmol/mg tissue, $p < 0.01$) and OVX rats (360.12 ± 49.06 vs 90.75 ± 5.60 nmol/mg tissue, $p < 0.01$) compared with control.

Similarly to the percentage of ORO positive area, OVX exacerbated hepatic TG levels in both normal (90.75 ± 5.60 vs 37.30 ± 17.07 nmol/mg tissue, $p < 0.01$) and HFHF diet (360.12 ± 49.06 vs 115.28 ± 43.31 nmol/mg tissue, $p < 0.01$) compare with same diet conditions in non-OVX rats.

The augmentation of TG in liver was significantly lowered by genistein administration in both non-OVX (70.64 ± 31.29 vs 115.28 ± 43.31 nmol/mg tssue, $p < 0.01$) and OVX (42.85 ± 15.90 vs 360.12 ± 49.06, $p < 0.01$) as compared with NASH groups (Figure 4.10).

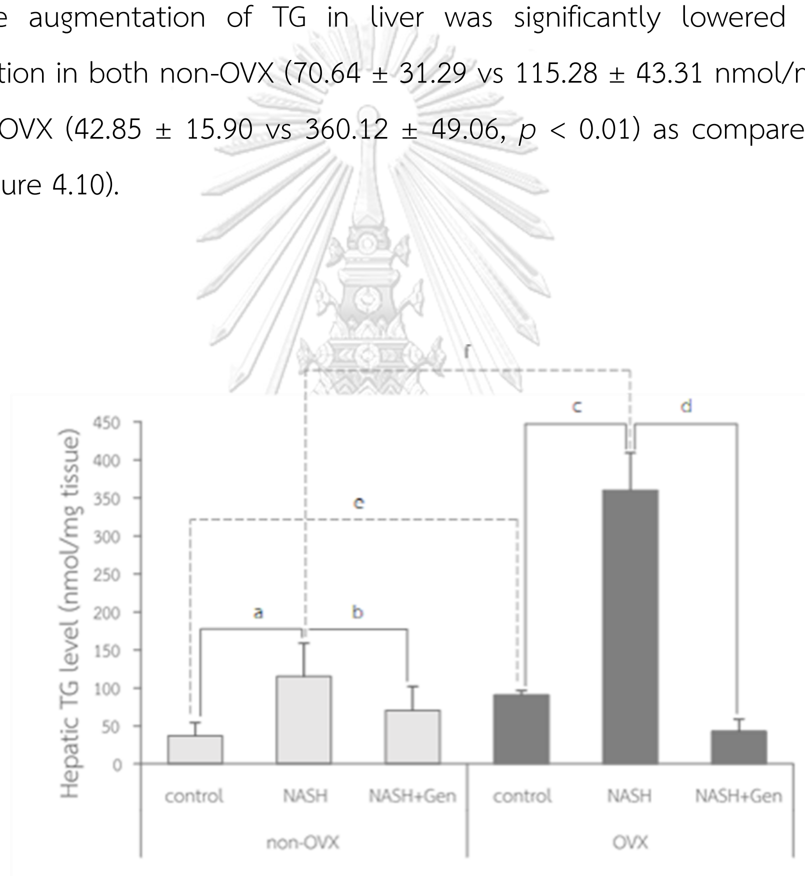


Figure 4.10 Hepatic triglyceride (TG) level by colorimetry analysis. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

Hepatic free fatty acid (FFA) levels increased in both normal (13.89 ± 0.09 vs 3.62 ± 0.77 nmol/mg tissue, $p < 0.01$) and HFHF diet (13.11 ± 1.65 vs 9.07 ± 2.27 nmol/mg tissue, $p < 0.01$) with OVX rats when compared with non-OVX. HFHF diet could elevated hepatic FFA levels only in non-OVX rats (9.07 ± 2.27 vs 3.62 ± 0.77 nmol/mg tissue, $p < 0.01$) compared with control.

The hepatic FFA levels in NASH group significant reduced by receiving the genistein only in OVX rats (13.11 ± 1.65 vs 6.50 ± 0.60 nmol/mg tissue, $p < 0.01$) (Figure 4.11).

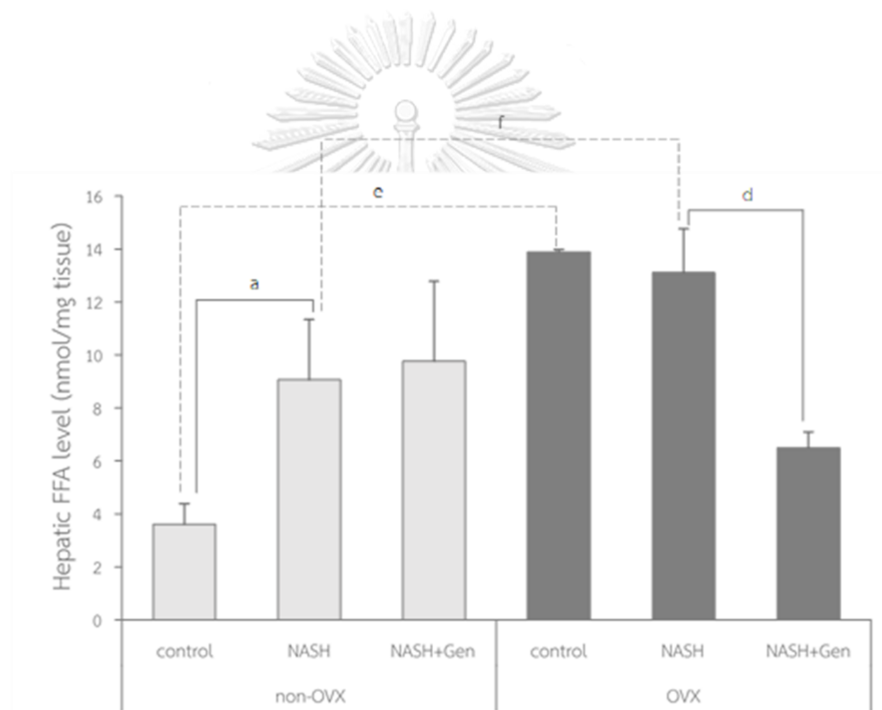


Figure 4.11 Hepatic free fatty acid (FFA) level by colorimetry analysis. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

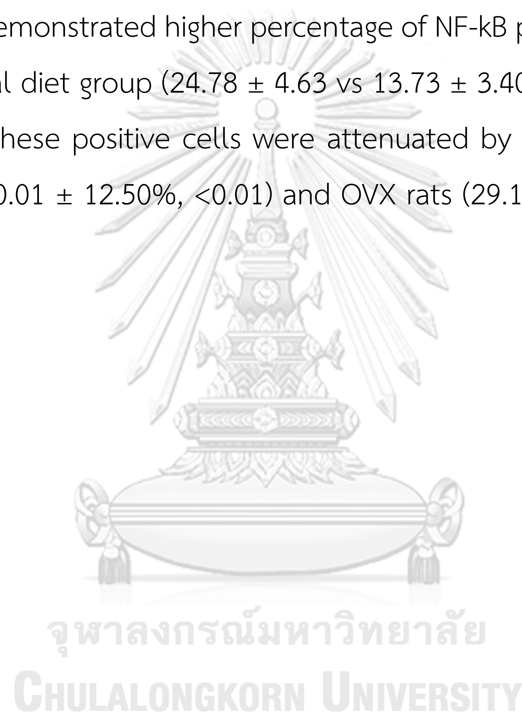
4.8 Immunohistochemistry analysis

NF-kB; the transcription factor which is the mediator in inflammatory response pathway, was detected by immunohistochemistry (IHC) analysis. Brown color on cells' nucleus is present as the activated activity of NF-kB (Figure 4.12A).

As shown in Figure 4.12B, the number of NF-kB positive cells which expressed as percentage were increased by HFHF induced NASH in both non-OVX (50.01 ± 12.50 vs 13.73 ± 3.40%, $p < 0.01$) and OVX rats (40.57 ± 6.57 vs 24.78 ± 4.63%, $p < 0.01$) when compared with control groups.

OVX rats demonstrated higher percentage of NF-kB positive cells than non-OVX rats in both normal diet group (24.78 ± 4.63 vs 13.73 ± 3.40%, $p < 0.01$).

However, these positive cells were attenuated by genistein in both non-OVX (28.04 ± 8.19 vs 50.01 ± 12.50%, <0.01) and OVX rats (29.12 ± 6.84 vs 40.57 ± 6.57%, < 0.01).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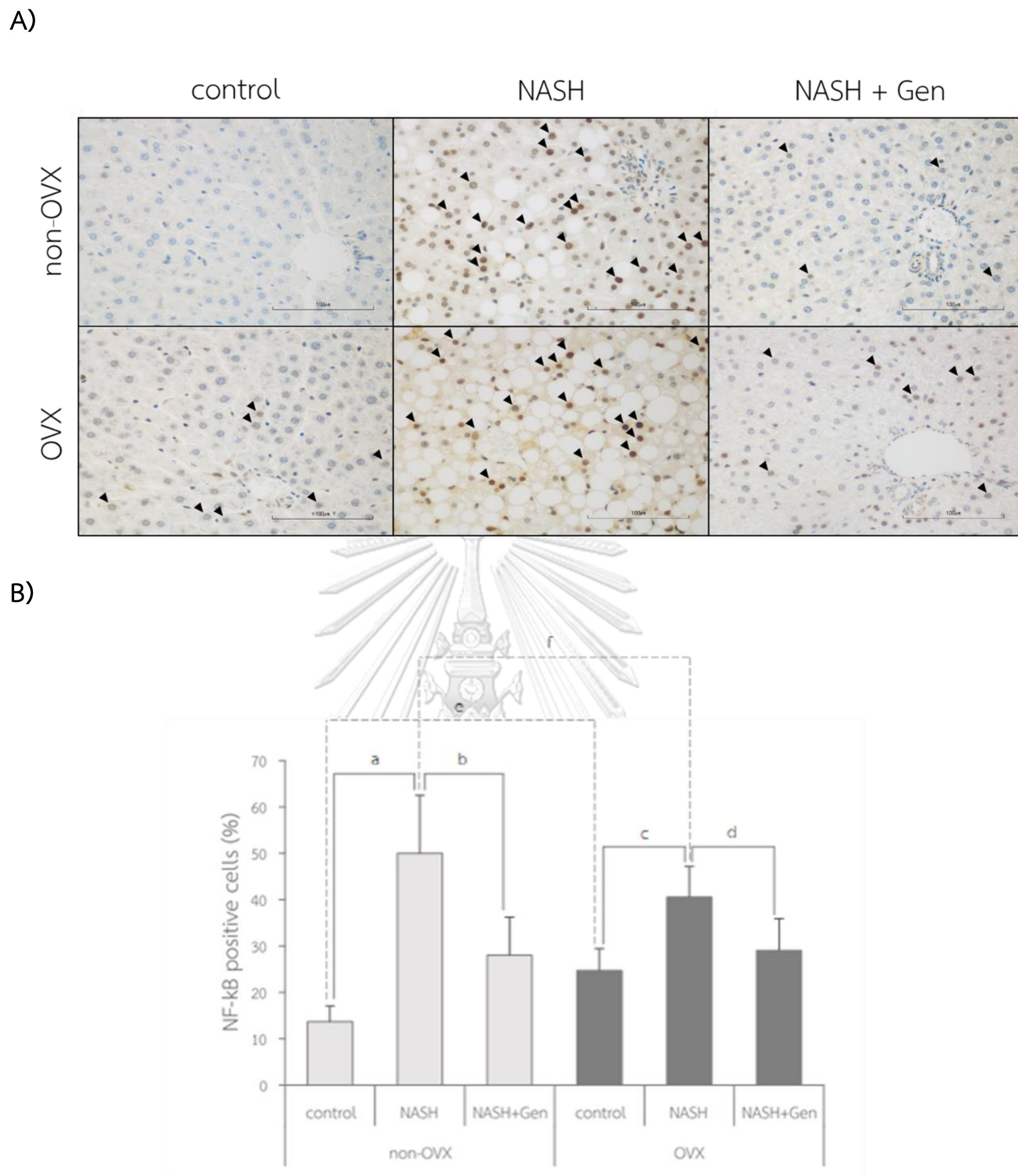


Figure 4.12 NF-kB positive cells in liver tissue. A) paraffin embedded sections were performed by IHC method. NF-kB positive cells were indicated with arrows. The original magnification is 400x. B) the percentage of NF-kB positive cells number. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

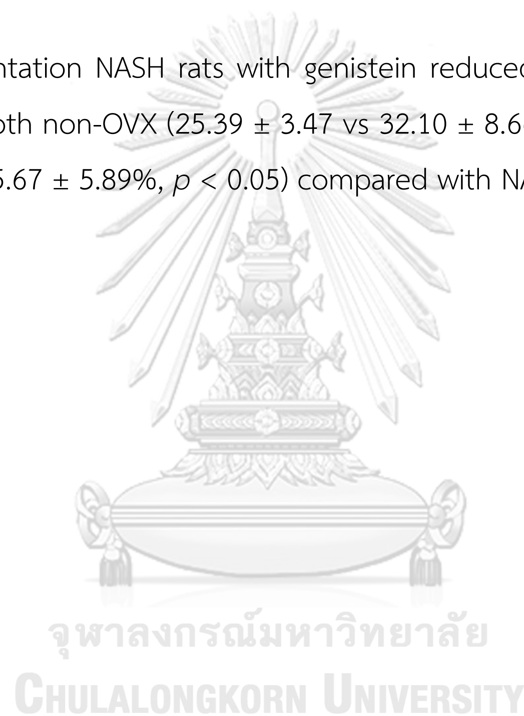
e; P < 0.05 compared between control group of non-OVX and OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

PPARγ, the transcription factor that regulates fat metabolism and inflammation, was detected. As shown in Figure 4.13A, liver sections stain with PPARγ antibody expressed higher positive cells in both non-OVX and OVX with HFHF diet-induced NASH rats. These positive cells were alleviated via genistein treated.

The percentage of PPARγ positive cells number was shown in Figure 4.13B. OVX rats with normal diet demonstrated the enhancement of PPARγ positive cells compared to non-OVX rats (29.35 ± 1.47 vs 15.47 ± 3.62%, $p < 0.01$). HFHF could increase positive cells number of PPARγ only in non-OVX rats (32.10 ± 8.66 vs 15.47 ± 3.62%, $p < 0.01$).

Supplementation NASH rats with genistein reduced the expression of PPARγ positive cells in both non-OVX (25.39 ± 3.47 vs 32.10 ± 8.66%, $p = 0.01$) and OVX rats (10.10 ± 3.09 vs 25.67 ± 5.89%, $p < 0.05$) compared with NASH group.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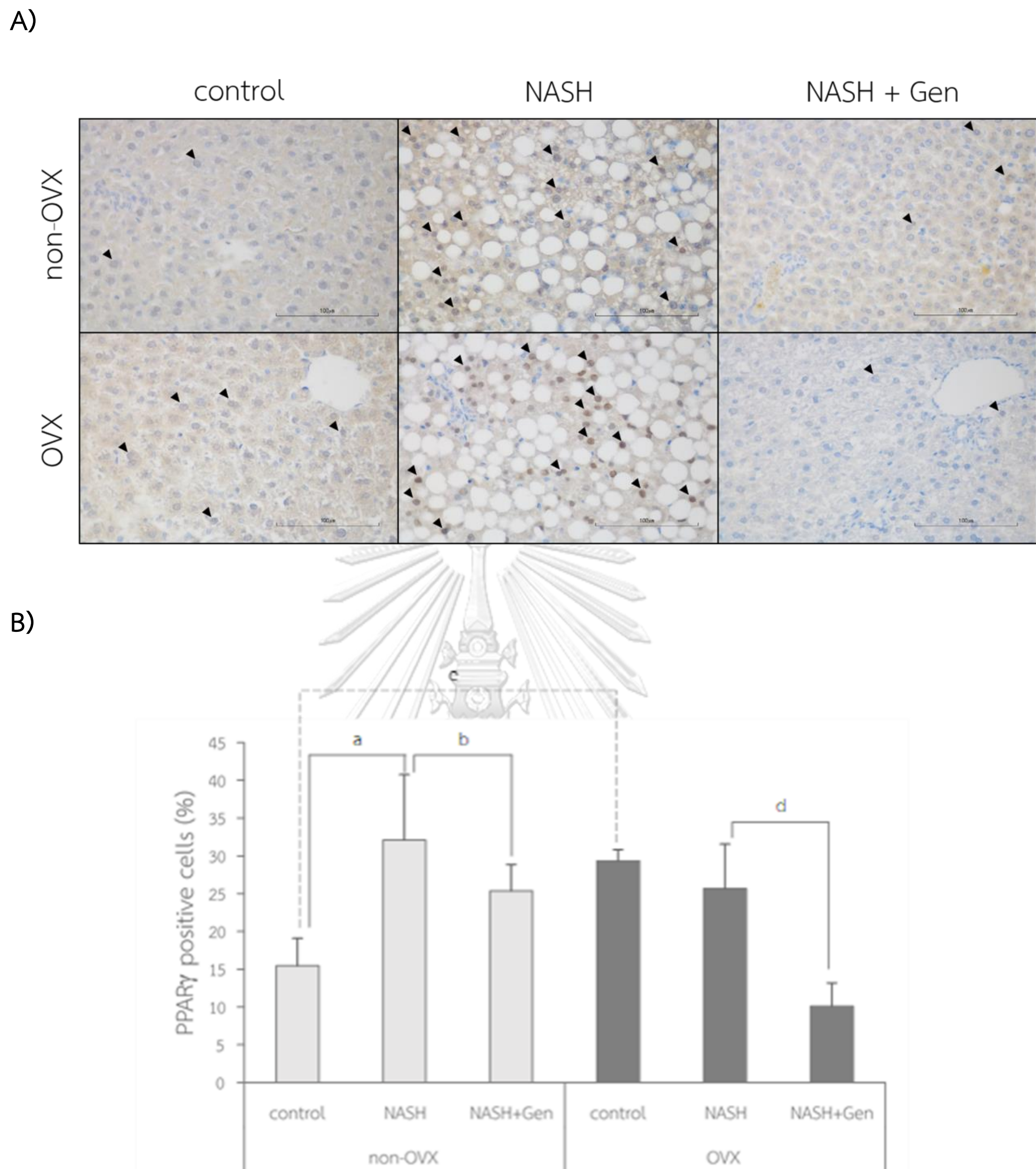

Figure 4.13 PPARγ positive cells in liver tissue. A) IHC of PPARγ from paraffin embedded liver section. PPARγ positive cells were indicated with arrows. The original magnification is 400x. B) the percentage of PPARγ positive cells number. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

e; P < 0.05 compared between control group of non-OVX and OVX rats

4.9 TUNELS analysis

Hepatocytes apoptosis was performed via TUNELs method. The TUNELs positive cells demonstrated the most in OVX rats with HFHF induced NASH group (Figure 4.14A) and genistein could decline the numbers of them.

The percentage of TUNELs positive cells was higher in NASH than control groups in both non-OVX (27.90 ± 8.55 vs 1.10 ± 1.06%, $p < 0.01$) and OVX rats (45.03 ± 5.13 vs 1.55 ± 1.62%, $p < 0.01$).

HFHF induced NASH with OVX rats showed more severity in hepatocytes apoptosis than non-OVX rats (45.03 ± 5.13 vs 27.90 ± 8.55%, $p < 0.01$).

Genistein administration attenuated hepatocytes apoptosis as shown with reduced the TUNELs positive cells percentage in both non-OVX (2.80 ± 1.07 vs 27.90 ± 8.55%, $p < 0.01$) and OVX groups (1.50 ± 0.56 vs 45.03 ± 5.13%, $p < 0.01$) when compared with NASH groups (Figure 4.14B).

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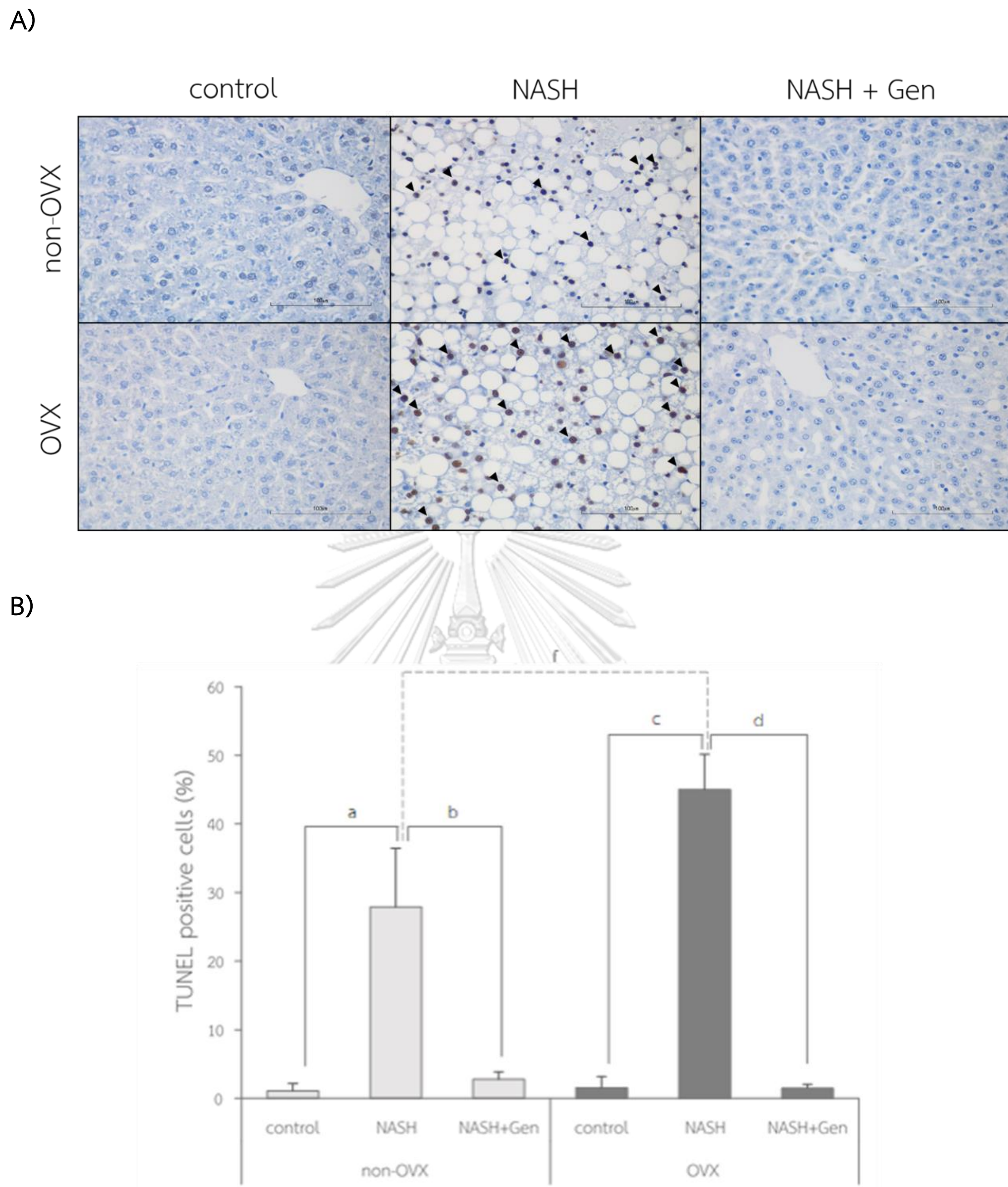

Figure 4.14 TUNELs positive cells in liver tissue. A) hepatocytes apoptosis in paraffin embedded liver section. TUNELs positive cells were indicated with arrows. The original magnification is 400x. B) the percentage of TUNELs positive cells number. Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

4.10 Western blot analysis

The protein expression of PPARγ in liver tissue; shown as relative ratio, was not different between HFHF induced NASH and control groups in both non-OVX (2.21 ± 0.68 vs 1.86 ± 0.62, p = 0.410) and OVX rats (2.22 ± 0.41 vs 2.30 ± 0.49, p = 0.834).

However, NASH treated with genistein could reduce hepatic protein expression of PPARγ only in non-OVX rats (1.01 ± 0.64 vs 2.21 ± 0.68, p < 0.01). Although, the discrepancy of hepatic PPARγ protein expression between NASH and NASH+Gen of OVX rats was not exhibited, the diminishing trend was observed (Figure 4.15).

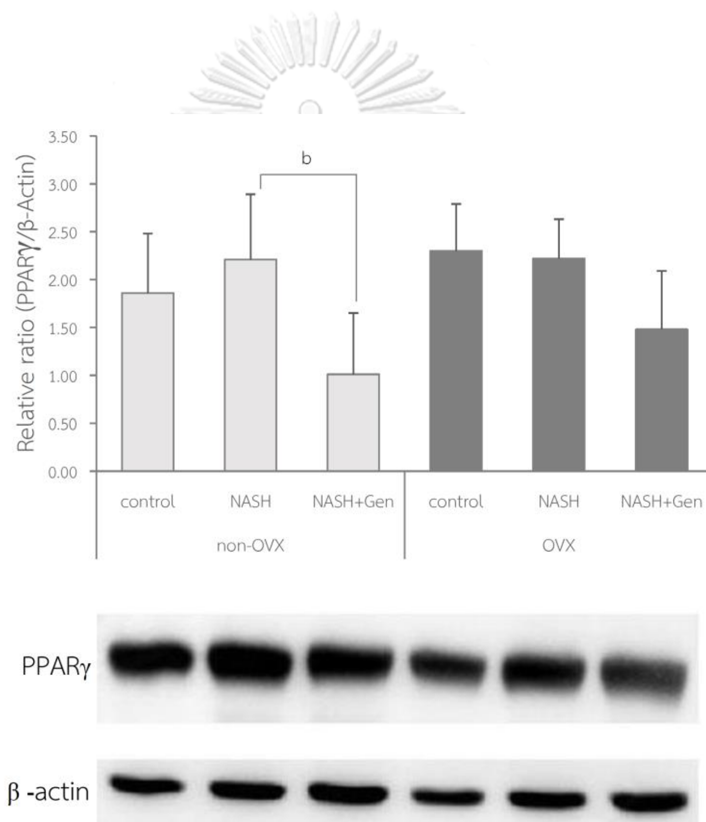

Figure 4.15 Western blot analysis of PPARγ protein expression in liver tissue. Value represent mean ± SD.

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

Adiponectin; protein derived from adipocyte which called adipokine, has been reported to protective effect against NASH. Unfortunately, the relative ratio of adiponectin expression in liver was not different between control and NASH groups in both non-OVX (0.29 ± 0.16 vs 0.68 ± 0.35, p = 0.215) and OVX rats (0.55 ± 0.26 vs 0.71 ± 0.27, p = 0.604).

Although, only increasing trend of adiponectin in NASH treated with genistein was observed in non-OVX rats (1.19 ± 0.40 vs 0.68 ± 0.35, p = 0.120), the significant increase was shown in OVX rats (1.45 ± 0.83 vs 0.71 ± 0.27, p < 0.05) (Figure 4.16)

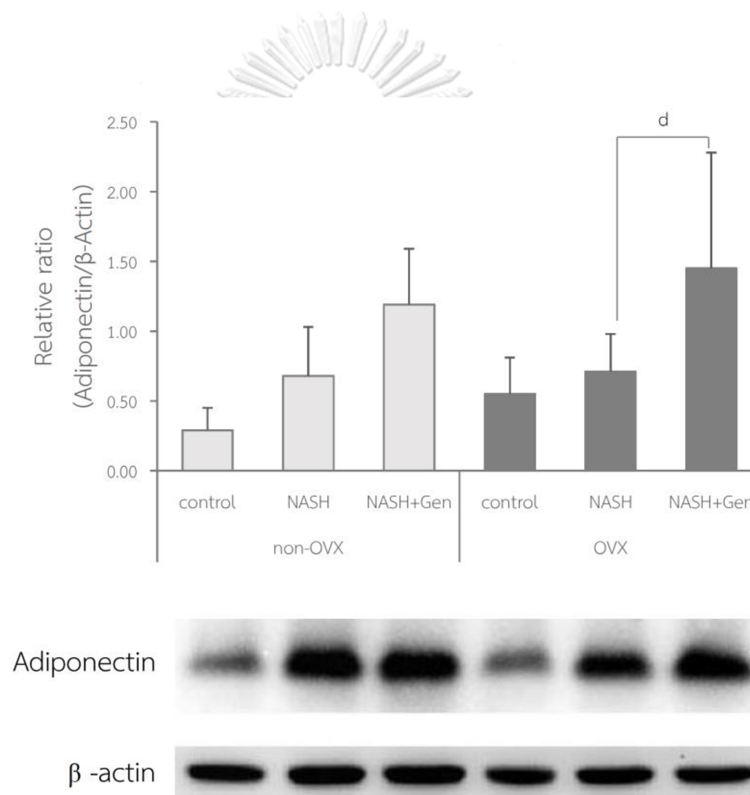

Figure 4.16 Western blot analysis of adiponectin protein expression in liver tissue. Value represent mean ± SD.

d; P < 0.05 compared between NASH and NASH+Gen group of OVX rats

Genistein has the estrogenic effect via binding with higher affinity with ERβ than ERα subtypes. The relative ratio of ERα expression was not different between non-OVX and OVX rats fed with normal diet (0.07 ± 0.02 vs 0.10 ± 0.03, $p = 0.42$). However, ERα expression in liver was significantly higher in both non-OVX (0.31 ± 0.03 vs 0.70 ± 0.02, $p < 0.01$) and OVX rats (0.24 ± 0.04 vs 0.10 ± 0.03, $p < 0.01$) fed with HFHF diet-induced NASH when compared with corresponding controls.

In contrast, ERα expression suppressed by genistein administration in only non-OVX rats compared with NASH group (0.24 ± 0.01 vs 0.31 ± 0.03,$p < 0.05$) (Figure 4.17A).

Another estrogen receptor; ERβ, was significantly reduced in OVX with NASH rats compared with control group (0.28 ± 0.06 vs 0.39 ± 0.05, $p < 0.05$). The statistical different was not observed in non-OVX rats (0.24 ± 0.08 vs 0.30 ± 0.11, $p = 0.35$), however, the decreasing trend demonstrated.

Unfortunately, genistein treatment could not alter hepatic ERβ expression in both non-OVX (0.19 ± 0.02 vs 0.24 ± 0.08, $p = 0.41$) and OVX (0.22 ± 0.03 vs 0.28 ± 0.06, $p = 0.32$) rats as compared with corresponding NASH groups. (Figure 4.17B)

The summary results of this study are shown in Table 4.4.

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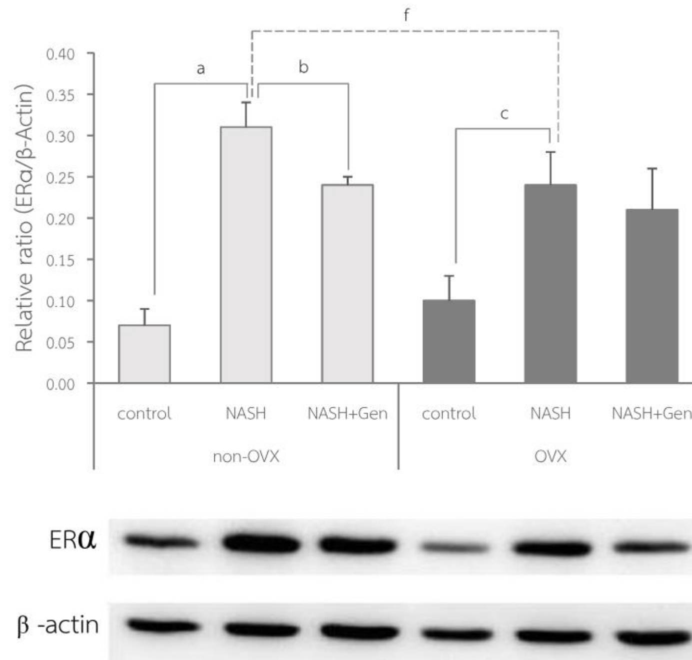
A)



B)

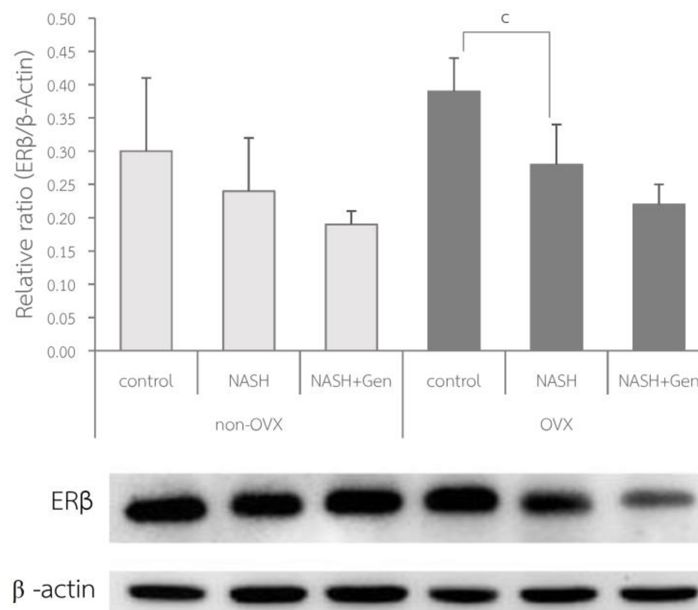


Figure 4.17 Western blot analysis of estrogen receptor (ER) subtypes protein expression in liver tissue; ERα (A) and ERβ (B). Value represent mean ± SD.

a; P < 0.05 compared between control and NASH group of non-OVX rats

b; P < 0.05 compared between NASH and NASH+Gen group of non-OVX rats

c; P < 0.05 compared between control and NASH group of OVX rats

f; P < 0.05 compared between NASH group of non-OVX and OVX rats

Table 4.4 The summary of all results in this study.

Parameters	Experimental groups					
	Non-OVX			OVX		
	control	NASH	NASH+Gen	control	NASH	NASH+Gen
Histopathological score[g]						
Steatosis	0.00±0.00	1.88±0.83[a]	1.38±0.92	0.00±0.00	3.00±0.00[c,f]	1.00±0.53[d]
Lobular inflammation	0.00±0.00	1.25±0.46[a]	1.00±0.53	0.75±0.71[e]	1.50±0.53[c]	0.50±0.53[d]
Ballooning	0.00±0.00	1.88±0.35[a]	1.25±0.46[b]	0.63±0.74[e]	1.75±0.46[c]	1.38±0.52
NASH score	0.00±0.00	5.00±0.93[a]	3.63±1.30[b]	1.38±1.30[e]	6.25±0.71[c,f]	2.88±1.13[d]
Liver enzymes (U/l) : AST	507.94±193.36	573.31±276.98	443.50±169.64	421.94±169.78	603.63±262.26	686.56±296.33
ALT	56.48±27.33	59.22±58.05	28.37±13.15	48.00±12.68	67.18±41.18	26.58±16.45[d]
Serum TNFα (pg/mL)	62.63±18.07	124.13±15.57[a]	105.84±29.77	124.13±16.04[e]	171.63±22.34[c,f]	73.08±19.31[d]
Hepatic MDA (nmol/mg protein)	0.12±0.01	0.14±0.03[a]	0.12±0.01	0.12±0.01	0.14±0.02	0.11±0.01[d]
Oil-red-O (%positive area)	11.17±1.86	67.42±4.33[a]	16.60±7.07[b]	23.24±7.63[e]	81.07±1.44[c,f]	12.84±3.96[d]
Hepatic TG (nmol/mg tissue)	37.30±17.07	115.28±43.31[a]	70.64±31.29[b]	90.75±5.60[e]	360.12±49.06[c,f]	42.85±15.90[d]
Hepatic FFA (nmol/mg tissue)	3.62±0.77	9.07±2.27[a]	9.77±3.01	13.89±0.09[e]	13.11±1.65[f]	6.50±0.60[d]
IHC (%positive cell)						
NF-kB	13.73±3.40	50.01±12.50[a]	28.04±8.19[b]	24.78±4.63[e]	40.57±6.57[c,f]	29.12±6.84[d]
PPARγ	15.47±3.62	32.10±8.66[a]	25.39±3.47[b]	29.35±1.47[e]	25.67±5.89[f]	10.10±3.09[d]
TUNELs	1.10±1.06	27.90±8.55[a]	2.80±1.07[b]	1.55±1.62	45.03±5.13[c,f]	1.50±0.56[d]
Western blot (relative ratio)						
ERα	0.07±0.02	0.31±0.03[a]	0.24±0.01[b]	0.10±0.03	0.24±0.04[c,f]	0.21±0.05
ERβ	0.30±0.11	0.24±0.08	0.19±0.02	0.39±0.05	0.28±0.06[c]	0.22±0.03
PPARγ	1.86±0.62	2.21±0.68	1.01±0.64[b]	2.30±0.49	2.22±0.41	1.48±0.61
Adiponectin	0.29±0.16	0.68±0.35	1.19±0.40	0.55±0.26	0.71±0.27	1.45±0.83[d]

Data are shown as mean ± SD. n = 8 in each groups.

[a]Comparison between control and NASH groups of non-OVX rats.

[b]Comparison between NASH and NASH with genistein groups of non-OVX rats.

[c]Comparison between control and NASH groups of OVX rats.

[d]Comparison between NASH and NASH with genistein groups of OVX rats.

[e]Comparison between control groups of non-OVX and OVX rats.

[f]Comparison between NASH groups of non-OVX and OVX rats.

[g]Pathology were scored into steatosis (0-3); lobular inflammation (0-3); and ballooning (0-2) following to the Brunt’s criteria, then, summed for a maximum total score of 8 for NASH score

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CHAPTER V
DISCUSSION AND CONCLUSIONS

High-fat high-fructose diet generated liver injuries and pathogenesis of NASH

NASH has been recognized as one of the most common chronic liver disease. Notwithstanding, the mechanisms of NASH are not fully understood. Two-hit hypothesis is the foremost theory that proposed to explain the pathogenesis of NASH, however, NASH has more complexity than clarify with "two" hits hypothesis. Accordingly, the multiple hits theory was proposed for more precisely explanation of NASH pathogenesis (6). Nonetheless, fat accumulation in liver is the essential initiation step in further pathogenesis of progressive inflammation and fibrosis from simple steatosis to NASH (131). Recent studies considered that dietary cholesterol is a potential risk factor for NASH advancement in both rats and human (132, 133). However, high fat with low carbohydrate consumption for long-term did not recreate the NAFLD (49) and responsible for only 15% of intrahepatic lipid (8). Contradictory, massive eating up of carbohydrate develop hepatic steatosis in a short duration. Fructose is the monosaccharide that extensive components in carbohydrate. In consideration of the major site for fructose metabolism, continue consuming high fructose may implicate the build-up of free fatty acid in liver via the activation of *de novo* lipogenesis (DNL) and inhibition of lipid oxidation. The key enzyme that responsible for the interaction of fructose metabolism and hepatic fat accumulation might be fructokinase, which converted fructose into fructose-1-phosphate (F1P) (134). Consequently, fructose-1-phosphate further enter glycolysis yielding excessive of acetyl-CoA which promote DNL, finally, leads to accumulate triglyceride in liver.

Hepatic lipid accumulation is connected to oxidative stress and inflammation; the consequences of lipotoxicity (131), leads the transition of simple steatosis to NASH. Free radicals, lipid peroxidation, and reduced antioxidants reported to elevate in human and animals models of NAFLD (135). High fructose influx to liver from diet consumption contribute to the elevation of metabolic stress by ATP depletion, since,

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fructose metabolism require large amount of ATP. These are involved with the production of many pro-inflammatory cytokines (such as TNF-α, IL-6, and NF-kB, etc.) acceleration which potential in the progression of NAFLD to NASH. Previous studies reported the correlation between NASH, oxidative stress, and inflammation. Rats fed *ad libitum* of high fat diet induced NASH observed the enlargement of MDA level (26). Furthermore, individuals who suffered from NASH had higher level of MDA than healthy (136). By the same token, the persistent of NF-kB activation was shown in animal models and patients with NASH (13). Tumor necrosis factor (TNF-α) shown to increase in NASH individuals (14) and positively associated with histological severity of liver damage (15). These reported are consistent with our finding. The induction of MDA level; the one of oxidative stress biomarkers, and TNF-α, NF-kB level; inflammatory molecules, in both non-OVX and OVX rats was occurred by HFHF consumption.

Our study exhibited that the pathogenesis of NASH could be reproducible with HFHF diet in both non-OVX and OVX rats. The histopathology features; steatosis, lobular inflammation, and hepatocellular ballooning, are deteriorate with HFHF diet consumption. Hepatic lipid accumulation which examined by oil red o stained was significantly augmented, additionally, hepatic triglyceride and free fatty acid level also induced by HFHF diet. Furthermore, oxidative stress (MDA levels), inflammation (TNF-α and NF-kB), and hepatocytes apoptosis were also enhanced with HFHF diet in both non-OVX and OVX rats.

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The liver enzymes; aminotransferases (AST and ALT), which indicate the liver injury, should increase in HFHF diet-induced NASH group. Our aminotransferases level are unchanged, in accordance with Liu J and colleagues studied (137). They suggested stable level of liver enzymes in NASH rats for 8 weeks, thereafter, these enzymes level were different at 16 and 24 weeks of high fat diet induced NASH. Likewise, 16 weeks of mice fed with HFHF diet showed signs of NASH but not different in ALT level (138). Previous studied reported the requirement of pyridoxal-5’-phosphate or vitamin B6 for liver enzymes activity and lacking of vitamin B6 showed to decrease ALT level (139). Furthermore, vitamin B6 level in plasma inversely associated with HF diet consumption which directly stimulate inflammation in obesity (140). Although, genistein improved

liver damage, the non-alteration of liver enzymes between NASH and NASH received genistein may be related to vitamin B6 insufficiency due to HF diet consumption.

In this present study, our results exhibited that HFHF diet induced the large amount of hepatocytes death in both non-OVX and OVX rats. Lipotoxicity is the key factor that triggered hepatocytes apoptosis, since, fatty acids are the mediator of apoptotic cell death signaling pathway (141). With regard to previous studied, exposure of free fatty acids in human liver demonstrated the up-regulation of Fas protein, which involved in the extrinsic programming of cell death, resulting to hepatocytes apoptosis (142). In addition, inflammatory mediators, especially TNF-α, can stimulate the activation of extrinsic cell death pathway through binding with Fas-associated protein with death domain (FADD) resulting to caspase 8 activation (143). Moreover, TNF-α signaling pathway is associated with c-Jun N-terminal kinase (JNK) activation (144). These are contributing factors of hepatocytes apoptosis.

NASH is well-known as the hepatic manifestation of metabolic syndrome which strongly associated with obesity, insulin resistance, and dyslipidemia (145). In this study, although, the typical pathologic and histopathological features of NASH were achieved with HFHF diet, we failed to increase body weight of rats. In MI rabbit feeding with high-fat high-fructose diet remained unchanged in body weight (146). Furthermore, additional drinking water with high percentage of fructose can be unchanged (147) or increased (148, 149) body weight of animals model. These can imply that body weight of rats altered without regard to the delivery mode of fructose. Additionally, fructose consumption causes terrible changes in liver liberated from obesity (54). Nevertheless, body weight reduction in this study may relate with type 2 diabetes, by a reason of, HFHF diet can induce the prominent of insulin resistance (146). However, diet-induced NASH by high fructose together with high cholesterol did not modify the insulin resistance (147) and our study did not monitor insulin resistance. Thereupon, the alteration of body weight with HFHF diet-induced NASH is still controversial and need more studies to elucidate.

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Estrogen deficiency exacerbated the pathogenesis of NASH

Chronic liver diseases are consequences of many factors and have pathological variation between men and women (150). Animal models and epidemiological investigates have shown influence of gender and reproductive states in the onset of NASH. Men had increased risk of having greater severity of NASH than menstrual women, but the different disappeared in postmenopausal women (19) and this effect is independent from obesity which commonly observed in postmenopausal status (151). These suggest the influence of estrogen in liver diseases. Estrogen is the primary female sex hormone that essential for normal metabolism in women, by means of, estrogen involved in many biological activities including lipid metabolism and inflammatory inhibition. Moreover, more than 1000 genes of human liver express as a sex-bias and most of their biological pathways are related in lipid metabolism (152). Patients with Turner syndrome have excessive visceral fat and lipid droplets in liver (153). The aromatase-deficient (ArKO) mice has been shown the spontaneously increased lipid accumulation in liver (154). In the liver of mice fed with high-fat diet, estrogen (E2) treatment suppressed the key lipogenic gene SCD1 expression, which accompanied by the reduction of triglyceride accumulation in liver and white adipose tissue (155). Moreover, E2 signals are related to LDL receptor expression, which resulting to the reduction of serum LDL-cholesterol. Treatment of ovariectomized rats with estrogen (E2) and estrogen receptor alpha (ERα) agonists have been shown to decrease body weight and total cholesterol level (24). In addition, estrogen therapy decreased the expression of genes involved in lipogenesis including acetyl-coenzyme A (CoA) carboxylate-α and -β, sterol regulatory element-binding protein 1c (SREBP-1c), stearoyl-CoA desaturase, lipoprotein lipase (LPL), FAS, fatty acid desaturase, and peroxisome proliferator-activated receptor (PPARγ) in postmenopausal women adipose tissue (156). For these reasons, estrogen deficiency leads to dysregulation of metabolism and associate with the alteration of lipid accumulation throughout our body which leads to the progression of NASH.

In present study indicates the comparable level of hepatic MDA, FFA, PPARγ, and adiponectin between rats fed with standard diet and HFHF diet-induced NASH in bilateral ovariectomized rat groups. Besides, diet-induced NASH rats with HFHF

demonstrated the exacerbation of pathological changes in liver by estrogen deficiency, as they were shown in higher NASH activity scores, hepatic fat accumulations (ORO, TG, FFA), inflammations (TNF-α, NF-kB), hepatocytes apoptosis, and PPARγ expression in OVX rats than non-OVX rats. Moreover, these phenomena were also observed between OVX and non-OVX rats fed with standard diet. These are exhibiting that, even in absence of nutrient factors, estrogen deficiency is relevant to the development of NASH.

Estrogen also influences the inflammatory and immune system, normally, it has evidence reported that ERα able to interact with the c-Rel subunit of NF-kB, preventing downstream molecules to promote expression of IL-6; one of the pro-inflammatory cytokines (157). Likewise, estrogen present the repression effect of IL-1 in liver. Additionally, the diet-induced atherosclerosis *in vivo* hepatic NF-kB activity was inhibited by estrogen supplementation in an ER-dependent manner (158). These has been attracted to the possible action of estrogen on anti-inflammatory properties in NASH. Accordingly, estrogen deficiency has been involved with the inflammatory processes stimulation which is the important circumstances in NASH pathogenesis. Ovariectomized mice reported to enhance TNF-α production from T-cell (159) which can further activate NF-kB signaling pathway for more inflammation. Lack of estrogen enhanced several hepatic inflammatory gene expressions and aggravated NASH in mice fed with high-fat and high-cholesterol diet (16). Menopausal women who have longer duration of estrogen deficiency associated with the greater risk of having more intense hepatic fibrosis (160). Together with increase fat accumulation and pro-inflammatory cytokines production, these emphasized the important role of estrogen deficiency in progression of NASH.

Genistein ameliorated liver injuries and pathogenesis of NASH

Genistein is the one of soybeans isoflavones that can mimic the physiological functions of estrogen via binding to both subtypes of estrogen receptor. Actions of genistein reported to alleviate hyperlipidemia through the activation of estrogenic pathway in several animal models (98, 99). In vitro study, genistein markedly inhibited upregulation of gene expression that involved with fatty acid synthesis and stimulated

the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) which abolished by fatty acid (161). Moreover, ApoE knockout mice demonstrated the effect of genistein on downregulation of proteins that has an important role in fatty acid metabolism in liver, and scavenger receptors involved in oxidized LDL uptake, CD36 and scavenger receptor A expression (28).

Genistein administration in our study demonstrated the significant improvement of histopathology scores in steatosis and lobular inflammation in ovariectomized rats and decreased hepatocellular ballooning score in non-OVX rats fed with HFHF diet. In addition, genistein attenuated hepatic lipid accumulation, TG and FFA level in NASH with or without estrogen deficiency. Oxidative stress (MDA level) and serum TNF-α level were alleviated by genistein, interestingly, these could observe only in OVX with NASH rats. Genistein reduced the expression of hepatic NF-kB in both NASH with or without estrogen deficiency. Certainly, genistein has been linked to the abated of hepatocytes apoptosis in NASH rats.

One of the main hallmarks of the NASH progression is inflammation which can be modulated by genistein. It has been found that genistein showed antioxidant effect on decreasing renal inflammation in streptozotocin-induced diabetic mice (106). Oxidative stress in liver of NASH db/db mice induced by methionine-choline-deficient (MCD) diet was diminished by genistein supplementation (110). Genistein has been accounted for lipid peroxidation; MDA level reduction in serum of NASH rats (23). In addition, in rodents with alcoholic liver disease or nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, isoflavones may improve PPARα-mediated fatty acid oxidation, alleviate hepatic steatosis, reduce CYP2E1-mediated oxidative stress overproduction, and attenuate progression of NAFLD (162). Genistein decreased the plasma level of TNF-α and IL-6 in rats fed with fructose (107), and also inhibited IL-1β, IL-6, and TNF-α mRNA levels. Furthermore, macrophages and neutrophils which contributing to the inflammatory response are attenuated their activation by genistein (108). Sustainable of oxidative stress and inflammation are resulting to enhancement of hepatocytes apoptosis. Consistent with this statement, genistein which has the anti-oxidative stress and anti-inflammatory properties in NASH, can derive subside of hepatocytes apoptosis.

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Genistein decreased the severity of NASH due to down-regulated hepatic PPARγ protein expression

PPARγ (peroxisome proliferator-activated receptor gamma) is belong to the ligand-activated nuclear receptor transcription factor superfamily, which plays indispensable role in lipid storage, glucose metabolism, and adipocyte differentiation. Therefore, the modulation of PPARγ expression might affect the pathogenesis of NASH. PPARγ activation may associate with the buildup of obesity and NAFLD as a consequence of PPARγ expression may associate with the enlargement in liver steatosis (69, 163). Interestingly, the PPARγ agonist; rosiglitazone, a potent insulin sensitizer, reported to remarkable exacerbate oxidative stress and liver steatosis (164) and did not modulate the reduction of NAFLD in HF mouse models (165). These indicated the possible action of PPARγ in steatosis production. Excessive of high lipid containing dietary intake brought out the induction of triglyceride in liver, followed by decline in PPARα and increased in PPARγ protein expression (71). Likewise, mice fed with high fructose diet demonstrated the hepatic steatosis in accordance with the extended of hepatic PPARγ expression (54). With this respect, it is interesting to note that the increasing of PPARγ in HFHF fed animals linked to the augmentation of sterol regulatory element-binding protein 1c (SREBP-1c); the target gene of PPARγ that implicates in lipogenesis. The activation of SREBP-1C facilitates others lipogenic genes that converts pyruvate into FA, which inhibits CPT-1 with by-product; malonyl co-A. These promote the re-esterification of FA to triglyceride and concentrate in liver. In addition, the abnormal high expression of hepatic PPARγ presented in NAFLD patients (166). However, there is a previous study urged that PPARγ-deficient ($PPAR\gamma^{+/-}$) mice developed more severe steatohepatitis than wild-type mice fed with methionine- and choline-deficient (MCD) diet (73). Thereby, the different responsive of PPARγ expression in NASH development requires more informations to elucidate.

This study exhibited that hepatic PPARγ significantly elevated in non-OVX rats fed with HFHF diet-induced NASH. On the contrary, we observed a similar degree of hepatic PPARγ expression in OVX rats between control and NASH groups, points the role of estrogen in NASH progression. Genistein showed the protective effect on NASH by reducing the expression of PPARγ in both non-OVX and OVX with NASH rats.

It is knowledge that genistein has anti-oxidation, anti-inflammatory, anti-apoptosis, and anti-proliferation of cell properties. Recently, genistein has also been displayed to be a PPARγ agonist (26, 67) and essential for NASH protection. However, our finding data do not support that genistein is an agonist of PPARγ. The present results are in agreement with several previous studies which demonstrated the suppressive effect of genistein on PPARγ protein expression. The supplementation of genistein in C57BL/6J mice fed with HF showed down-regulated the expression of lipogenic or adipogenic transcription factors, including PPARγ (167). Genistein also attenuated hepatic steatosis by reducing the PPARγ target gene; monoacylglycerol O-acyltransferase 1, mRNA level in apolipoprotein E-deficient (ApoE(-/-)) mice fed a HF diet (28). The alleviation effect of genistein on PPARγ expression may associate with MAPK activation (168, 169), as a result of MAPK stimulation leads to PPARγ phosphorylation and suppresses transcriptional activities of PPARγ (170). These, most likely, are tempting to speculate that genistein is a PPARγ antagonist and protects against NASH.

Genistein improved NASH via up-regulated adiponectin protein expression in liver

Adiponectin is an anti-inflammation, anti-diabetic, and anti-atherogenic polypeptide that released by adipocytes. This kind of adipokines is highly correlated with insulin sensitivity (171). Epidemiology studies revealed the strongly association of NASH and obesity, particularly visceral obesity. The enlargement of white adipose cells in obesity interrupted hormones, inflammatory cytokines, and adipokines, which altered the normal homeostasis resulting to wide progression of disorders. Serum adiponectin reported to negative correlation with obesity, thereby, it level may modify in NASH. Comparison between healthy and NASH patients showed 50% lower of serum adiponectin in NASH than healthy individuals (172) and this lowering level in serum associated with NAFLD (173, 174). Moreover, diminishing levels of adiponectin in NASH patients demonstrated severity of inflammation, which related to fatty liver and steatohepatitis development (175). NASH patients did not only show the declination

of adiponectin in serum but also the declination of mRNA expression in liver tissue (176).

In contrast to these respects, the results of this study could not exhibit the alteration of adiponectin between control and NASH rats with both non-OVX and OVX groups. In this present study, both non-OVX and OVX rats fed on HFHF diet did not alter protein expression of hepatic adiponectin, although, the typical features of NASH has been achieved. However, oral administration of genistein increased the expression of adiponectin in liver of NASH, especially in OVX rats. This phenomena might be caused of animal model in present study unable to gain weight.

Previous studies reported that adiponectin has a potential action to improve NASH. In *ob/ob* mice, adiponectin attenuated steatosis, inflammation and escalated serum level of ALT (177). Stimulation of adiponectin receptors increased activities of AMP kinase and PPARα; the key mediator in β-oxidation, in genetic modulating mice (178). Nevertheless, adiponectin level may be modulated by genistein, since, it has been reported that genistein effected on adiponectin. In mice fed with HF diet, dietary intake of genistein exhibited hepatic steatosis and adiposity reduction, which related to the elevation of adiponectin and reduction of leptin in adipose tissue (167). Although, it can be argued that, adiponectin expression is regulated by PPARγ (179), genistein unable to stimulate PPARγ transcriptional activity in both adipocyte (180) and C57BL/6L mice (167). These finding are compatible with this present study which established conversely level of PPARγ and adiponectin with oral administration of genistein, suggesting that genistein might not increase adiponectin directly via PPARγ activation. Previous studies in NASH patients could not find the correlation between circulation adiponectin levels and hepatic expression of adiponectin, proposing different regulation mechanism on the expression of adiponectin in liver (176). *In vitro* study found the stimulatory effect of genistein on upregulated adiponectin expression in liver via TNF-α inhibition (180). This effect activated through JNK signaling pathway inhibition. Additionally, genistein inhibits TNF-α-induced endothelial inflammation in C57BL/6 mice (181) and in liver of NASH muse model (26). It is tempting to consider that regulated mediator for hepatic adiponectin might be proinflammatory cytokines, especially TNF-α. In concordance with these findings, present study clarified that

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genistein significantly reduced TNF-α in NASH with OVX rats accompanied with significantly increased adiponectin. This can be speculated that genistein administration in our study might increase adiponectin in NASH rats due to TNF-α inhibition.

Genistein effected on estrogen receptors expression.

Estrogen is a potential hormone that has the pleiotropic hormone and involved in varieties organ regulation. Many metabolic disorders found the association between estrogen and mechanism of diseases including NASH. The declination of estrogen levels is related to interruption of lipid or glucose metabolism (182). Whereas, ERα likely displays the protective effect on metabolic diseases, the function of ERβ is unclear. However, the different biological functions of ERα and ERβ were identified and demonstrating tissue-specific effects. Transgenic mouse model revealed that αERKO mice are diabetic and insulin resistant, on the contrary, βERKO mice exhibited insulin responsibility and do not have exceed fat accumulation in their body (183). OVX CD-1 rats exhibited the reduction of hepatic ER mRNA and returned to same level of control within 1-3 days (184). ERβ deficiency in female mice fed on HF diet advocate the accumulation of white adipose tissue via stimulation of PPARγ signaling, indicating the role of ERβ on negative PPARγ regulation (185). These mechanisms might be mediated by common co-activators competition (186).

HFHF diet-induced NASH demonstrated upregulation of ERα expression in both non-OVX and OVX rats and administration of 16 mg/kg BW genistein by gavage significantly downregulated ERα in non-OVX rats. Unfortunately, these respect were not observed in ERβ, which the difference among 6 groups were not exhibited. Furthermore, the alteration of both ER subtypes after ovariectomized were not existed. These results consistent with previous studies that found the similarity level of ERα expression in liver between 12 weeks OVX and control groups (187).

In liver, male hamster feeding with HF diet exerted downregulation of ERα mRNA expression (98). Moreover, patients with NASH had lower ERα expression percentage in liver than simple steatosis individuals (188). C57BL/6J mice fed with HF diet-induced NAFLD and showed downregulation of ERα, while ERβ were upregulated

(167). This appears the cause of the compensatory mechanism for the downregulation of ERα. Therefore, it might explain the opposite alteration of ERs in NASH groups.

Genistein, phytoestrogen which binds with higher affinity to ERβ than ERα, exhibits contrasting effect on ERs. Genistein administration in mice with 100 mg/kg BW increased significantly ERα expression in uterine (189). HF feeding together with genistein reported to upregulate both hepatic ERs mRNA expression and alleviate hyperlipidemia (98). However, *in vivo* studies, there are several responsive patterns of ERs to genistein (190, 191). Nevertheless, these studies announced higher concentration and prolonged exposure to genistein, finally, suppressed both ERs expression. Thereby, ERs response to genistein in NASH are still controversial. The tissue-specificity, duration of administration, and concentration of genistein may be taking to account for these distinct actions.

Conclusions

In conclusion, this study demonstrates that estrogen deficiency is a contributing factors to be NASH. Genistein is a potent phytoestrogen to improve pathology of NASH through anti-lipid accumulation, anti-oxidation, anti-inflammation, and anti-apoptosis properties. Besides, the increment of the protective adipokine which is adiponectin. These are postulated the effective roles of genistein against NASH.

Accordingly, genistein might be the one of effective natural products that has the protective effect on NASH and might be useful for non-alcoholic steatohepatitis treatments, especially, in estrogen deficiency individuals.

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