ผลของเจนิสทีนต่อภาวะออกซิเดทีฟสเตรสและการอักเสบในหนูไมซ์ที่เป็นตับอ่อนอักเสบเฉียบพลัน

นางสาวจำลองลักษณ์ ศรีโคว์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Effects of Genistein on Oxidative Stress and Inflammation In Acute Pancreatitis Mice

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จำลองลักษณ์ ศรีโคว์ : ผลของเจนิสทีนต่อภาวะออกซิเดทีฟสเตรสและการอักเสบในหนูไมซ์ที่เป็นตับอ่อนอักเสบเฉียบพลัน (Effects of Genistein on Oxidative Stress and Inflammation In Acute Pancreatitis Mice) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดวงพร วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ. ประสงค์ ศิริวิริยะกุล, 91 หน้า.

เจนิสทีนเป็นสารในกลุ่มไอโซฟลาโวนที่พบมากที่สุดในถั่วเหลือง มีโครงสร้างคล้ายคลึงกับฮอร์โมนเอสตราไดออล มีฤทธิ์ต้านอนุมูลอิสระและต้านการอักเสบ ในการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของเจนิสทีนต่อภาวะออกซิเดทีฟสเตรส การอักเสบและการเปลี่ยนแปลงทางพยาธิสภาพของหนูไมซ์ที่เป็นโรคตับอ่อนอักเสบเฉียบพลันจากการชักนำด้วยแอล-อาร์จีนีน (L-arg) โดยหนูไมซ์เพศผู้สายพันธุ์ ICR ถูกแบ่งเป็น 4 กลุ่ม (กลุ่มละ 6 ตัว) ได้แก่ กลุ่มควบคุม (Con) สัตว์ทดลองได้รับ 2% ไดเมทิลซัลฟอกไซด์ (DMSO) โดยการฉีดเข้าทางช่องท้อง (IP) วันละ 1 ครั้ง เป็นเวลา 4 วัน กลุ่มที่เป็นโรคตับอ่อนอักเสบเฉียบพลัน (AP) ได้รับ L-arg ที่ละลายในน้ำเกลือ 0.9% ขนาด 350 มิลลิกรัม (มก)/น้ำหนักตัว 100 กรัม โดยการฉีดเข้าทางช่องท้อง 2 ครั้ง เวลาห่างกัน 1 ชั่วโมง เพื่อชักนำให้เกิดโรค กลุ่มที่ชักนำให้เกิดโรคตับอ่อนอักเสบเฉียบพลันร่วมกับได้รับเจนิสทีนขนาดต่ำ (LG) โดยหนูได้รับเจนิสทีนขนาด 10 มิลลิกรัม/กิโลกรัม (มก/กก) และกลุ่มที่ชักนำให้เกิดโรคตับอ่อนอักเสบเฉียบพลันร่วมกับได้รับเจนิสทีนขนาดสูง (HG) ได้รับเจนิสทีนขนาด 100 มก/กก 2 ชั่วโมงก่อนการชักนำให้เกิดโรคด้วย L-arg หลังจากนั้นได้รับเจนิสทีนวันละ 1 ครั้งต่ออีก 3 วัน น้ำหนักสัตว์ทดลองถูกเปรียบเทียบระหว่างจุดเริ่มต้น (Day 0) และจุดสิ้นสุดของการทดลอง (Day 4) ส่วนตับอ่อนและซีรัมของสัตว์ทดลองถูกเก็บหลังจากได้รับ L-arg 72 ชั่วโมง ผลการทดลองแสดงให้เห็นว่าเจนิสทีนทั้งขนาดต่ำและขนาดสูงสามารถลดความรุนแรงของโรคตับอ่อนอักเสบเฉียบพลันได้จากผลการทดลองที่แสดงให้เห็นว่าสามารถฟื้นฟูการลดลงของน้ำหนักสัตว์ทดลองได้ ลดระดับเอ็นไซม์ AMY ในในซีรัม ไซโตไคน์ IL-6, CPR, MPO, MDA ในตับอ่อน รวมถึงลดการบาดเจ็บของพยาธิสภาพตับอ่อน งานวิจัยนี้ช่วยเพิ่มความเข้าใจเกี่ยวกับผลของเจนิสทีนต่อภาวะออกซิเดทีฟสเตรส การอักเสบ รวมถึงพยาธิสภาพของตับอ่อนในหนูไมซ์ที่เป็นโรคตับอ่อนอักเสบจากการชักนำด้วย L-arg

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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JUMLONGLUK SRIKO: Effects of Genistein on Oxidative Stress and Inflammation In Acute Pancreatitis Mice. ADVISOR: PROF. DUANGPORN WERAWATGANON, M.D., CO-ADVISOR: ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D., 91 pp.

Genistein (GEN), the most abundant isoflavones in soy, has similar structure to human estradiol. It has been reported the potent properties of GEN on antioxidant and anti-inflammation. The aim of this study was to investigate the effects of GEN on oxidative stress, inflammatory, and histopathology in acute pancreatitis induced by L-arginine in mice. Male ICR mice were randomly divided into 4 groups (6 mice in each group). Control (Con) group: mice were injected with 2% dimethyl sulfoxide (DMSO) once daily for 4 days, via IP. Acute pancreatitis (AP) group: mice were injected 2 times (1 hour interval) of 350 mg/100 g body weight L-arginine which dissolved in 0.9% normal saline to induce acute pancreatitis. AP + low dose genistein (LG) group: mice were received 10 mg/kg GEN, while AP + high dose GEN (HG) group: mice were received 100 mg/kg GEN, dissolved in 2% DMSO administered by IP injection 2 hours before the induction of L-arginine, then daily injection of GEN for 3 days. Body weight change was compared between at the beginning and the end of study. Serum and pancreas were collected 72 hours after induction of L- arg. The result showed that, low and high doses of genistein were able to attenuate severity of acute pancreatitis which characterized by restored body weight loss, decreased serum AMY, inflammatory cytokines (IL-6, CPR, and MPO), pancreatic MDA, and decreased histological damage. In the present study increased understanding of the effects of genistein on oxidative stress, inflammation as well as pancreatic histopathology in L-arg induced acute pancreatitis mice.

Field of Study: Medical Science
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATION

AP	Acute pancreatitis
AIHA	Arginase inhibitor (+) – S- 2- amino -6 – ionoacetamidohexanoic acid
AMY	Amylase
℃	Degree Celsius
CCK	Cholecystokinin
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
ER	Estrogen receptor
GEN	Genistein
GPx	Glutathione peroxidase
GST	Glutathione S-transferases
HRT	Hormone replacement therapy
IL-1β	Interleukin 1 beta
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
L-arg	L-arginine
LDL	Low-density lipoproteins
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
MPO	Myeloperoxidase
NaOH	Sodium hydroxide
NASH	Nonalcoholic steatohepatitis

LIST OF ABBREVIATION (Continue)

NF-kB	Nuclear factor *kappa B*
NO	Nitric oxide
NOS	Nitric oxide synthase
O.D.	Optical density
ODC	Ornithine decarboxylase
pg/ml	Picrogram per milliliter
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor alpha
SAM	S - adenosylmethionine
SAMDC	S - adenosylmethionine decarboxylase
SAP	Severe acute pancreatitis
SD	Standard deviation
SPINK 1	Serine protease inhibitors
SOD	Superoxide dismutase
SSAT	Spermidine/spermine N-acetyltrasferase
STZ	Streptozotocin
TBA	Thiobarbuturic Acid
U/L	Unit/liter
µl	Microliter
µm	Micrometer

CHAPTER I INTRODUCTION

1.1 Background and Rationale

Acute pancreatitis (AP) is a sudden inflammatory disorder of the pancreas, and is a potentially life-threatening disease. The severity of acute pancreatitis ranges from mild, found in 70-80% of cases, to severe, with a high mortality rate, due to complications from pathogenesis, found in 15-25% of case (1, 2).The etiologies and pathogenesis mechanism of AP were the subject of continuous investigation for many years in the past. Various studies believe that the pathology of AP rise from an early intra-acinar cells of inactive enzymes into active, leading to autodigestive pancreas. This encouraging the synthesis and release of many pro-inflammatory cytokines and chemokines, including oxidative stress, causing local inflammation (1-3). AP is characterized by interstitial edema, acinar cells necrosis, hemorrhages, and neutrophil infiltration. Moreover, inflammatory mediators trigger its development from local to systemic inflammation, potentially resulting in multiple organ dysfunction syndrome, arising from the excess of secretion of pro-inflammatory mediators into the circulation (4, 5). Over the past three decades considerable progress has been made, but the treatment of AP remains supportive, and for the time being there are no specific treatments that can alter the course of the disease. This lack of target therapy is mainly due to our incomplete understanding of the underlying mechanism of AP (2).

Phytoestrogens are natural chemical compounds derived from plants, which have structures and functions similar with estrogens generated within the endocrine system. Genistein (4', 5, 7 - trihydroxylisoflavone) is a phytoestrogen that belongs to the category of isoflavones. The remarkable pharmacological activity of genistein is phytoestrogenic, because it has a similar structure with estradiol (6). In addition, GEN has been extensively used as an antioxidant, and acts both directly and indirectly as an antioxidant agent. Its direct antioxidant potencies are influenced by its structure, acting as a free radical scavenger due to its ability to donate hydrogen from phenolic hydroxyl groups to deleterious free radical molecules (7, 8). It can also increase the activity of antioxidant enzymes, including superoxide dismutase, glutathione reductase, and glutathione peroxidase in TPA-induced H_2O_2 formation and superoxide anion (O_2^-) generation by xanthine/xanthine oxidase in HL-60 cells and the mouse skin tumorigenesis model (9). Furthermore, several studies have shown the anti-inflammation properties of GEN. It can effectively decrease many inflammatory

mediators related with inflammation; for instance, C-reactive protein (CRP) tumor necrosis factor (TNF- α) and transforming growth factor TGF-β1 in streptozotocin (STZ) induced diabetic rats (10). In addition, reduced interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and nuclear factor kappa B (NF-kB) in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages (11, 12). Hence, the aim of this study was to determine the effects of genistein on oxidative stress and inflammatory status in AP mice.

1.2 Research question

Can genistein attenuate AP in mice models?

1.3 Research objectives

1) To determine the effect of genistein on oxidative stress in mice with acute pancreatitis

2) To determine the effect of genistein on inflammatory cytokine in acute pancreatitis in mice

3) To determine the effect of genistein on histopathology of acute pancreatitis in mice

1.4 Hypothesis

Genistein can attenuate acute pancreatitis through the inhibition of oxidative stress and inflammation.

1.5 Assumption

All animals are not different.

1.6 Key words

Acute pancreatitis, genistein, oxidative stress, inflammation, L-arginine

1.7 Research design

Animal experimental design

1.8 Expected benefit and application

The results will increase understanding of the effects of genistein on oxidative stress, inflammation as well as pancreatic histopathology in L-arg induced acute

pancreatitis mice and it will be useful for further studies and application on acute pancreatitis treatment.

CHAPTER II LITERATURE REVIEWS

2.1 PANCREAS

The pancreas is a glandular organ, and is located in the abdominal cavity behind the stomach with the head of the pancreas surrounded by the duodenum. The pancreas has two main functions; it is an endocrine gland producing several important hormones by small islands of cells, called the islets of langerhans, which play an important role in controlling glucose levels. The second function of the pancreas is producing enzymes from the exocrine gland that help with the digestion of food, assisting the breakdown of many categories of nutrients, including carbohydrates, proteins, and lipids. These exocrine cells release their enzymes into a series of progressively larger tubes (called ducts) that finally join together to form the main pancreatic duct. The main pancreatic duct runs the length of the pancreas and drains the fluid produced by the exocrine cells into the duodenum (13).

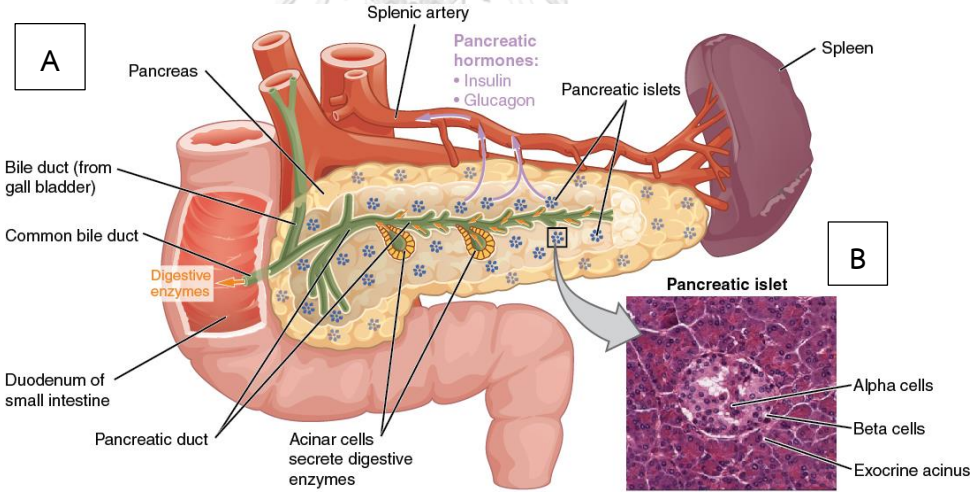


Figure 2.1 Anatomic structure of pancreas, (A) normal gross anatomy of pancreas, (B) histological slide.

2.2 ACUTE PANCREATITIS

Acute pancreatitis (AP) is an abnormal condition of the pancreas, presenting as a sudden inflammatory disorder. The etiologies and pathogenesis mechanism of AP have long been investigated. The most common causes of AP include alcohol abuse,

gallstones, and other causes, such as hypercalcemia, drugs and infections (14). The clinical diagnosis of AP is based on at least 2 of 3 criteria, including abdominal pain, usually acute, constant, and most often radiating to the back, localized to the epigastric area; serum amylase (AMY) and/or lipase rising 3 times the upper limit of normal; and characteristic findings of AP on a computed tomography (CT) scan (15).

2.2.1 PATHOLOGY OF ACUTE PANCREATITIS

In normal physiological conditions of pancreas, the main functions of the pancreas are producing enzymes and hormones. In the pancreas exocrine secretion, enzymes are produced within acinar cells, including amylolytic, lipolytic, and proteolytic enzymes, into the pancreatic duct by stimulation of the ingestion of food, the vagal nerves, vasoactive intestinal peptide, gastrin releasing peptide, secretin, cholecystokinin (CCK), and encephalins. AMY, the major amylolytic enzyme, hydrolyzes starch to oligosaccharides. The lipolytic enzymes include lipase, phospholipase A, and cholesterol esterase. Proteolytic enzymes, which include trypsin, chymotrypsin, carboxypeptidases, aminopeptidases, and elastases act on peptide bonds of proteins and polypeptides. To prevent autodigestion of the pancreas, the major protease of the pancreatic juice secretes as an inactive form precursor and is stored in vesicles known as zymogens, separate from lysosome granules. In addition, the synthesis of pancreatic secretory trypsin inhibitors and serine protease inhibitors (SPINK1) and also the acidic pH and a low calcium concentration in the zymogen granules prevent against premature activation of the proenzymes (14). These precursor enzymes reach the duodenum where trypsinogen, the proenzyme for trypsin, is activated by the brush border enzyme enterokinase. Trypsin then simplifies the conversion of the other pro-enzymes to their active form.

Various studies believe that the pathology of AP (Figure 2.2) rises from the early intra-acinar cells of inactive enzymes into active forms or the premature activation of proenzymes, leading to auto-digestion of acinar cells (1-3). The exact mechanism by which the various etiologies of pancreatitis cause this premature activation are not well understood. It appears that auto-digestion triggers a local inflammation response, and encourages the synthesis and release of many pro-inflammatory cytokine and chemokine, including oxidative stress, which causes local inflammation characterized by interstitial edema; acinar cells necrosis, hemorrhages, and neutrophil infiltration. Moreover, inflammatory mediators increase the severity of disease, triggering its development from local to systemic inflammation, resulting from

the excess of secretion of pro-inflammatory mediators into the circulation. It can lead to distal organ failure (4, 5).

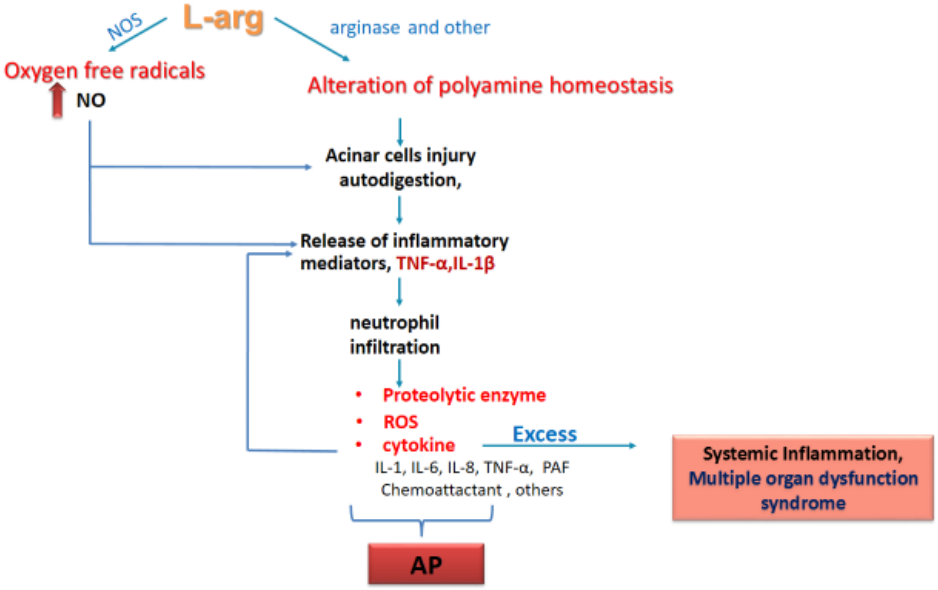


Figure 2.2 Mechanism of L-arginine induced acute pancreatitis

2.3 INFLAMMATORY MEDIATORS / CYTOKINES

Cytokines are small secreted proteins produced and released by a number of cell types, although predominantly by leukocytes. Cytokine acts as a chemical messenger, and has a specific effect on the interactions and communications between cells (16). TNF-α is a cytokine involved in the systemic inflammation, and is generally secreted by tissue damage and macrophage upon activation by a variety of stimuli (17). Local enhancing concentrations of TNF-α cause heat, swelling, redness and pain, activate the expression of new adhesion molecules, and increases the mobilization and effecter function of neutrophils and their adhesiveness to endothelial cells. Furthermore, it can enhance the production of other inflammatory mediators, such as IL-1, IL-6, IL-8, and itself, including chemokines whereby stimulation of macrophages cause amplification of the inflammatory response in AP (18). Cytokines appear to play a pivotal role related to the pathogenesis of pancreatitis. Experimental studies of TNF showed that it exhibits an early peak at two hours after the inducement of acute pancreatitis, and pre-treatment with anti-TNF factors can reduce the severity of disease. In addition, C-reactive protein (CRP), an acute phase reactant synthesized by the hepatocytes and is usually elevated in inflammatory conditions. The production of CRP is stimulated by cytokines including myeloperoxidase (MPO) in blood, considered to be a marker of neutrophil activity significantly increased in AP (19).

2.4 OXIDATIVE STRESS/ REACTIVE OXYGEN SPECIES

Oxidative stress has been defined as an imbalance between antioxidants and free radicals, with an increase in levels of free radicals or insufficient antioxidant enzymes. Free radicals are unstable chemicals, any atom or molecule that has a single unpaired electron in an outer shell, are highly reactive, and are produced in biologic systems under normal physiologic as well as pathophysiologic condition.

They are divided into reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are highly reactive and capable of extracting electrons, whereby oxidizing a variety of biomolecules vital to cell and tissue function, can interact with polyunsaturated fatty acids, and lipid peroxidation often occurs in response to oxidative stress (20). ROS are released from tissue damage and recruiting inflammatory cells, and can activate the inflammatory cascade to produce pro-inflammatory cytokine via the regulation of many signal molecules, such as nuclear factor (NF-kB), activator-protein-1 (AP-1), mitogen-activated protein kinases (MAPK), pro-inflammatory cytokine product, which in turn amplifies the inflammatory cascade (21).

2.5 BIOLOGICAL MARKER OF ACUTE PANCREATITIS

2.5.1 Amylase/ Lipase

To diagnose AP, the investigation of amylase and lipase levels are the most commonly performed laboratory tests, as they are secreted by the acinar cells of the pancreas. AMY is a glycoside hydrolase primarily produced in the pancreas (p-isoamylase) and salivary glands (s-amylase), although small amounts are also produced in other tissues. It helps to digest starch, glycogen and related polysaccharides. In acute pancreatitis, the blood levels of amylase rapidly rise within 3-6 hr. of the onset symptoms, with a half-life of 10-12 hr., and remain elevated for 3-5 days, until excreted by the kidney. Amylase levels are mostly measured by total amylase from both isoenzymes. Normal amylase levels typically range from 20-300 U/L (22) and the cut-off for diagnosis of AP is 3 times above the upper limit of normal (23). Lipase, which plays an important role in fat digestion, typically increases within 4-8 hr. of onset of pancreas, and peaks at 24 hr., while serum concentration remains elevated for 8-14 days, and normalizes in 8-14 days. The cut-off for diagnosis of AP is 4 times above the upper limit of normal. It is more useful than amylase when presentation or investigation has been delayed by more than 24 hr., and has a greater sensitivity than

amylase (24). However, both amylase and lipase can also increase from other causes (25).

2.5.2 C-Reactive Protein (CRP)

CRP is an acute phase reactant synthesized by the hepatocytes and is usually elevated in inflammatory conditions. The production is stimulated by cytokines, such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin 1beta (IL-1-beta). The demerit of CRP as marker is its delayed peak (48–72 hours) and its nonspecific nature as inflammatory marker. Before measurement of CRP, other inflammatory conditions should be ruled out. (26).

2.5.3 Myeloperoxidase (MPO)

Neutrophil sequestration in inflamed tissues is quantified by measuring the tissue myeloperoxidase (MPO) activity. MPO has been implicated in promoting tissue damage in various inflammatory diseases. MPO in blood is considered to be a marker of neutrophil activity. It is a hemoprotein that is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages. MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite and is secreted by activate neutrophils during inflammatory condition (27).

2.6 L-ARGININE INDUCED ACUTE PANCREATITIS

Because of ethical constraints, experimental studies of the pathogenesis, pathological process or experimental treatment in humans is unacceptable. Thus, experimental models on animals are necessary to advance understanding of the mechanisms involved.

There are many ways to induce AP. The experimental models of AP included invasive and non-invasive models. The invasive models such as pancreatic drug injection, where a solution (such as activate pancreatic enzyme, bile) is perfused along the pancreatic duct, in opposition to the normal flow of pancreatic juice (28). Edematous pancreatitis produces a severe form of induced pancreatitis, depending on the pressure and volume of solution (29). Duct ligation model, this method works by directly ligating the common bile duct as it enters the duodenum causing bile reflux into the pancreatic duct and inducing acute pancreatitis (30). Non-invasive models, such as cerulein-induced AP, (31-33), induced by an overdose of cerulein via intravenous or intraperitoneal (IP) injection. Cerulein is a hormone that is analogous to

cholecystokinin (CCK), which incumbency is to induce pancreatic enzyme secretion by acinar cells. The understanding about the mechanism of cerulein's ability to induce AP is unclear, but evidence shows that localization of the zymogen with contained inactive enzymes and lysozyme enzymes leads to the auto-activity of enzymes within acinar cells (34). Using L-arginine (L-arg) to induce AP is one of the few non-invasive models of AP. This method is very popular because the technique is easily implemented, inexpensive and similar in its course and histological changes to human pancreatitis (35).

L-arg is an essential amino acid, and the influence of an excessive dose of L-arg injection induced acinar cells injury was first discovered by Mizunuma, in 1984, with IP injection single dose of L-arg 500 mg/100 g BW in rats. The result showed selective damage on pancreatic acinar cells without any morphological change in other tissue (lung, heart, intestine, testis, spleen and thymus) including in islets of langerhans (36). Observation by Tani *et al*, in 1990, after IP administration of a high dose of L-arg in rats, biochemical detection. At 6 hr. showed the rise of lipase, amylase, and anionic trypsin (ogen). Light microscope observation showed the alteration of histology, with a number of vacuolization of the cytoplasms within acinar cells or acinar cell necrosis. Observation by electron microscope showed abnormal mitochondria that were swollen, round, and fused to each other, as well as broken cristae, and rough endoplasmic reticulum showing unusual characteristics, with loss of ribosomes and dilated membranes. At 72 hr., pancreatic damage reached its maximum (70-80%), unable to regenerate acinar cells and replaced by adipose tissue, including inflammatory cells infiltrate into the interstitium (35).

Other effective single doses, 450, 400, 300, 250 mg/ 100 g BW, or double doses (2 x 250, 2 x200 mg/100 g body weight) have been reviewed by Hegyi et al., and were found to also induce AP. In addition, doses less than 200 mg/ 100g were unlikely to produce the disease characteristics, and more than 500 mg in one dose killed animals (37). Metabolism of L- arg was shown in Figure 2.3. There are two key enzymes that associated with L-arg catabolism in different pathways. L-arg is catalyzed into nitric oxide (NO) and L-citrulline by nitric oxide synthase (NOS), whereas, is hydrolyzed into L-ornithine and urea by arginase. Polyamine, putrescine, spermidine, and spermine are direct downstream metabolite of L-ornithine, which are regulated by the activity of ornithine and S - adenosylmethionine decarboxylase (ODC and SAMDC respectively), whereas polyamine catabolism is controlled by rate-limiting spermidine/spermine N-acetyltrasferase (SSAT) (38). Although the induction of L-arg induced AP is greatly

appreciated, understanding of the extraction mechanism of L-arg induced AP is not clear.

There is some evidence to support the presumable involvement of L-arg induced AP with nitrosative and oxidative stress, which are direct metabolites of L-arg (39, 40). Various studies demonstrate that the effect of many anti-oxidant (reduce reactive oxygen/nitrogen species) drugs ameliorate the severity of L-arg induced AP, although also anti-inflammatory properties in these drugs, such as lowsone (41), melatonin (42), trimetazidine (43), syringic acid (44) and oxymatrine (45).

Alteration of polyamine homeostasis and activated polyamine catabolism was assumed to be associated with the incidence of AP. The amine putrescine, spermidine, and spermine have an important functions to stabilize ribosomes and nucleic acids and affect nucleic acid synthesis (46). The pancreas contains the highest concentration of spermidine in the mammalian body. In a study by Rkonczay Jr Z *et al.* (2008), rats injected with 3.5 g/kg L-arg resulted in much greater increases in serum ornithine vs citrulline concentration. This demonstrated that a large dose of L-arg is mainly metabolized via arginase rather than by NOS (40). The pancreas is rather sensitive to acute declines in polyamine levels, as indicated by the activation of polyamine catabolism in transgenic rats overexpressing spermidine/ spermine *N*1-acetyltransferase, the key enzyme in polyamine catabolism caused polyamine depletion, spermidine and spermine resulted in severe acute pancreatitis (SAP) (47-49) and treatment with methylated polyamine analogues after the induction of pancreatitis showed ameliorate severity of pancreatic damage (50). In addition, the inhibition of arginase activity by arginase inhibitor (+) – S- 2- amino -6 - ionoacetamidohexanoic acid (AIHA) showed accelerated severity of AP. The above indicated that L-arg induced AP is likely involved in the alteration of polyamine homeostasis. Because protein metabolism is most active in pancreatic acinar cells, it is likely that acinar cells are the first target of L-arg over dose, resulting in degeneration or necrosis. However, role of polyamine homeostasis process to the integrity of the pancreas is still unknown, and requires further study (51).

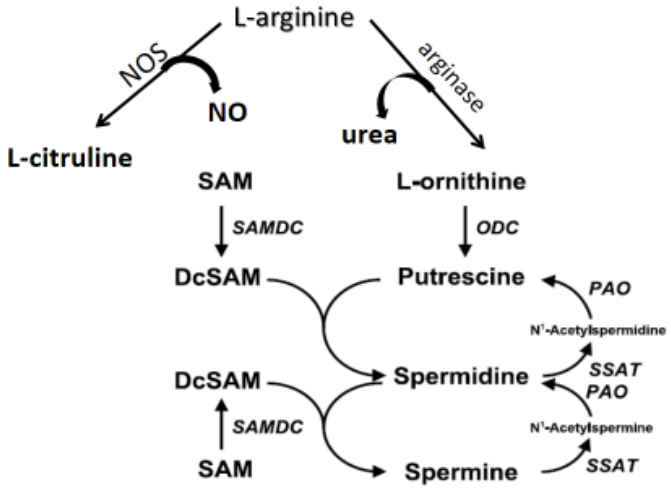


Figure 2.3 Key enzymes involve in the metabolism of L-arginine. Nitric oxide synthase catalyzes the conversion of L-arginine to nitric oxide and L-citrulline. Arginase hydrolyzes L-arginine to L-ornithine and urea. Late-limiting enzymes of polyamine metabolism. Ornithine decarboxylase (ODC) and S-adenosylmethionine (SAM) decarboxylase (SAMDC) mediate polyamine biosynthesis, whereas polyamine catabolism is controlled by spermidine/spermine N1-acetyltransferase (SSAT). DcSAM indicates decarboxylated SAM; PAO, polyamine oxidase (51).

2.7 GENISTEIN

2.7.1 Genistein as a Phytoestrogen

Phytoestrogens are natural chemical compounds derived from plants, with similar structure and function as estrogens generated within the endocrine system (52). The three major classes of phytoestrogens are coumestans, prenylflavonoids and isoflavones (53). Of the three, isoflavones are widely distributed in the plant kingdom, and the main source of isoflavones are legumes, especially soybeans (54). Isoflavones have also been identified as the most prevalent phytoestrogens and have the best-known effect on animal and human health. Genistein, 4', 5, 7- trihydroxylisoflavone, is a phytoestrogen, belongs to the category of isoflavones, is biosynthetically the simplest of the isoflavonoid compounds, and has the ability to act as a central intermediary to produce complex isoflavones (figure 2.4) . One remarkable pharmacological activity of genistein is phytoestrogenic, because there are similarities of structure between genistein and estradiol (Figure 2.5), distance between 4, and 7 – hydroxy group (11.5 A) and diphenolic ring, resulting in genistein's ability to interact with estrogen receptors and sex hormone binding proteins in animal and human bodies (6). Estrogen receptors (ER) have 2 type of receptors, alpha and beta ER. The estrogenic

activity of genistein has shown both weak estrogenic and weak anti-estrogenic effects, depending on affinity to ER. Normally genistein affinity shows about 100 folds weaker than that of estradiol, and prefers binding in beta ER rather than alpha ER (55). Treatment with genistein in menopausal women demonstrated that it could increase bone density and significantly reduce hot flashes (56).

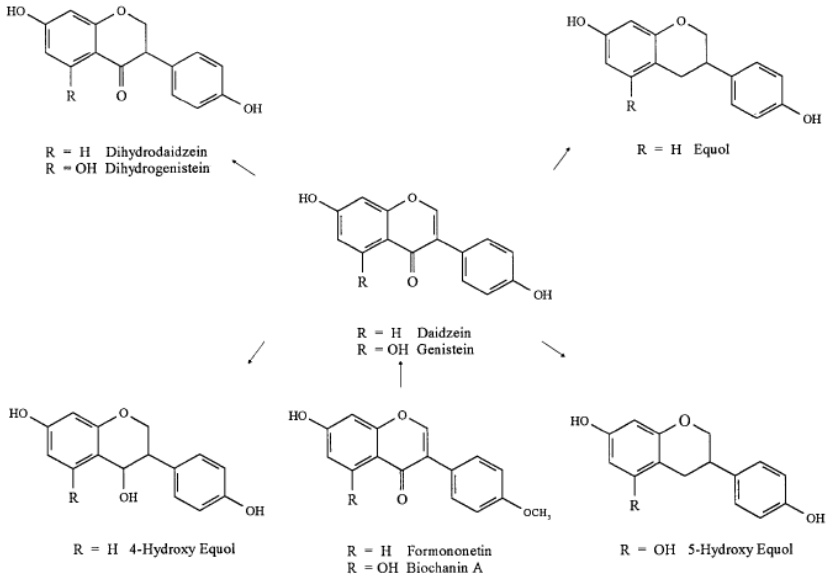
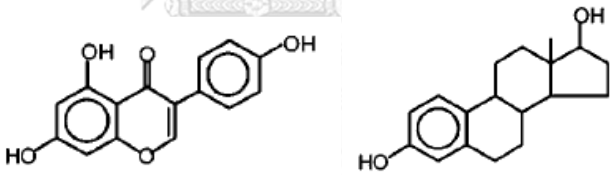


Figure 2.4 Structures of the isoflavone metabolites (8)



Genistein estradiol

Figure 2.5 Structural similarity between genistein and estrogen (6)

2.7.2 Genistein as an Antioxidant

Genistein has been extensively used as an antioxidant and shown to act as both directly and indirectly antioxidant. Its directly antioxidant potencies were influence by its structure, this molecule acts as a free radical scavenger because its ability to donate hydrogen from phenolic hydroxyl groups to deleterious free radical molecules. With all hydroxyl groups of genistein structure at C-4,5,7 positions, C-4 showed most effective antioxidant (8, 57). Antioxidant activity of genistein exerts inhibition of lipid peroxidation in liposome system, and can suppress both metal-ion induced peroxidation and peroxyl-radical-induced peroxidation (8). In Kapiotis., *et al*, genistein administration inhibited the oxidation of low-density lipoproteins (LDL) triggered by copper ions or superoxide/nitric oxide radicals as well as *in vivo* study (58).

It can also protect endothelial cells damaged via inhibiting the oxidation of LDL. It provides the antioxidant function both cell-frees system, intact cells and mammalian organisms. In addition, its potency can induce increasing in the activity of antioxidant enzymes, including superoxide dismutase (SOD), glutathione *S*-transferase (GST), and glutathione peroxidase (GPx) (9).

2.7.3 Genistein as an Anti-inflammation

The anti-inflammation properties of genistein have been shown by several studies. The anti-inflammation effects of genistein, as shown in the *In vitro* study lipopolysaccharide (LPS)-stimulated BV2 microglia, genistein inhibited the increase of released and expression of inflammatory cytokine, including IL-1β and TNF-α, prostaglandin E2, cyclooxygenase-2 and was able to suppress the production of nitric oxide (NO) by inhibiting inducible NO synthase (iNOS) (59). Experiment LPS- treated RAW 264.7 macrophages. Y.C. Liang *et al*. (1999), demonstrated that the anti-inflammation activity of genistein inhibited NO and PGE2 production by modulating cyclooxygenase-2 (COX-2) and iNOS expression (60) as well as decreasing the production of TNF-α and IL-6, and nuclear factor NF-kB. In addition, this compound administration has a significant anti-inflammatory on high-fat diet induce nonalcoholic steatohepatitis (NASH) rats (61). Furthermore, Suresh K. *et al*. study showed that genistein has the ability to protect streptozotocin (STZ) -induced diabetic cardiomyopathy in rats, by reduced inflammation, decreased expression of CRP, TNF-α and a slight increased antioxidants enzyme (10).

CHAPTER III MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Male ICR mice weighing about 30-40 grams were purchased from the Nation Laboratory Animal Center, Salaya Campus, Mahidol University, Nakhon Pathom, Thailand. L-arginine (L-arg) and genistein were purchased from Sigma Aldrich Co (Pvt Ltd, USA). Double dose of 350 mg/ 100 g BW of L-arginine was administered to mice by intraperitoneal (IP) injection, an interval of 1 hr., to induced AP. Genistein 10 and 100 mg/kg was administered to mice by IP injection, an interval of 1 time per day. The experimental protocol was approved by Ethical Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The mice were acclimatized at least 1 week in a controlled temperature room at 25 ± 1 ºC with 12:12 hour light-dark cycle and were fed *ad libitum*.

3.1.2 Chemicals preparation

L-arginine preparation

L-arginine (L- arg), powder (Sigma Aldrich Co Pvt Ltd, USA) was dissolved in 0.9% saline and the *p*H was adjusted to 7 with 5*N* HCl and was administered in a dose of 350 mg/100g BW, two IP injection with an interval of 1 hr.

Genistein preparation

Genistein (Cayman Chemical Company, USA) was dissolved in 2% dimethyl sulfoxide (DMSO) that was freshly prepared for the experiment. Genistein 10 and 100 mg/kg were administered to mice by IP injection, an interval of 1 time per day.

Serum amylase reagent and equipment (R&D system, Inc., USA)

Reagents

- **Test strips**
- **Reflotron pipetted**
- **Reflotron pipetted tips**
- **0.9 % NaCl**
- **Components per test:**

  Indolyl-α, D-maltoheptaoside 81 µg: α- glucosidase (yeast rec.) ≥ 3.1 U;

2-methoxyl-4-morpholinophenyldiazoniumtetrachlorozincate 6.8 µg; buffer.

IL-6 reagent and equipments (R&D system, Inc., USA)

- **Mouse IL-6 Microplate**: 96 well polystyrene microplates (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse IL-6.
- **Mouse IL-6 Standard:** Recombinant mouse IL-6 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*
- **Mouse IL-6 Control:** Recombinant mouse IL-6 in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.
- **Mouse IL-6 Conjugate:** 23 mL/vial of a polyclonal antibody against mouse IL-6 conjugated to horseradish peroxidase with preservatives.
- **Assay Diluent RD1-14:** 12 mL/vial of a buffered protein solution with preservatives. *Contains a precipitate. Mix well before and during use.*
- **Calibrator Diluent RD5T:** 21 mL/vial of a buffered protein solution with preservatives.
- **Wash Buffer Concentrate:** 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*
- **Color Reagent A:** 12 mL/vial of stabilized hydrogen peroxide.
- **Color Reagent B:** 12 mL/vial of stabilized chromogen (tetramethylbenzidine).
- **Stop Solution:** 23 mL/vial of diluted hydrochloric acid.
- **Plate Sealers:** Adhesive strips.

CRP reagent and equipment (R&D system, Inc., USA)

- **Mouse CRP Microplate:** 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse CRP.
- **Mouse CRP Standard:** 2 vials of recombinant mouse CRP in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*

- **Mouse CRP Control:** 2 vials of recombinant mouse CRP in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.
- **Mouse CRP Conjugate:** 12 mL of a polyclonal antibody specific for mouse CRP conjugated to horseradish peroxidase with preservatives
- **Assay Diluent RD1W:** 11 mL of a buffered protein solution with preservatives.
- **Calibrator Diluent RD5P Concentrate:** 21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*
- **Wash Buffer Concentrate:** 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*
- **Color Reagent A:** 12 mL of stabilized hydrogen peroxide.
- **Color Reagent B:** 12 mL of stabilized chromogen (tetramethylbenzidine).
- **Stop Solution:** 23 mL of diluted hydrochloric acid.
- **Plate Sealers:** 4 adhesive strips.

Pancreatic malondialdehyde (MDA) assay Thiobarbuturic Acid (Cayman Chemical Company, USA)

- **TBA Acetic Acid-** (Item No. 10009200)
- **TBA sodium hydroxide (10x)** – (Item No. 10009201)
- **TBA malondialdehyde Standard**-(item No. 10009202)
- **TBA SDS Solution**-(item No. 10009203)
- **9-well Solid Plate**
- **96-well Cover Sheet**

3.2 EXPERIMENTAL PROTOCOLS

Male ICR mice were divided into 4 groups (6 each).

1. **Control group (Con group):** mice were received IP injection of 2 % DMSO once daily for 4 days.

2. **Acute pancreatitis group (AP group):** mice were received two IP injection of 350 mg/ 100 g body weight of L-arg dissolved in 0.9% normal saline, pH 7.0, at an interval of 1 hr. to induce acute pancreatitis.
3. **AP + low dose genistein group (LG group):** mice were received 10 mg/kg genistein in DMSO administered by IP injection 2 hr. before induced with L-arg then once daily for 3 days.
4. **AP + high dose genistein group (HG group):** mice were received 100 mg/ kg genistein in DMSO administered by IP injection 2 hr. before induced with L- arg then once daily for 3 days.

The body weight change (gram) were compared between beginning and the end of the study in each group. At the end of the study, all mice were sacrificed 72 hr. after induced with L- arg by using IP injection of an overdose thiopental sodium (>50 mg / kg body weight). The abdominal wall was opened and whole pancreas were rapidly removed and washed with cold normal saline. Pancreas was collected and divided into 2 pieces, the first one was frozen in lipid nitrogen and stored at -80 °C for MDA measurement. The remaining pancreas was fixed in a 10% formalin solution for histological and immunohistochemistry examination for MPO. Subsequently, blood samples were drawn by cardiac puncture and allowed to clot by being kept at room temperature for 2 hr.; afterwards, it was centrifuged at 1000 x g for 20 min, after which serum were collected for AMY enzyme, IL-6, and CRP testing.

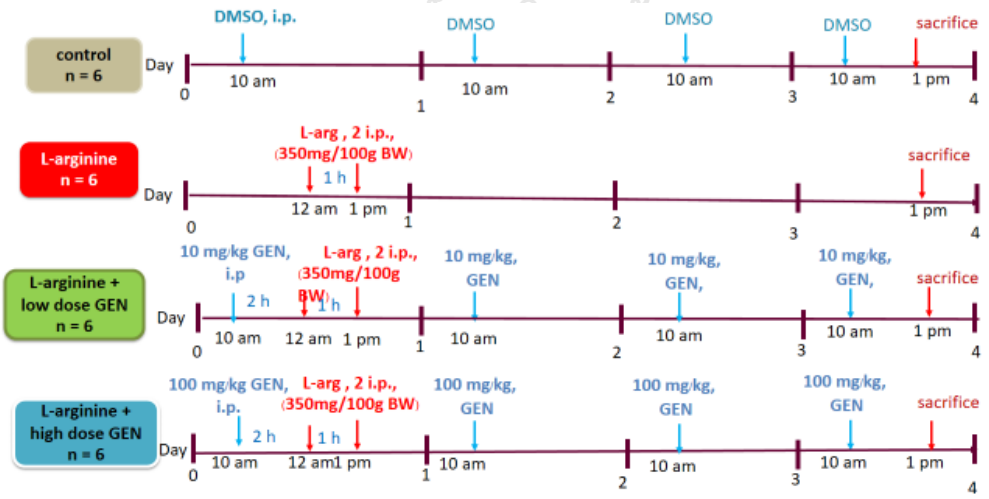


Figure 3.1 Experimental design

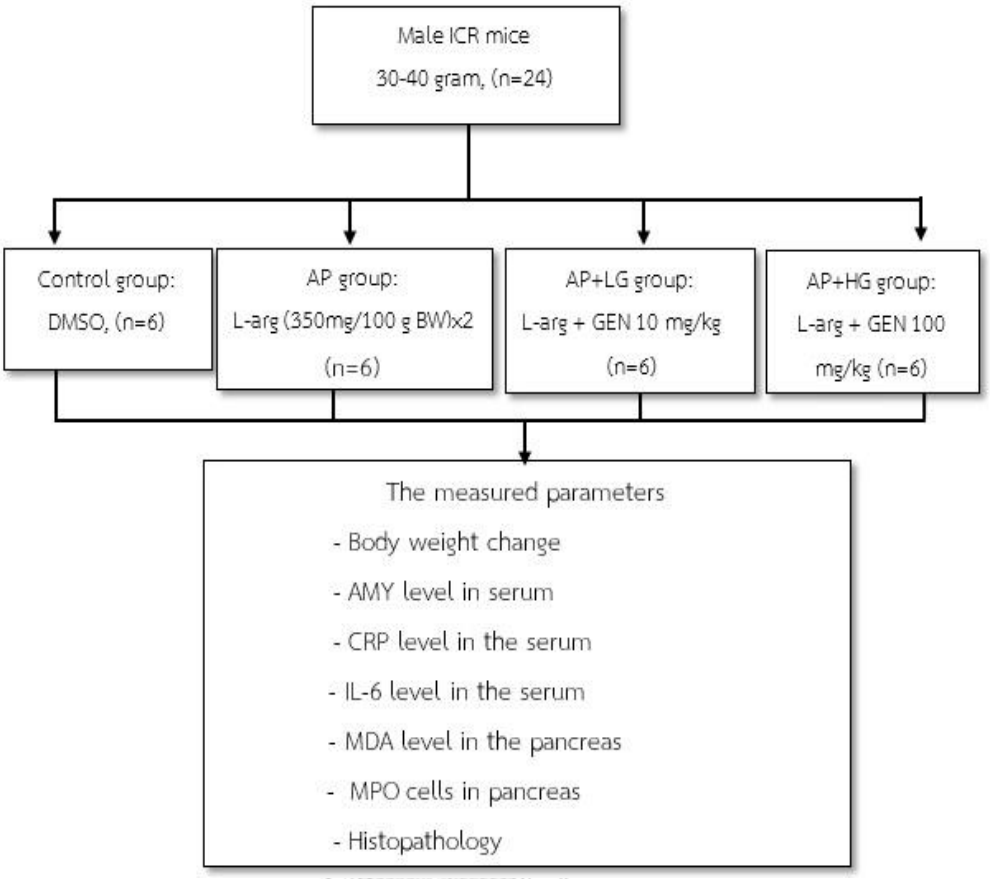


Figure 3.2 Schematic diagram of experimental

3.3 DATA COLLECTION

3.3.1. Body weight change

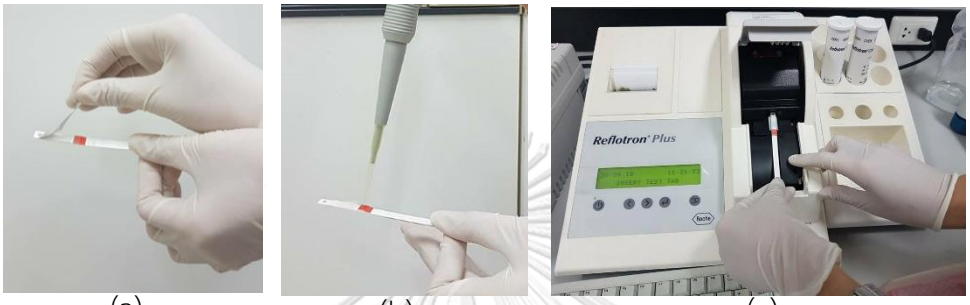
The body weight of animals was recorded. Levels of body weight change (gram) were calculated by BW at the end of study minus BW at the beginning.

3.3.2 Serum amylase (AMY) assay

Assay

- Remove a test strip from container. Tightly recap the container immediately after removing a test strip.
- Peel off the aluminium protective foil, taking care not to bend the test strip (a).
- All Reflotron tests require a sample volume of 30 µL.
- Apply the required volume of sample onto the centre of the red application zone using a pipette (e.g. Reflotron pipette) – being careful not to touch the application zone. Avoid air-bubble (b).

- Open the flap or sliding cover. Within 15 seconds of applying the sample, place the test strip onto the guide, and slide it forward horizontally until it locks into place. Close the sliding cover or flap (c).
- The test parameter abbreviation is shown on the display, if the test strip has been correctly inserted and the magnetic code has been read. The result is displayed depending on the setting of the instrument.



(a) (b) (c)

Figure 3.3 Construction of serum amylase assay

Principle of the assay: After application of test strip, the sample flows into reaction zone, where, in the case of blood sample, the separation of the erythrocytes from the plasma occurs.

The **α**-amylase contain in the sample and the enzyme **α**-glucosidase contained in the test cleave the substrate indolyl-**α**, D-maltohepataoside yielding indoxyl and glucose. The indoxyl is coupled with diazonium derivative to form a purple dye. The amount of dry formed per unit of time is directly proportional to the **α**-amylase activity.

Indolyl-**α**, D-maltoheptaoside $\xrightarrow{\alpha\text{ -amylase}}$ indoxyl + glucose

indoxyl + 2 methoxyl-4-morpholinophenyl-diazoniumtetrachlorozincate $\xrightarrow{\alpha\text{- glucosidase}}$ purple dye

Figure 3.4 Complex of product, indoxyl + glucose, with A-amylase and **α**- glucosidase

The enzyme activity is measured kinetically at a wavelength of 567 nm and 37°C, and is displayed after about 170 seconds in U/L.

Calculation

The amylase activity is calculated automatically from the measurements taken, as well as function and conversion factors read from the magnetic strip on the lower

face of each test strip. The enzyme activity is shown for 25°C, 30°C or 37°C in U/L or µkat/L, depending on the way the instrument has been set.

Measuring range

29-860 U/L.

If the α-amylase activity measured is above the measuring range for the Reflotron Amylase assay (indicated by an asterisk next to the displayed value or the message DILITE AMY), the sample may be diluted 1+2 with physiological saline solution. Multiply the result by a factor of 3.

3.3.3 Serum IL-6 assay

Serum IL-6 assay was determined using a commercially available mouse IL-6 assay kit (R&D system, Inc., USA)

Principle of the assay: This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse IL-6 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse IL-6 bound in the initial step. The sample values are then read off the standard curve.

Procedure: Reagent preparation

1. Bring all reagents to room temperature before use.
2. Mouse IL-6 control - Reconstitute the control with 1.0 mL deionized or distilled water. Assay the control undiluted.
3. Wash buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.
4. To prepare enough wash buffer for one plate, add 20 mL of wash buffer concentrate into deionized or distilled water to prepare 500 mL of wash buffer.
5. Substrate solution - Color reagents A and B should be mixed together in equal volumes within 15 min of use. 100 µL of the resultant mixture is required per well.

6. Mouse IL-6 standard - refer to the vial label for reconstitution volume.
7. Reconstitute the mouse IL-6 standard with calibrator diluent RD5T. Do not substitute other diluents. This reconstitution produces a stock solution of 500 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 min with gentle agitation prior to making dilutions.
8. Pipette 200 µL of calibrator diluent RD5T into each tube. Use the stock solution to produce dilution series (below). Mix each tube thoroughly before the next transfer. The mouse IL-6 standard (500 pg/mL) serves as the high standard. Calibrator diluent RD5T serves as the zero standard (0 pg/mL).

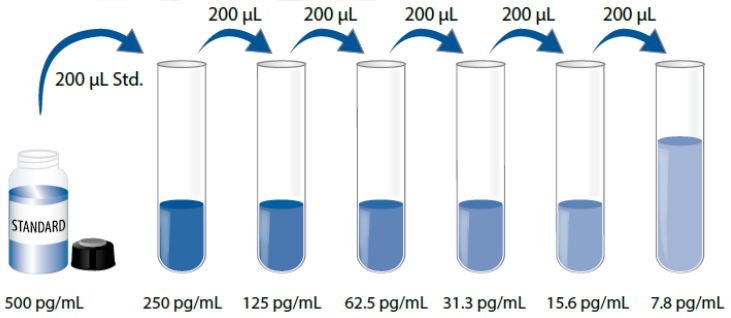


Figure 3.5 Using the stock solution to produce a dilution series

Assay procedure

1. Bring all reagents and samples to room temperature before use.

2. Prepare reagents, samples, and standard dilutions as directed by the previous sections.

3. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

4. Add 50 µL of Assay Diluent RD1-14 to the center of each well. RD1-14 contains undissolved material. Mix well before and during its use.

5. Add 50 µL of Standard, Control, or sample to the center of each well. Cover with the adhesive strip provided. Mix by gently tapping the plate frame for 1 minute. Incubate for 2 hr. at room temperature. A plate layout is provided as a record of samples and standards assayed.

6. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

7. Add 100 μL of Mouse IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hr. at room temperature.

8. Repeat the aspiration/wash as in step 5.

9. Add 100 μL of Substrate Solution to each well. Incubate for 30 min at room temperature. Protect from light.

10. Add 100 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

11. Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm.

Calculation of results

- Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

- Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

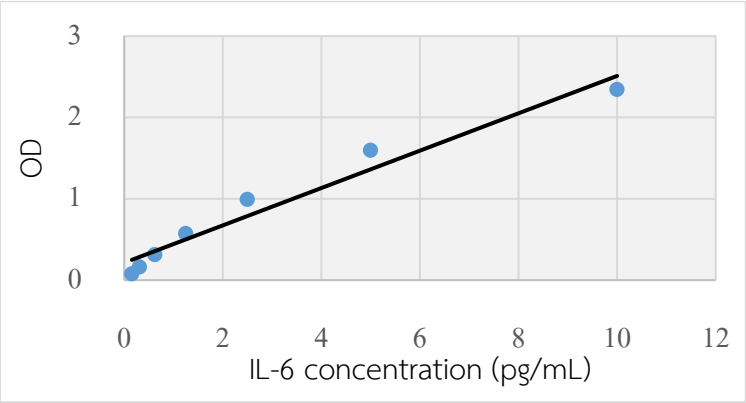


Figure 3.6 Example of standard IL-6 curve: plot of corrected absorbance at 450 nm versus IL-6 concentration

$$[\text{IL-6}] = \left[\frac{(\text{Absorbance at 450}) - (\text{Y-intercept})}{\text{Slope}}\right] \times \text{Dilution factor}$$

*If sample have been diluted, the concentration read form the standard curve must be multiple by dilution factor.

3.3.4 C-Reactive Protein (CRP) assay

Serum CRP was determined using a commercially available mouse C-reactive protein assay kit (R&D system, Inc., USA)

Principle of the assay: This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse CRP has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any CRP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse CRP is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of CRP bound in the initial step. The sample values are then read off the standard curve.

Reagent preparation

1. Bring all reagents to room temperature before use.
2. Mouse CRP control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.
3. Wash buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add

20 mL of Wash Buffer concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

4. Substrate solution - Color reagents A and B should be mixed together in equal volumes within 15 min of use. Protect from light. 100 µL of the resultant mixture is required per well.
5. Calibrator diluent RD5P (diluted 1:5) - Add 20 mL of calibrator diluent RD5P concentrate to 80 mL of deionized or distilled water to prepare 100 mL of calibrator diluent RD5P (diluted 1:5).
6. Mouse CRP standard - Refer to the vial label for reconstitution volume. Reconstitute the mouse CRP standard with calibrator diluent RD5P (diluted 1:5). This reconstitution produces a stock solution of 10 ng/mL. Allow the stock solution to sit for a minimum of 5 min with gentle mixing prior to making dilutions. Use polypropylene tubes.
7. Pipette 200 µL of calibrator diluent RD5P (diluted 1:5) into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted mouse CRP standard (10 ng/mL) serves as the high standard. Calibrator diluent RD5P (diluted 1:5) serves as the zero standard (0 ng/mL).

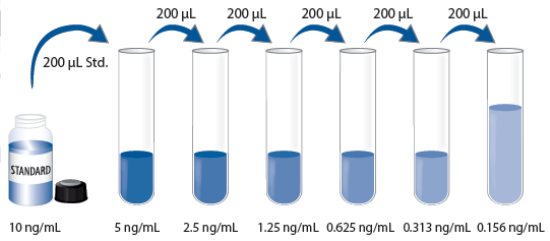


Figure 3.7 Using the stock solution to produce a dilution series

Assay procedure

1. Bring all reagents and samples to room temperature before use. It is recommended that all standards, control, and samples be assayed in duplicate.
2. Prepare all reagents, standard dilutions, control, and samples as directed in the previous sections.
3. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

4. Add 50 μL of assay diluent RD1W to each well.
5. Add 50 μL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hr. at room temperature. A plate layout is provided to record standards and samples assayed.
6. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with wash buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. Add 100 μL of mouse CRP conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hr. at room temperature.
8. Repeat the aspiration/wash as in step 5.
9. Add 100 μL of substrate solution to each well. Incubate for 30 min at room temperature on the bench top. Protect from light.
10. Add 100 μL of stop solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm.

Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse CRP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

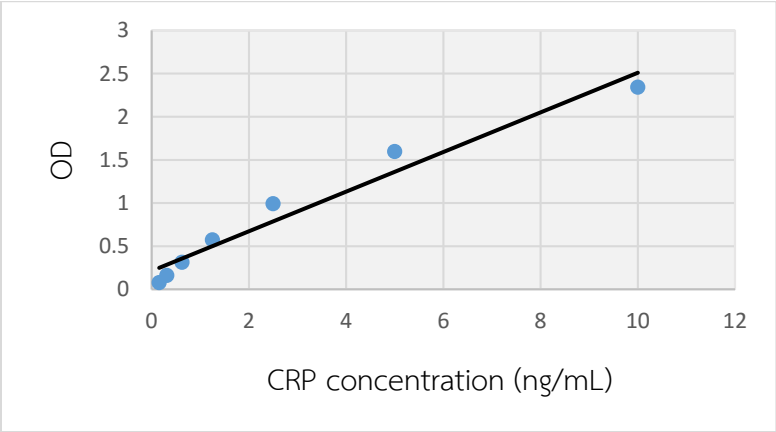


Figure 3.8 Example of standard CRP curve: plot of corrected absorbance at 450 nm versus CRP

$$[CRP] = \frac{[(\text{Absorbance at 570}) - (\text{Y-intercept})]}{\text{Slope}} \times \text{Dilution factor}$$

*If sample have been diluted, the concentration read form the standard curve must be multiple by dilution factor.

3.3.5. Pancreatic malondialdehyde (MDA) assay

MDA level were measured from the homogenized tissue using a commercial assay kit (Cayman Chemical Company, USA).

Principle MDA assay

MDA is a naturally occurring product of lipid peroxidation, is used as an indicator of oxidative stress in cell and tissue. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100 °c) and acidic condition is measured colorimetrically at 530-540 nm.

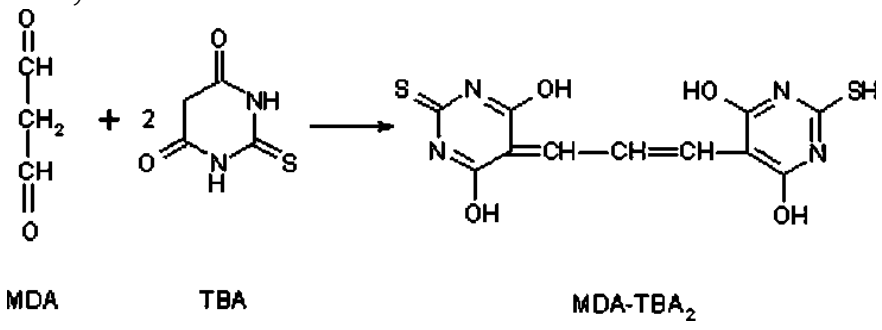


Figure 3.9 Reaction of MDA with TBA

Reagent Preparation

1. **Thiobarbuturic Acid (Item No. 10009199)**

   The vial contained 2 g Thiobarbuturic Acid (TBA). It is ready to use to prepare the color reagent.

2. **TBA Acetic Acid- (Item No. 10009200)**

   Each vial contains 20 ml concentrated acetic acid. Slowly add both vials (40 ml) of TBA Acetic Acid to 160 of HPLC-grad water. This diluted Acetic Acid Solution is use in preparing the color Reagent**.** The diluted Acetic acid solution is stable for least three months at room temperature.

3. **TBA sodium hydroxide (10x) – (Item No. 10009201)**

   The vial contained a solution of sodium hydroxide (NaOH). Dilute 20 ml of TBA NaOH with 180 ml of HPLC-grade water. This diluted NaOH solution is used in preparing the color reagent. The diluted NaOH solution is stable for least three months at room temperature. Store the diluent NaOH solution in plastic container suitable for corrosive materials.

4. **TBA malondialdehyde Standard-(item No. 10009202)**

   The vial contains 500 µM malondialdehyde (MDA) in water. It is ready to use to prepare the standard curve.

5. **TBA SDS Solution-(item No. 10009203)**

   The vial contains a solution of sodium dodecyl sulfate (SDS) The solution is ready to use as supplied.

6. **To prepare the Color Reagent**:

   The following amount of amount of color reagent is sufficient to evaluate 24 sample. Adjust the volume accordingly if more or less samples are going to be assayed. Weight 530 mg of TBA (Item No. 10009199) and add to ≥ 150 ml beaker containing 50 ml of diluted TBA acetic acid solution. Add 50 ml of diluted TBA sodium hydroxide and mix until the TBA is completely dissolved. The solution is stable for 24 hr.

Assay procedure

Tissue homogenate

1. Weight out of pancreatic tissue approximately 25 mg into a 1.5 ml centrifuge tube.
2. RIPA Buffer 250 µM was added in (prepare from Item No. 10010236) containing
3. protease inhibitor of choice.
4. The tissue was homogenized or sonicated on ice.
5. The tube was centrifuged at 1,600 x g for 10 min at 4°c, supernatant was used for analysis.

Performing the assay

1. Label vial caps with standard number or sample identification number.
2. Standard preparation

Dilute 250 µM of the MDA standard (Item Mo. 10009202) with 750 µl of water to obtain a stock solution of 125 µM. Take eight clean glass test tubes and label them A-H. Add the amount of 125 µM MDA stock solution and water to each tube as described in the **Table 3.1**

Tube	MDA (µl)	Water (µl)	MDA concentration (µl)
A	0	1,000	0
B	5	995	0.626
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

Table 3.1 MDA colorimetric standards

Assay procedure

1. Label vial caps with standard number or sample identification number.
2. Add 100 µl of sample or standard to appropriately labeled 5 ml vial.
3. Add 100 µl of SDS solution to vial and swirl to mix.
4. Add 4 ml of the color reagent forcefully down side of each vial.

5. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.
6. Add vials to vigorously boiling water. Boil vials for one hours.
7. After 1 hr., immediately remove the vials and place in ice bath to stop
8. Reaction. Incubate on ice 10 min.
9. After 10 min, centrifuge the vials for 10 min at 1,600 x g at 4 °C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.
10. Vials are stable at room temperature for 30 min.
11. Load 150 µl (in duplicate) from each vial to either the clear plate.
12. Read the absorbance at 530-540 nm.

Calculation of results

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of standard A (100 µl) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of MDA concentration (see Table 3.1, on page 30).
4. Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown on page 31 in figure 3.10.

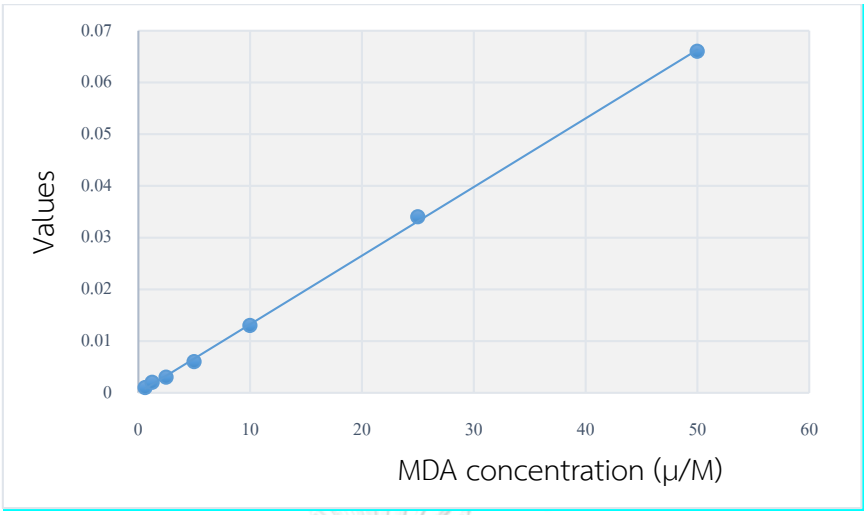


Figure 3.10 Example standard MDA curve: plot of corrected absorbance at 532 nm versus MDA concentration

$$\text{MDA } (\mu M) = \frac{[(\text{Corrected absorbance}) - (\text{y-intercept})]}{\text{Slope}} \times \text{Dilution factor}$$

*If sample have been diluted, the concentration read form the standard curve must be multiple by dilution factor.

3.3.6 Histopathological examination

Preparation for pancreas pathology

1. Freshly pancreas tissues were fixed in 10% paraformaldehyde for 24-48 hr. at room temperature.

2. Pancreas tissues were dehydrated through 70%, 80%, 95% alcohol, 5 min each, followed with 3 times of 100% alcohol, 5 min each.

3. Tissues were immersed with xylene for 2 times, 5 min each, followed with paraffin for 3 times, 5 min each.

4. Tissues were embedded in a paraffin block (The paraffin tissue block can be stored at room temperature for years).

5. Pancreas blocks were cut into 5 µm on a microtome and float in a 40°C water bath containing distilled water and dry the tissue section at 45 °C.

6. Tissue sections were stained with hematoxylin for 10 min, washing in running tap water before double staining with Eosin (E) for 1.5-2 minutes, washing, and dehydration by dipping the slide in graded series of ethanol (low-high%).

7. Slides were immersed in xylene for 3-5 time, and covered slide with coverslip glass by mounting media.

8. An experienced pathologist were evaluated all samples while being blinded to the experiment. All fields in each section were examined for grading of neutrophil infiltration, edema, and necrosis. According to the criteria described by H. E. V. DE COCK *et al*. in 2007 (62).

Score	Inflammation	Edema and Fat Necrosis
0	No neutrophils present	Not present
1	Mild neutrophilic inflammatory infiltrate affecting maximum 25% of the pancreatic parenchyma	Mild, <25% of the parenchyma involved
2	Moderate neutrophilic inflammation affecting 25–50% of the parenchyma	Moderate, present in 25–50% of the parenchyma
3	Severe neutrophilic inflammation affecting >50% of the pancreatic parenchyma	Severe, >50% of the parenchyma involved

Total score 0 = Normal, 1-3 = Mild, 4-6 = Moderate, 7-9 = Severe

3.3.7 Myeloperoxidase cells infiltration assay

The tissue microarrays sections in combination with immunohistochemistry were used for myeloperoxidase cells infiltration study.

Preparation of pancreatic tissue microarrays and immunostaining protocol

Preparation of pancreatic tissue microarrays (step 1-6)

1. Pancreas relevant areas were marked on the hematoxylin and eosin stained section slides, corresponding to the pancreas issues embed in a paraffin block.
2. Pancreas tissues embed in a paraffin block from histopathological examination steps were used to be donor block.
3. A blank recipient block (paraffin block) with a size of 45 × 20 × 15 mm was prepared, and drilled the holing needle, core size 2.0 mm.
4. Working place on the donor block were punched by the sampling needle and retrieved. When the sampling needle moves precisely above the recipient hole, the core needle is exactly pressed to squeeze out the tissue core and then inserted it into the recipient hole, 6-8 chips/mouse from donor block were inserted into the recipient block by repeating this process.
5. After insertion of all the samples, the recipient blocks were heated in an oven at 58-65 ℃ for 1 hr., to melt together the paraffin of the tissue chip and recipient block and the tissue core was flattened by a glass slide and then cooled down.

6. Recipient blocks were cut into 5 µm on a microtome and float in a 40°C water bath containing distilled water and dry the tissue section at 60 °C slides at room temperature until ready for use in step of immunostaining. (63, 64)

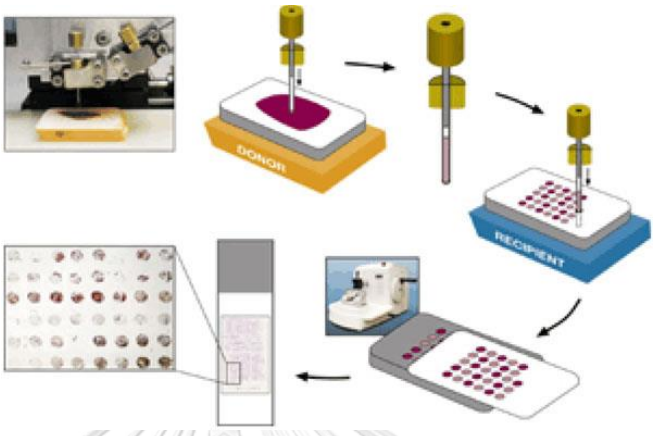


Figure 3.11 Construction of tissue microarray

Immunostaining protocol (Step 7-15):

7. Tissue microarray sections were immersed in xylene for 2 times for 5 min each, to deparaffinize.

8. Tissue section were transferred to 100% alcohol, for 2 times, 3 min each, and then once through 95%, 70% and 50% alcohols respectively for 3 min each.

9. Tissues were covered with 3% H_2O_2 solution in methanol at room temperature for 25 min on the dark to block endogenous peroxidase, and then washed with PBS for 2 times, 5 min each.

10. Tissue section were covered with antibody against MPO (dilution 1:500; Dako, Denmark) 90 min at room temperature, and then washed with PBS for 2 times, 5 min each.

11. Tissues were covered with anti-goat secondary antibody (dilution 1:1000; Dako, Denmark) and incubate for 30 min at room temperature, and then washed with PBS for 2 times, 5 min each.

12. Tissue were incubated with DAB substrate sections (freshly prepared, 0.05% DAB - 0.015% H_2O_2 in PBS) for < 5 min until suitable staining develops, and then rinsed sections with water.

13. Tissue section were counterstained with hematoxylin for 2 min, and then rinse the slides.

14. Stained slides were immersed in xylene for 3-5 times and covered glass with mounting media.

15. Myeloperoxidase cells infiltrating into pancreas were conducted by quantitation of MPO positive cell in 10 randomly high power fields (x400) (65).

Calculation of results

All slides were observed by two observers who was not aware of the sample identity. The number of myeloperoxidase positive cells in each section were calculated by counting the number of positive stained cells in 10 fields /6 cores/mouse of TMA slides at a magnification of x 400. Values present means ± SD of result from the experiment (66).

3.4 Statistic analysis

All data were presented as mean and standard deviation (SD). For comparison among groups of animals, one-way analysis of variance (one-way ANOVA) and LSD's post-hoc comparisons test. Descriptive statistics was used for the histological examination of the pancreas. Differences were considered statically significant at $p<0.05$.

CHAPTER IV RESULTS

4.1 The effect of genistein on body weight change

The results of weight change were showed in **Table 4.1 and Figure 4.1**. The level weight changed in Con group showed normally increased (Con: 1.75±0.19 g). These were significant decreased in AP group when compared with Con group (AP: -1.46±0.37g *vs*. Con*:* 1.75±0.19 g, p=0.000) and significantly increased in LG (LG: 0.30±0.66g *vs*. AP:-1.46±0.37g, p=0.000) and HG (HG: 0.41±0.54g *vs.* AP: -1.46±0.37 g, p=0.000) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 0.30±0.66g *vs*. HG: 0.41±0.54g, $p \geq 0.05$).

4.2 The effect of genistein on serum amylase (AMY)

The results of serum AMY were showed in **Table 4.1and Figure 4.2**. The serum AMY in Con group showed normal (Con: 5,714±201 g). These were significantly increased in AP group when compared with Con group (AP: 13,860±5,918 U/L *vs*. Con: 5,714±201 U/ L, p=0.002) and slightly decreased but no significant in LG (LG: 11,283±4,226 *vs*. AP: 13,860±5,918 U/L, $p \geq 0.05$) while significantly decreased in HG (HG: 8,728±3,213 *vs.* AP: 13,860±5,918 U/L, p=0.037) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 11,283±4,226 *vs.* HG: 8,728±3,213 U/L, $p \geq 0.05$).

4.3 The effect of genistein on serum IL-6 and serum CRP

The results of serum IL-6 were showed in **Table 4.1 and Figure 4.3.** Serum IL-6 in Con group showed normal level (18.59±18.90 pg/ mL). These were significantly increased in AP group when compared with Con Group (AP: 124.68±106.07 *vs.* Con: 18.59 ±18.90 pg/ mL, p=0.005) and significantly decreased in LG (LG: 16.61±11.24 *vs.* AP: 124.68±106.27 pg/ mL, p=0.004) and HG (HG: 52.58± 42.70 *vs.* AP: 124.68±106.27 pg/ mL, p=0.045) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 16.61±11.24 *vs.* HG: 52.58±42.70 pg/ mL, $p \geq 0.05$). The result of serum CRP were showed in **Table 4.1 and Figure 4.4.** Serum CRP in Con group showed normal level (Con: 8,068±3,065 pg/ mL). These were significantly increased in AP group when compared with Con Group (AP: 11,687±3,691 *vs.* Con: 8,068± 3,065 pg/ mL, p=0.044) and significantly decreased in LG (LG:

8,094±1,795 *vs.* AP: 11,687±3691 pg/mL, p=0.045) and HG (HG: 7,607±2,757 *vs.* AP: 11,687±3,691 pg/mL, p=0.025) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 8,094±1,795 *vs.* HG: 7,607±2,757 pg/mL, $p \geq 0.05$).

4.4 The effect of genistein on pancreatic MDA

The results of pancreatic MDA were showed in **Table 4.1 and Figure 4.6.** Pancreatic MDA in Con group showed normal level (0.065±0.007 nmol/mg protein). These were significantly increased in AP group when compared with Con Group (AP: 0.119±0.062 *vs.* Con: 0.065±0.007 nmol/mg protein, p=0.008) and significantly decreased in LG (LG: 0.069±0.004 *vs.* AP: 0.119±0.062 nmol/mg protein, p=0.012) and HG (HG: 0.070±0.013 *vs.* AP: 0.119±0.062 nmol/mg protein, p=0.014) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 0.069±0.004 *vs.* HG: 0.070±0.013 nmol/mg protein, $p \geq 0.05$).

4.5 The effect of genistein on myeloperoxidase positive cells

To investigate the effect of genistein on myeloperoxidase cells infiltration into the damage tissue, we were observed at immunohistological staining of MPO in pancreas. The results were showed in **Table 4.1 and Figure 4.5, 4.13-4.14**. The number of MPO cells infiltrating in the pancreas was markedly increased in AP group when compared with Con group (AP: 21±11 *vs*. Con: 0±0 cells of HPF, p=0.000) and this increase was significantly reduced when compared with LG (LG: 4±2 *vs.* AP: 21±11 cells of HPF, p=0.000) and HG (HG: 3±1 *vs*. AP: 21±11 cells of HPF, p=0.000) groups when compared with AP group. However, there was not different between LG and HG groups (LG: 4±2 *vs.* HG: 3±1 cells of HPF, $p \geq 0.05$).

4.6 The effect of genistein on histopathology

To investigate the effect of genistein on L-arg induced AP, we stained pancreatitis section with H&E**.** The summarized scores of pancreatic inflammations were presented in **Table 4.2-4.3** and **Figure 4.7-4.12**. There was no pancreatic injury in Con group. Administration of L-arginine induced extensive tissue damage characterized by neutrophil infiltration, edema, and acinar cell necrosis. Nevertheless, after treatment with genistein in LH and HG groups the damage was limited to mild and less histological damage, and significantly decreased histopathological score

compared with AP group, nevertheless, there was not different of histopathological score between LG and HG groups.

Table 4.1: The effects of genistein on inflammation in acute pancreatitis mice

Groups (n=6)	Parameters					
	Body weight change (g)	Serum amylase (U/L)	Serum IL-6 (pg/mL)	Serum CRP (ng/mL)	MPO positive (cells of HPF)	Pancreatic MDA (nmol/mg protein)
Con	1.75 ± 0.19	5,714 ± 201	18.59 ± 18.90	8,069 ± 3,065	0 ± 0	0.065 ± 0.007
AP	-1.46 ± 0.37^{a1}	13,860 ± 5,918^{a1}	124.68 ± 106.27^{a1}	11,687 ± 3,692^{a2}	21 ± 11^{a1}	0.119 ± 0.062^{a1}
LG	0.30 ± 0.66^{b1}	11,283 ± 4,227	16.61 ± 11.24^{b1}	8,095 ± 1,795^{b2}	4 ± 2^{b1}	0.069 ± 0.004^{b2}
HG	0.41 ± 0.54^{b1}	8,728 ± 3,214^{b2}	52.58 ± 42.70^{b2}	7,608 ± 2,758^{b2}	3 ± 1^{b1}	0.070 ± 0.013^{b2}

Result are express as mean ± SD; n=6 for each group.

Con: Control group, AP: Acute pancreatitis group, LG: AP + Genistein low dose group and HG: AP + Genistein high dose group

$^{a1}p < 0.01$ and $^{a2}p < 0.05$ compared with Con group.

$^{b1}p < 0.01$ and $^{b2}p < 0.05$ compared with AP group.

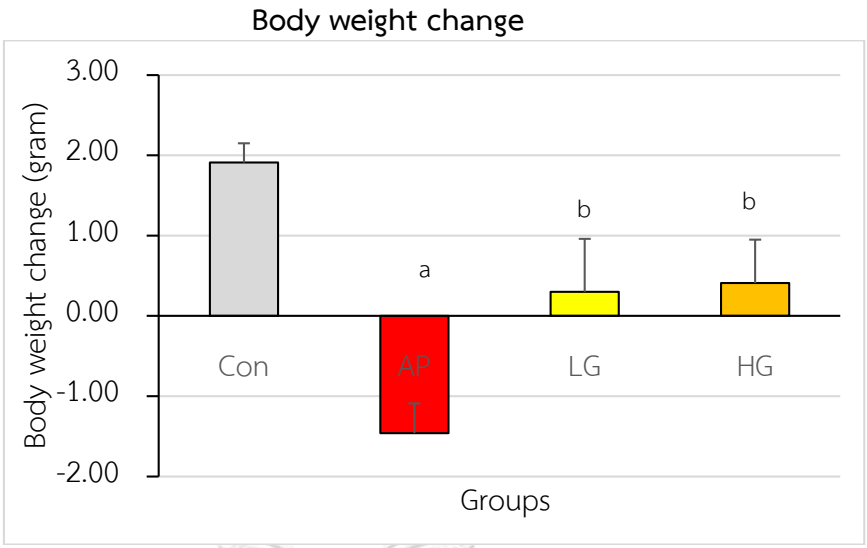
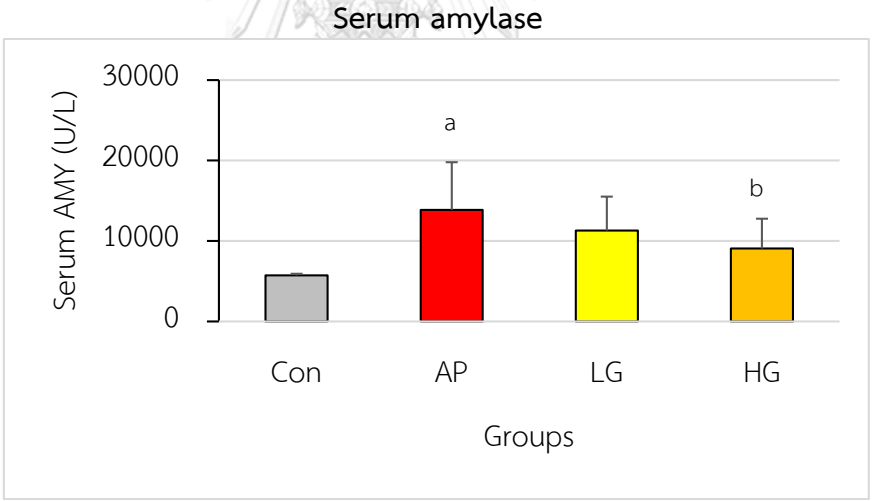


Figure 4.1 Effect of genistein on body weight change in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. [a]$p < 0.01$ compared with Con group, [b]$p < 0.01$ compared with AP group.



Figutre 4.2 Effect of genistein on serum AMY in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. [a]$p < 0.01$ compared with Con group, [b]$p < 0.05$ compared with AP group.

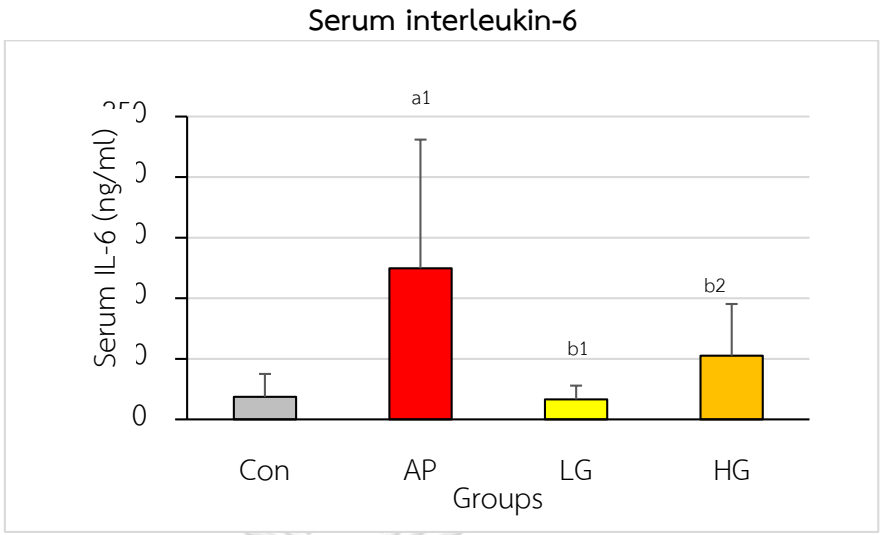


Figure 4.3 Effect of genistein on serum IL-6 in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. $^{a1}p < 0.01$ compared with Con group, $^{b1}p < 0.01$ and $^{b2}p < 0.05$ compared with AP group.

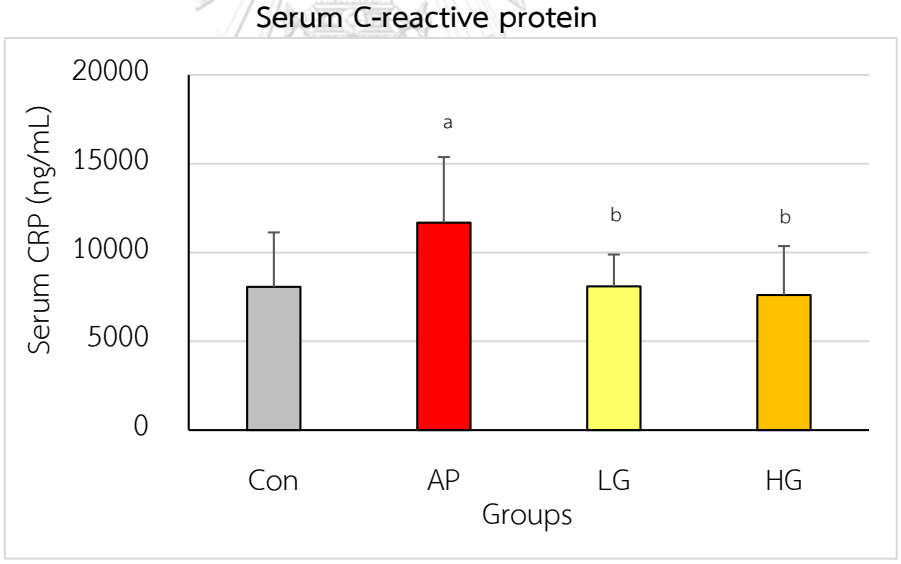


Figure 4.4 Effect of genistein on serum CRP in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. $^{a}p< 0.05$ compared with Con group, $^{b}p < 0.05$ compared with AP group.

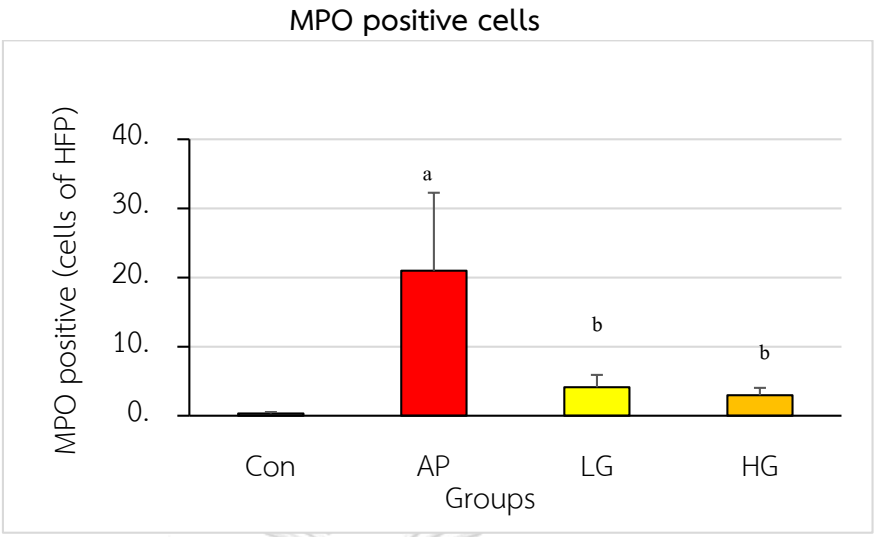


Figure 4.5 Effect of genistein treatment on MPO cell infiltration in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. [a]$p < 0.01$ compared with Con group, [b]$p < 0.05$ compared with AP group.

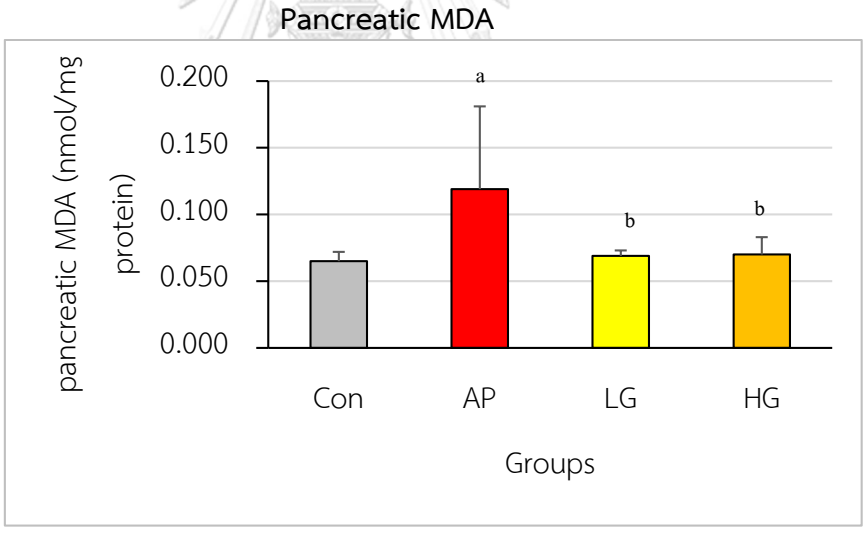


Figure 4.6 Effect of genistein treatment on pancreatic MDA in mice with acute pancreatitis, mean ± SD of 6 animals in each groups. [a]$p < 0.01$ and compared with Con group, [b]$p < 0.05$ compared with AP group.

Table 4.2: Summarized histopathology score of acute pancreatitis

Groups	N	pancreas pathology											
		Neutrophil infiltration				Edema				necrosis			
		0	1	2	3	0	1	2	3	0	1	2	3
Con	6	6	-	-	-	6	-	-	-	6	-	-	-
AP	6	-	3	1	2	-	2	3	1	-	2	3	1
LG	6	1	4	1	-	-	4	2	-	1	3	2	0
HG	6	5	-	1	-	-	6	-	-	3	2	-	1

Values are number of animals.

Each section were examined for pancreatic inflammation according to the criteria

described by H. E. V. DE COCK *et al*. in 2007 (63). The histological grading, scale ranging from 0-3 (0= Not present, 1= Mild, <25% of the pancreatic parenchyma, 2= Moderate, present in 25–50% of the parenchyma, 3= Severe, >50% of the parenchyma).

Table 4.3: Comparison of severity of histopathology

Parameters /groups	**N**	**Severity (N)**			
		Normal	Mild	Moderate	Severe
Con	6	6	-	-	-
AP	6	-	0	4	2
LG	6	-	4	2	-
HG	6	-	5	1	-

Total score 0=normal 1-3=mild 4-6= moderate 7-9= severe

Effect of genistein on the histopathology score in L-arginine induced acute pancreatitis, value are number of animals.

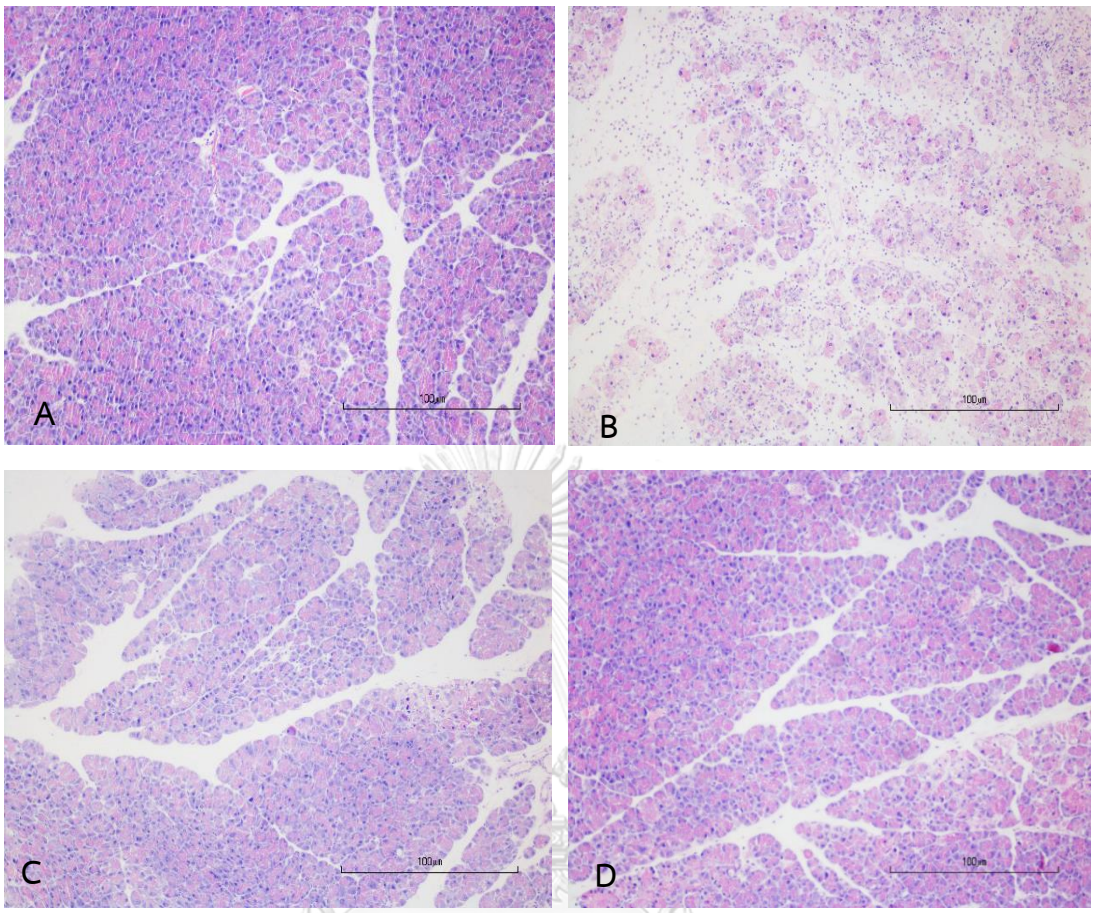


Figure 4.7 Pathological study the effect of genistein on L-arginine-induced acute pancreatitis (H&E, X10), (A) Control group showed normal histology, (B) Acute pancreatitis group showed extensive tissue damage characterized by neutrophil infiltration, edema, and acinar cells necrosis, treatment with genistein low dose (10 mg/kg) and (C) genistein high dose (100 mg/ kg) (D) showed less pancreatic damage.

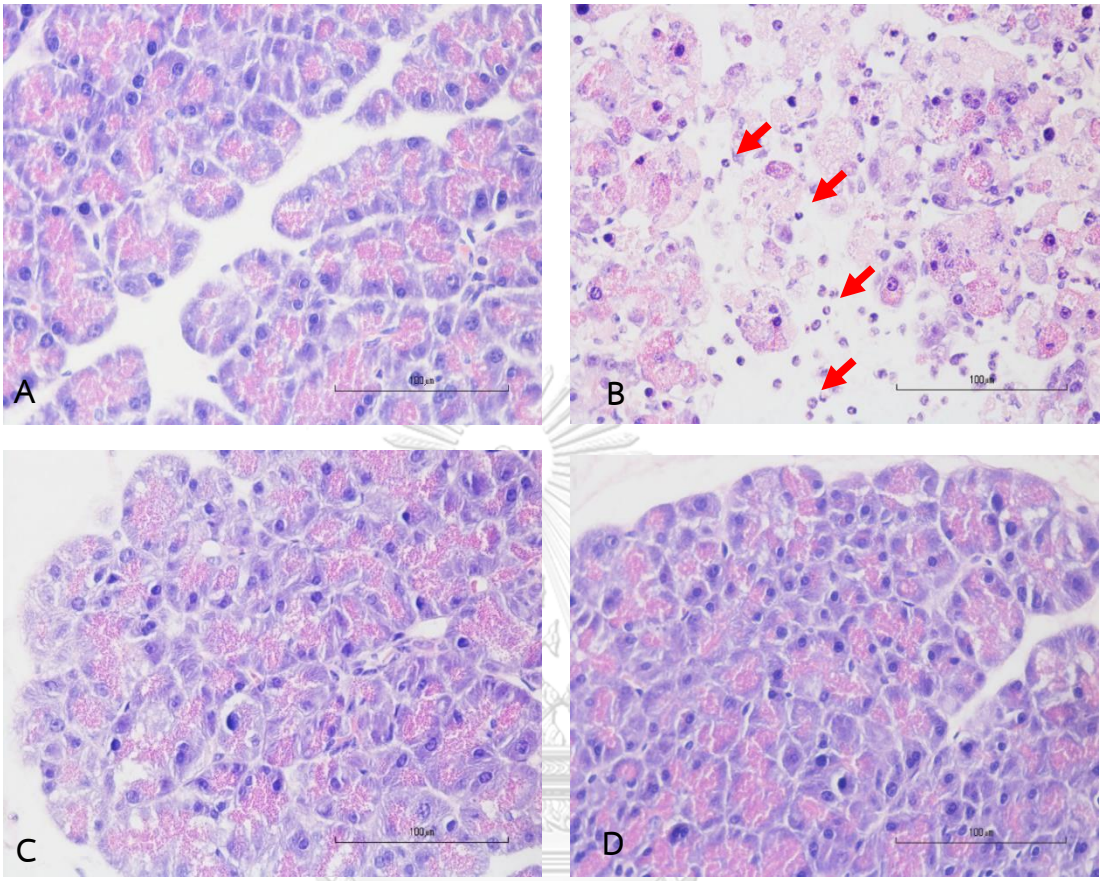


Figure 4.8 Histopathological study the effect of genistein on L-arginine-induced acute pancreatitis (H&E, X40) , (A) Control group showed normal histology, (B) Acute pancreatitis group showed extensive tissue damage characterized by neutrophil infiltration (arrow), edema, and acinar cells necrosis, treatment with genistein low (10 mg/ kg) and (C) high (100 mg/ kg) (D) doses showed less pancreatic damage.

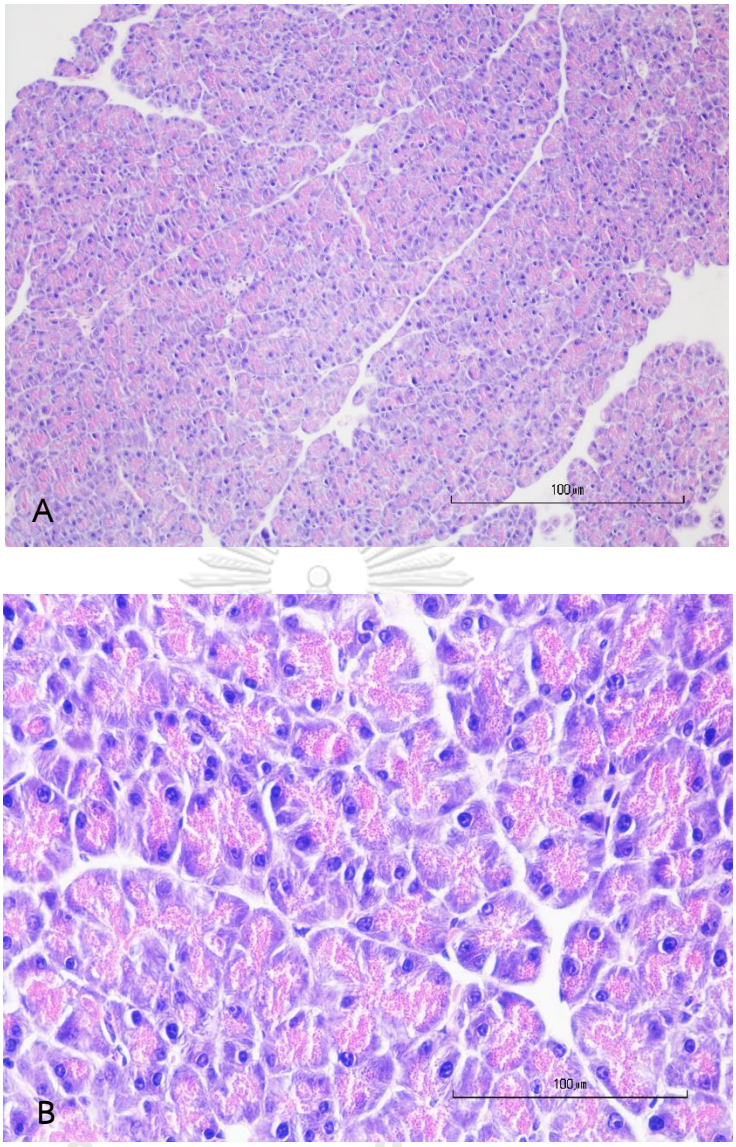


Figure 4.9 Pancreatic histopathology of H&E staining (A: X10 and B: X40); Control group showed normal pancreatic architecture, absent tissue damage.

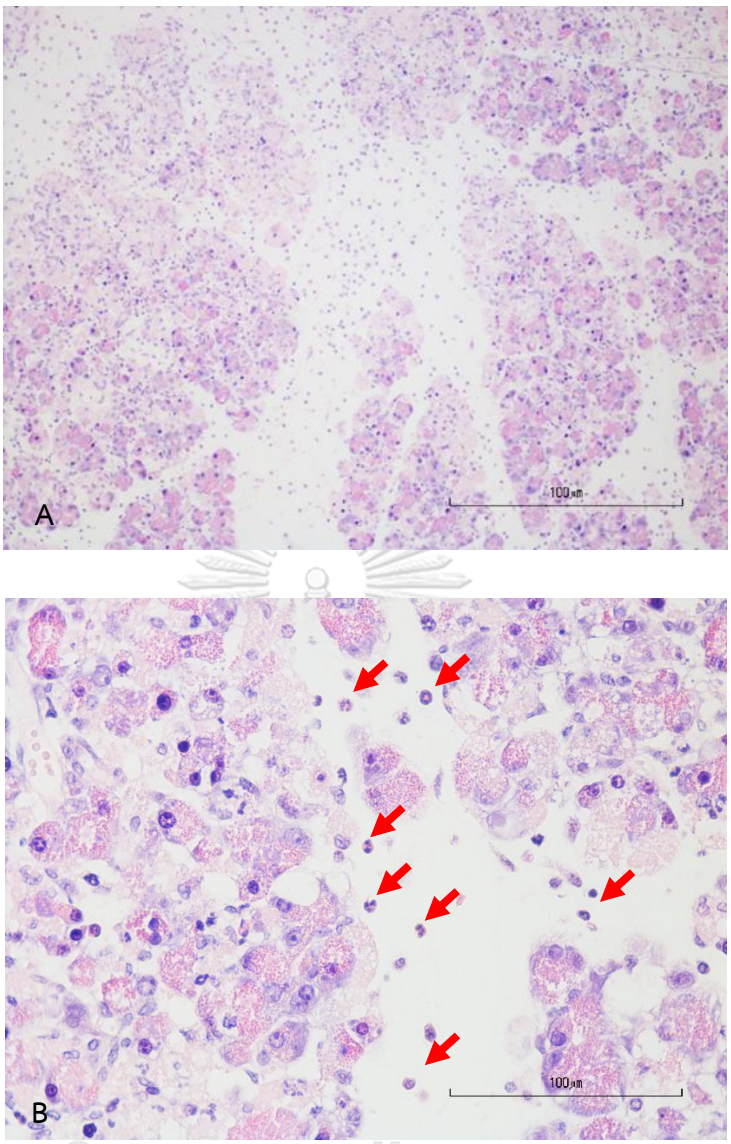


Figure 4.10 Pancreatic histopathology of H&E staining (A:X10 and B:X40); AP group showed extremely tissue damage characterized by neutrophil infiltration (Arrow), edema, and acinar cell necrosis.

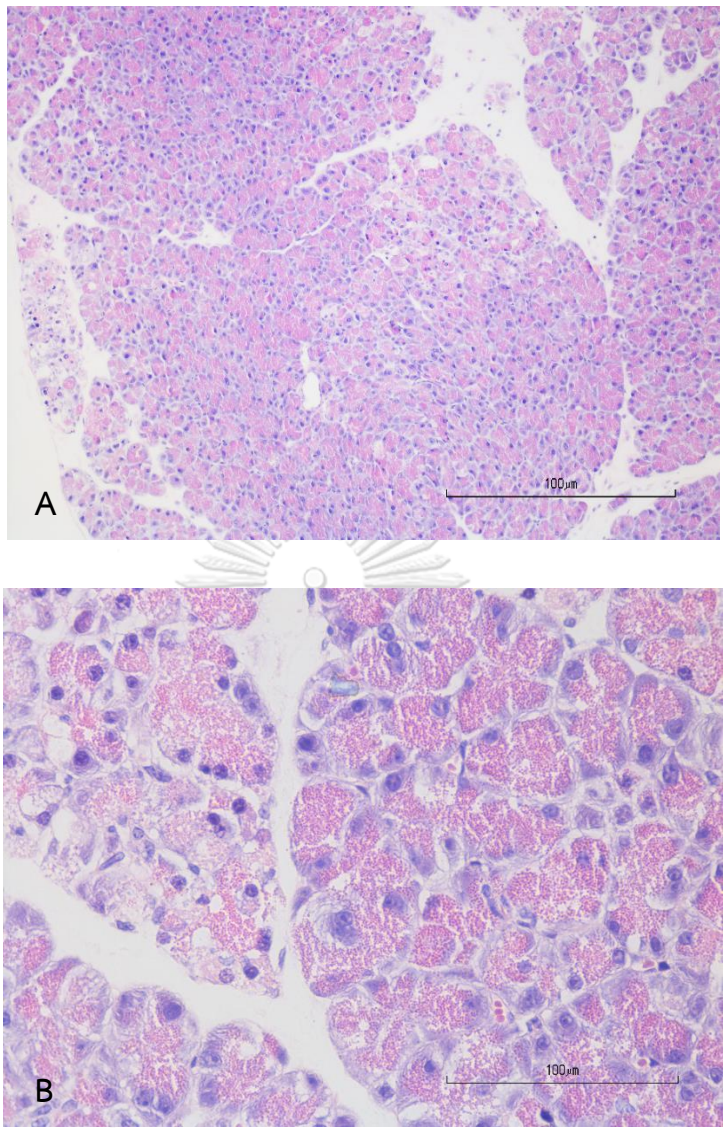


Figure 4.11 Pancreatic histopathology of H&E staining (A: X10 and B: X40); LG group effectively reduced of neutrophil infiltration, edema, and acinar cell necrosis.

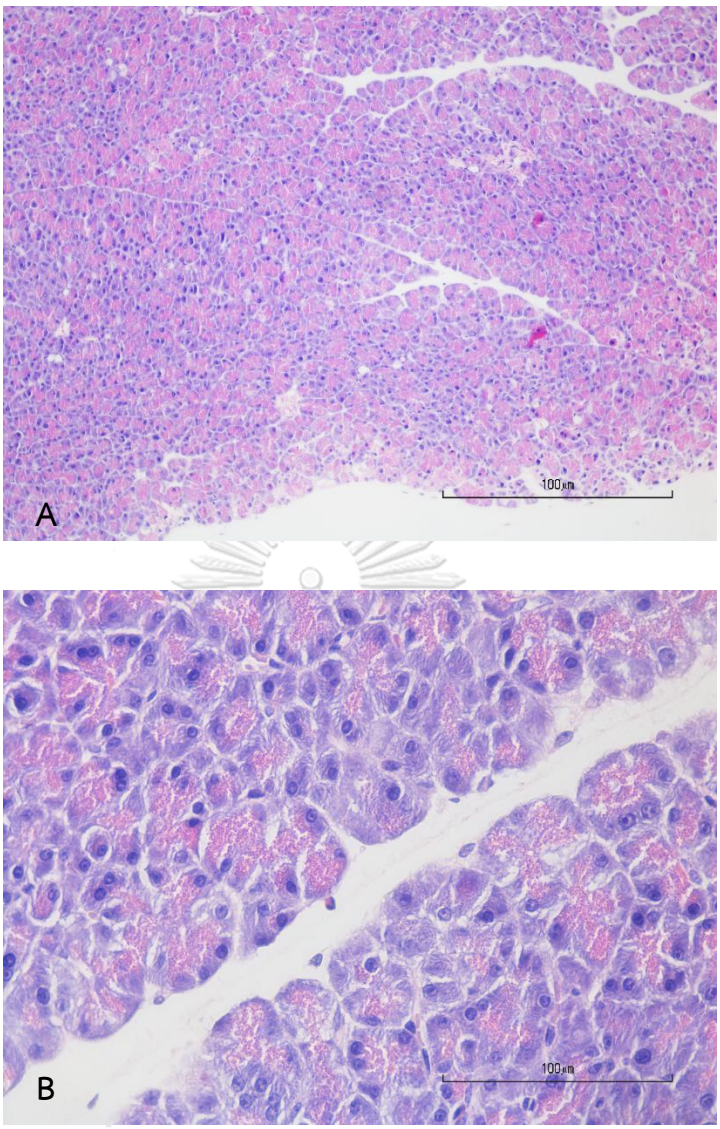


Figure 4.12 Pancreatic histopathology of H&E staining (A: X40 and B: X40); HG group effectively reduced of neutrophil infiltration, edema, and acinar cell necrosis.

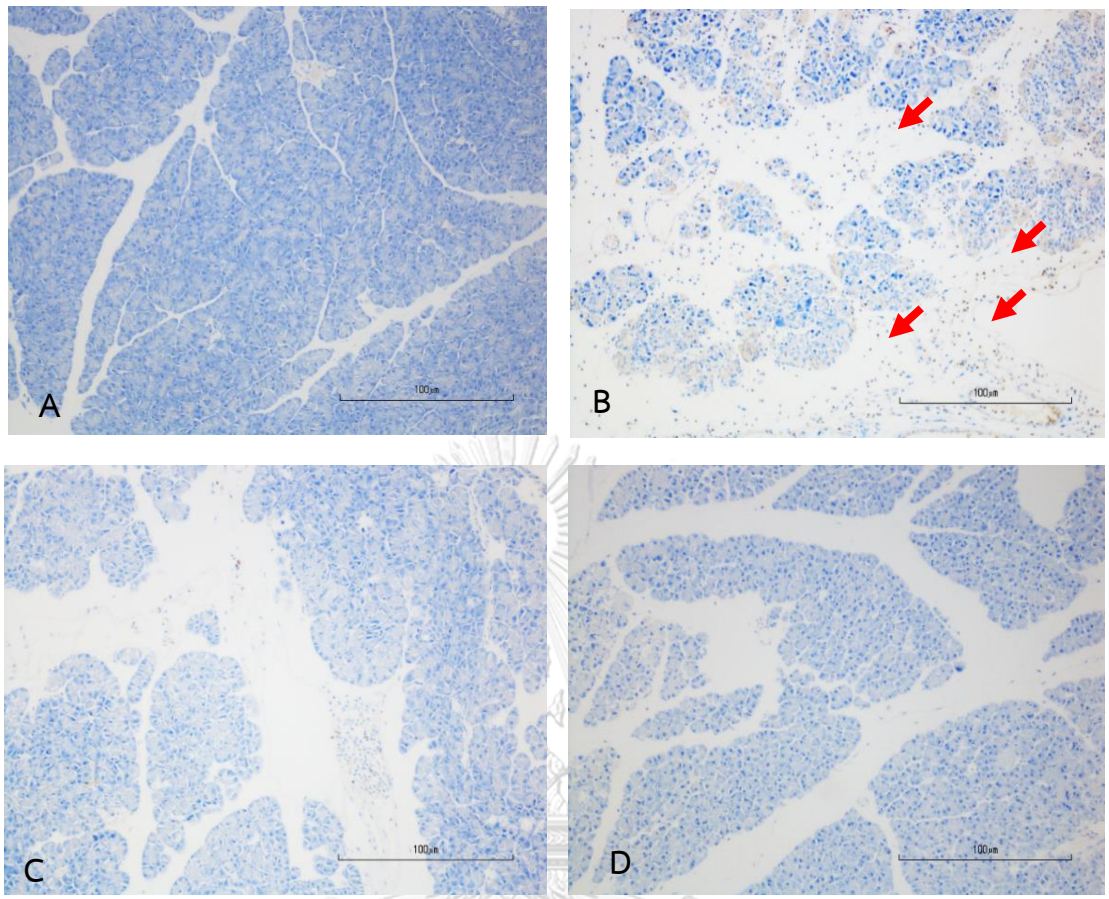


Figure 4.13 Immunohistochemistry of MPO in pancreas (X10). Con group (A): absent of MPO cell infiltrating into pancreas tissue (arrow). AP group (B): large of number MPO cells infiltrating into pancreas tissue. Treatment with low (C) and high (D) doses of genistein obviously reduced MPO cells infiltrating.

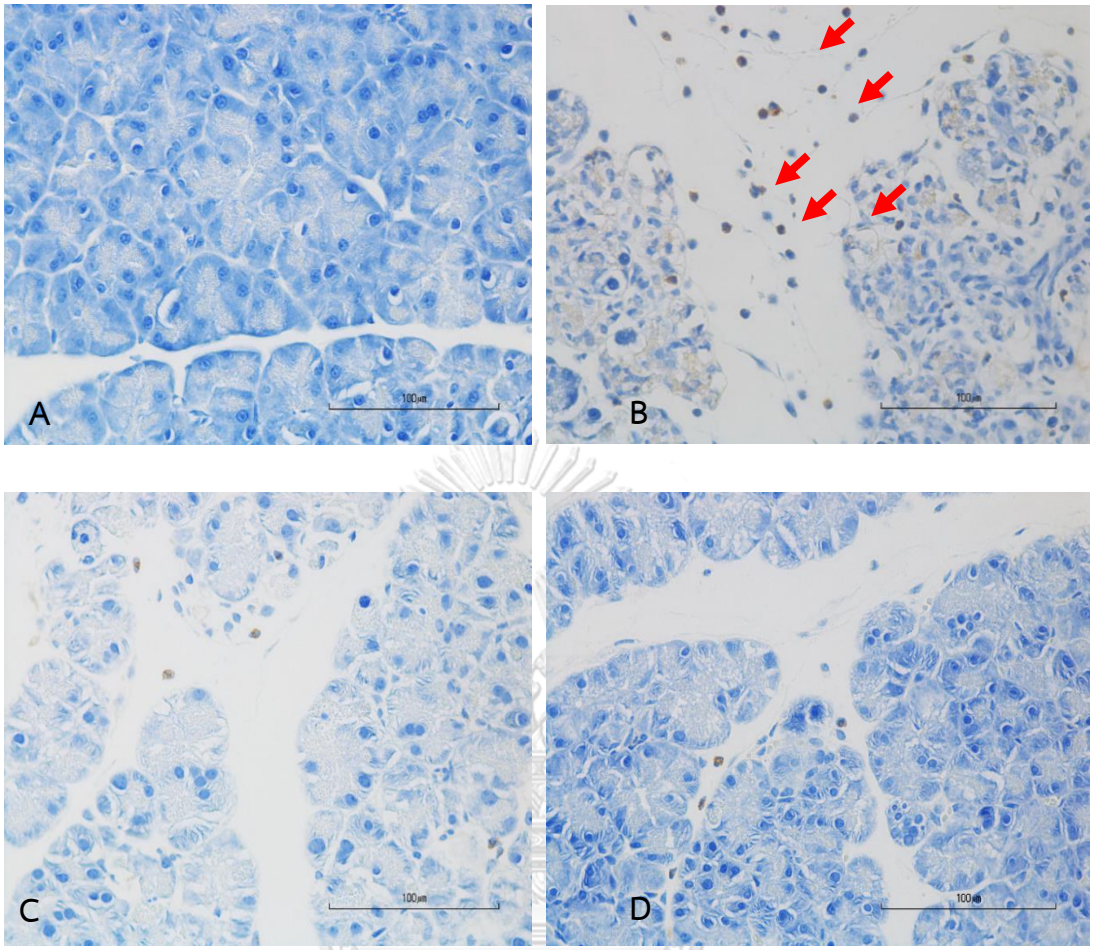


Figure 4.14 Immunohistochemistry of MPO in pancreas (X40). Con group (A): absent of MPO cell infiltrating into pancreas tissue. AP group (B): large of number MPO cells infiltrating into pancreas tissue (arrow). Treatment with low (C) and high (D) doses of genistein obviously reduced MPO cells infiltrating.

CHAPTER V

DISCUSSION AND CONCLUSION

Acute pancreatitis (AP) is a sudden inflammatory disorder of the pancreas, and is a potentially life-threatening disease (1, 2), which may lead to several systemic responses. Various studies believe that the pathology of acute pancreatitis rise from an early intra-acinar cells of inactive enzymes into active, leading to autodigestive pancreatic tissue. This encouraging the synthesis and releasing of many pro-inflammatory cytokines and chemokines, including oxidative stress, causing local inflammation (1-3). AP is characterized by interstitial edema, acinar cells necrosis, hemorrhages, and neutrophil infiltration.

In this study, L-arg was used to induce experimental acute pancreatitis. The influence of an excessive dose of L-arg injection induced acinar cells injury was first discovered by Mizunuma in 1984; i.p. injection of L-arg in rats. The result showed selective damaged on pancreatic acinar cells without any morphological change in other tissues. Thus, using L-arg to induce acute pancreatitis is one of the few noninvasive models of acute pancreatitis. This method is very popular because the technique is easily implemented, inexpensive and similar in its course including histological changes to human pancreatitis (35). From our pilot study, we injected various doses of L-arginine at 350, 400, and 450 mg/100 g BW to mice. We found that, although, at doses of 400 and 450 mg/100 g BW were able to demonstrate more severity of AP as showed in histopathology, but more than 50% mortality rate of mice was exhibited within 3 days. With this reason, the pancreatic tissue and blood samples could not collected in the appropriated time. Therefore, we considerate dose of 350 mg/100 g BW L-arg as suitable dose to induce AP in this study, this dose of L-arg effectively generated moderate-to-severe AP with low mortality rate. In addition, because AP disease reduces GI functions, rest bowel diet is one of medical treatment for AP. Therefore, administration of genistein by IP injection was decided as a good way to perform in this study.

Genistein (4', 5, 7 - trihydroxylisoflavone) is a phytoestrogen that belongs to the category of isoflavones. The remarkable pharmacological activity of genistein is phytoestrogenic because it has a similar structure with estradiol (6). In addition, genistein has been extensively used as an antioxidant, and acts both directly and indirectly as an antioxidant agent. Its direct antioxidant potencies are influenced by its structure, acting as a free radical scavenger due to its ability to donate hydrogen from

phenolic hydroxyl groups to deleterious free radical molecules. Genistein is well-known natural compound that has various biological functions. Because of it usefulness in treatment and prevention of disease. Various study have shown beneficial effects of genistein on oxidative stress and inflammation (58, 66). However, the role of genistein on acute pancreatitis are not yet clear.

This study is the first to report that low (10 mg/ kg) and high (100 mg/ kg) doses of genistein can inhibit the development of L-arginine induced AP. Genistein treatments resulted in decreasing body weight loss, serum amylase, serum IL-6, and serum CRP, MPO cell infiltration, pancreatic MDA, and improving pancreas pathology in mice with L-arginine induced AP. Nevertheless, this study were unable to show a dose dependent effect for all of these parameters.

The effect of genistein on serum AMY in acute pancreatitis mice

To diagnose acute pancreatitis, the investigation of AMY is the most commonly performed laboratory tests, as they are secreted by the acinar cells of the pancreas. The blood levels of AMY rapidly rise within 3-6 hours of the onset symptoms, with a half-life of 10-12 hours, and remain elevated for 3-5 days, and the cut-off for diagnosis of AP is 3 times above the upper limit of normal (23). Similarly, in accordance with previous reported, in the present study, induction of L-arg-induced acute pancreatitis significantly increased serum AMY at the end of the study (67). Administration of genistein decreased amylase level, moreover significantly decreased in HG groups when compared with AP group may indicate protective effect of genistein on pancreatic damage.

The effect of genistein on serum IL-6 and CRP in acute pancreatitis mice

The level of inflammatory cytokines was used to evaluated the degree of AP. Cytokines are inflammatory marker, produced and released by a number of cell types, although predominantly by leukocytes. Cytokines appear to play a pivotal role related to the pathogenesis of pancreatitis. One of the inflammatory cytokines that important in L-arg-induced acute pancreatitis is IL-6. IL-6 is a principle mediator which has a role in regulation of immune response and inflammatory process (68, 69). In addition, C-reactive protein (CRP), an acute phase reactant synthesized by the hepatocytes, is usually elevated in inflammatory conditions, moreover, CRP has been shown to be an accurate severity predictor (70). The production of CRP, which is stimulated by

cytokines in blood and considered as a marker of neutrophil activities. Recent studies had shown elevation of IL-6 and CRP in animal model of caeruline (71) and L-arginine induce acute pancreatitis (43). In the present study, levels of serum IL-6 and serum CRP proved to be increased in L-arg-treated group. However, treatment with genistein low and high doses effectively decreased levels of serum IL-6 and serum CRP indicated that genistein treatment probably due to its anti-inflammatory action.

The effect of genistein on myeloperoxidase cells infiltration in acute pancreatitis mice

Neutrophil sequestration in inflamed tissues is quantified by measuring the tissue myeloperoxidase infiltration. MPO has been implicated in promoting tissue damage in various inflammatory diseases. MPO in blood is considered to be a marker of neutrophil activity. It is a hemoprotein that is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages. MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite and is secreted by activate neutrophils during inflammatory condition (27). To investigate the effect of genistein on myeloperoxidase cells infiltration into the damage tissue, we were observed at immunohistological staining of MPO in pancreas, our study has demonstrated that genisein low and high doses ostensibly reduced inflammatory cell infiltration.

The effect of genistein on pancreatic MDA in acute pancreatitis mice

Oxidative stress has been defined as an imbalance between antioxidants and free radicals, with an increase in levels of free radicals or insufficient antioxidant enzymes. Free radicals are unstable chemicals, any atom or molecule that has a single unpaired electron in an outer shell, are highly reactive, and are produced in biologic systems under normal physiologic as well as pathophysiologic condition. ROS are highly reactive and capable of extracting electrons, whereby oxidizing a variety of biomolecules vital to cell and tissue function, can interact with polyunsaturated fatty acids, and lipid peroxidation often occurs in response to oxidative stress (20). MDA used as an indicator of oxidative stress in cell and tissue, naturally occurring, results from lipid peroxidation of polyunsaturated fatty acids. Polyunsaturated fatty acids, which are plentiful in plasma membrane and mitochondrial membrane, are among the most susceptible targets of ROS/RNS (72). Recent studies had shown elevation of MDA in animal model of L-arginine (73). However MDA was decreased by genistein. It was undersatnable that genistein could protect against the free radical mutilated oxidative

stress by scavenging of free radical that limits lipid peroxidation implicated in membrane damage and stabilizing action on cell membrane in mice with acute pancreatitis.

The effect of genistein on histopathology and body weight change in acute pancreatitis mice

Finally, histopathology of pancreas was determined in each treatment group. Accordingly with many previous studies, it is well known that the extent of pancreatic tissue damage in acute pancreatitis correlated with levels of inflammatory mediators (74, 75). The current study revealed that low and high genistein pretreatment significant attenuated the histological parameters; neutrophil infiltration, edema, and acinar cells necrosis (Table 4.1, Figure 4.7-4.12), probably due to its anti-inflammatory effect which can reduce inflammatory mediators. In addition, administration of L-arg shown impared body weight change from poor ingestion of food because of sickness, the enzymes autoactivation that released by acinar gland while progression of AP. However, this symptom was improved by genistein.

To our knowledge, our present study is the first to report that administration of genistein can inhibit the development of acute pancreatitis. The administration of L-arg significantly developed the acute pancreatitis characterized by increased level of body weight loss, serum AMY, serum IL-6, serum CRP and histopathology score. Genistein is able to counteract L-arg-induced change in laboratory parameters of acute pancreatits. Injection of genistein can beneficially decrease serum AMY, inflammatory cytokines (IL-6, CPR, MPO), pancreatic MDA, and prevent histological damage to the pancreas. These effects may be due to anti-inflammatory and anti-oxidant property of genistetin, nonetheless, this study were unable to show a dose dependent effect of genistein.

Anti-oxidant and anti-inflammatory effects of genistein in this study were reported that accordance with in a number of other studies. The anti-oxidant property if genistein is depend on its molecular structure, which act as a free radical scavenger to donate hydrogen from phenolic hydroxyl groups to deleterious free radical molecules. With all hydroxyl groups of genistein at C-4,5,7 positions, C-4 showed the most effective antioxidant indirectly (8, 57).

Anti-oxidant property of genistein has been shown in various studies. Choi C et al., 2003 revealed that genistein decreased the activation of NF-KB, which is

inducible by oxidative stress and regulates the expression of genes involved in immune and inflammation responses (76). Anti-oxidant effect of genistein was showed by reduction of inflammatory cells that secrete reactive oxygen and nitrogen by LDL oxidation (77). It has been demonstrated that genistein can suppress inhibition of lipid peroxidation in liposome system, and can suppress both metal-ion induced peroxidation and peroxyl-radical-induced peroxidation (8). In Kapiotis *et al*, genistein administration inhibited the oxidation of low-density lipoproteins (LDL) triggered by copper ions or superoxide/nitric oxide radicals (58). In addition, genistein effectively increase the activity of anti-oxidant enzymes, including superoxide dismutased, glutathione reductase, glutathione peroxidase in TPA-induced H_2O_2 formation and superoxide anion (O_2^-) generation by xanthine/xanthine oxidase HL-6-0 cells and the mouse skin tumorigenesis model (9). Genistein can inhibit hepatic malondialdehyde (MDA) and significantly increase glutathione (GSH) in alcohol-induced liver injury (78). *In vitro* study, genistein also protected proximal tubule epithelium damaged via inhibiting ROS and increase glutathione (66).

Genistein has been reported to exhibit anti-inflammatory properties. Guiyuan Ji et al., showed that genistein efficiently inhibit production of the TNF-α, IL-6 levels and NF-kB activation induced by LPS. Moreover, it was demonstrated that genistein prevented LPS-induced decrease in adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. These effects were obviously attenuated by an AMPK inhibitor. Taken together, genistein has ability to attenuate inflammatory responses via inhibition of NF-kB activation following AMPK stimulation in RAW 264.7 macrophages (12). In M. Blay et al. study, the results suggested that genistein modulates the inflammatory response in activated macrophages by inhibiting NO and PGE_2 and by modulating the expression of key genes defined by transcriptomic profiling (79). Experiment LPS-treated RAW 264.7 macrophages by Y.C. Liang *et al*. (1999), demonstrated that the anti-inflammation activity of genistein inhibited NO and PGE2 production by modulating cyclooxygenase-2 (COX-2) and iNOS expression (60) as well as decreasing the production of TNF-α and IL-6, and nuclear factor NF-kB. In addition, this compound administration has a significant anti-inflammatory on high-fat diet induce nonalcoholic steatohepatitis (NASH) rats (61). Furthermore, experimental streptozotocin (STZ)-induced diabetic cardiomyopathy, indicated that the anti-inflammatory activity of genistein reduced the increasing of CRP, TNF- α, and slightly increase anti-oxidant enzymes (10). In the present study the results showed accordance with previous studies, showing genistein decreased inflammatory markers

(IL-6, CRP, MPO) including oxidative stress marker (MDA), these factors are relate with occurring of AP disease.

The optimal dose of genistein on AP has not been reported. Previous data showed the dose-dependent manner of genistein, therefore, our study applied non-toxic doses of 10 and 100 mg/kg injected to AP mice (80). Our experimental is the first study that reported low (10 mg/kg) and high (100 mg/kg) doses of genistein could inhibit the development of AP induced by L-arginine. The most of all parameters showed significantly decreased, with non-significantly decreased only amylase level treated with LG. Amylase enzyme was reported to increase in the blood of after acinar cell injury, depending on the severity of the disease in pancreatitis patients. Nevertheless, amylase level may be normal in patients with AP. Elevation of serum amylase also depending on the onset time of the disease (81). Severity of histopathology revealed slightly different between AP mice treated with LG and HG groups, 4 mice demonstrated mild, while, 2 mice with moderate AP. Whereas, 5 mice exhibited mild AP and one mouse was modulate AP in LG group. It is probably lower than 10 mg/kg of genistein effectively attenuate severity of AP.

To achieve the best result, we decided the experiment by both pretreatment and treatment, however the occurrence of disease is unpredictable. Consequently, the experimental design for studying the treatment effect of genistein on AP should be further elucidated. Furthermore, hypothetically, regularly diet intake of soy products, should be also performed in the further study. Nevertheless, the effective doses of genistein in this study are much higher than in soy food products that daily intake of human. In the present study, 10 and 100 mg/kg genistein can attenuate severity of AP, confirmed by improved histopathology and reduced inflammatory cytokines (IL-6, CRP, MPO), and oxidative stress (MDA). The exact mechanism of genistein on AP should be explored in further study. Our study, informed the data for more understanding of the effects of genistein on oxidative stress, inflammation as well as pancreatic histopathology in L-arginine induced AP mice and could be useful for further studies and applications in AP treatment.

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APPENDIXS

APPENDIX A

Descriptive: body weight change

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	1.7450	.18609	.07597	1.5497	1.9403	1.51	1.97
AP	6	-1.4550	.37399	.15268	-1.8475	-1.0625	-1.82	-.94
LG	6	.2950	.66057	.26968	-.3982	.9882	-.42	1.38
HG	6	.4133	.53526	.21852	-.1484	.9751	-.07	1.39
Total	24	.2496	1.24257	.25364	-.2751	.7743	-1.82	1.97

ANOVA: body weight change

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31.025	3	10.342	46.098	.000
Within Groups	4.487	20	.224		
Total	35.511	23			

Multiple Comparisons: body weight change

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	3.20000*	.27346	.000	2.6296	3.7704
	LG	1.45000*	.27346	.000	.8796	2.0204
	HG	1.33167*	.27346	.000	.7612	1.9021
AP	Con	-3.20000*	.27346	.000	-3.7704	-2.6296
	LG	-1.75000*	.27346	.000	-2.3204	-1.1796
	HG	-1.86833*	.27346	.000	-2.4388	-1.2979
LG	Con	-1.45000*	.27346	.000	-2.0204	-.8796
	AP	1.75000*	.27346	.000	1.1796	2.3204
	HG	-.11833	.27346	.670	-.6888	.4521
HG	Con	-1.33167*	.27346	.000	-1.9021	-.7612
	AP	1.86833*	.27346	.000	1.2979	2.4388
	LG	.11833	.27346	.670	-.4521	.6888

*. The mean difference is significant at the 0.05 level.

APPENDIX B

Descriptive: AMY

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	5714.00	201.109	82.102	5502.95	5925.05	5472	6006
AP	6	13860.00	5918.256	2416.118	7649.17	20070.83	9000	25116
LG	6	11283.67	4226.538	1725.477	6848.19	15719.15	5824	17546
HG	6	8728.33	3213.615	1311.953	5355.85	12100.82	5824	13390
Total	24	9896.50	4824.010	984.697	7859.50	11933.50	5472	25116

ANOVA: AMY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	218948899.333	3	72982966.444	4.615	.013
Within Groups	316285720.667	20	15814286.033		
Total	535234620.000	23			

Multiple Comparisons: AMY

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-8146.000*	2295.959	.002	-12935.29	-3356.71
	LG	-5569.667*	2295.959	.025	-10358.95	-780.38
	HG	-3014.333	2295.959	.204	-7803.62	1774.95
AP	Con	8146.000*	2295.959	.002	3356.71	12935.29
	LG	2576.333	2295.959	.275	-2212.95	7365.62
	HG	5131.667*	2295.959	.037	342.38	9920.95
LG	Con	5569.667*	2295.959	.025	780.38	10358.95
	AP	-2576.333	2295.959	.275	-7365.62	2212.95
	HG	2555.333	2295.959	.279	-2233.95	7344.62
HG	Con	3014.333	2295.959	.204	-1774.95	7803.62
	AP	-5131.667*	2295.959	.037	-9920.95	-342.38
	LG	-2555.333	2295.959	.279	-7344.62	2233.95

*. The mean difference is significant at the 0.05 level.

APPENDIX C

Descriptive: IL-6

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	18.5887	18.90245	7.71689	-1.2482	38.4256	2.00	44.85
AP	6	124.6765	106.27239	43.38552	13.1505	236.2025	11.86	288.13
LG	6	16.6132	11.24313	4.58999	4.8142	28.4121	2.19	29.56
HG	6	52.5823	42.69891	17.43176	7.7726	97.3921	3.62	109.38
Total	24	53.1152	70.36499	14.36319	23.4026	82.8277	2.00	288.13

ANOVA: IL-6

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45874.701	3	15291.567	4.497	.014
Within Groups	68003.642	20	3400.182		
Total	113878.344	23			

Multiple Comparisons: IL-6

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-106.08783*	33.66592	.005	-176.3137	-35.8620
	LG	1.97550	33.66592	.954	-68.2504	72.2014
	HG	-33.99367	33.66592	.325	-104.2195	36.2322
AP	Con	106.08783*	33.66592	.005	35.8620	176.3137
	LG	108.06333*	33.66592	.004	37.8375	178.2892
	HG	72.09417*	33.66592	.045	1.8683	142.3200
LG	Con	-1.97550	33.66592	.954	-72.2014	68.2504
	AP	-108.06333*	33.66592	.004	-178.2892	-37.8375
	HG	-35.96917	33.66592	.298	-106.1950	34.2567
HG	Con	33.99367	33.66592	.325	-36.2322	104.2195
	AP	-72.09417*	33.66592	.045	-142.3200	-1.8683
	LG	35.96917	33.66592	.298	-34.2567	106.1950

*. The mean difference is significant at the 0.05 level.

APPENDIX D

Descriptive: CRP

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	8068.63	3065.244	1251.381	4851.86	11285.41	4698	12381
AP	6	11687.07	3691.954	1507.234	7812.60	15561.54	5594	15318
LG	6	8094.60	1795.023	732.815	6210.84	9978.36	6179	10508
HG	6	7607.77	2757.940	1125.924	4713.49	10502.05	4685	12617
Total	24	8864.52	3189.026	650.957	7517.91	10211.12	4685	15318

ANOVA: CRP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	64634466.113	3	21544822.038	2.546	.085
Within Groups	169272949.160	20	8463647.458		
Total	233907415.273	23			

Multiple Comparisons: CRP

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-3618.433*	1679.648	.044	-7122.12	-114.75
	LG	-25.967	1679.648	.988	-3529.65	3477.72
	HG	460.867	1679.648	.787	-3042.82	3964.55
AP	Con	3618.433*	1679.648	.044	114.75	7122.12
	LG	3592.467*	1679.648	.045	88.78	7096.15
	HG	4079.300*	1679.648	.025	575.62	7582.98
LG	Con	25.967	1679.648	.988	-3477.72	3529.65
	AP	-3592.467*	1679.648	.045	-7096.15	-88.78
	HG	486.833	1679.648	.775	-3016.85	3990.52
HG	Con	-460.867	1679.648	.787	-3964.55	3042.82
	AP	-4079.300*	1679.648	.025	-7582.98	-575.62
	LG	-486.833	1679.648	.775	-3990.52	3016.85

*. The mean difference is significant at the 0.05 level.

APPENDIX E

Descriptive: MPO

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	.3333	.20656	.08433	.1166	.5501	.00	.60
AP	6	21.0000	11.26197	4.59768	9.1813	32.8187	10.20	38.60
LG	6	4.1333	1.78736	.72969	2.2576	6.0091	2.20	6.80
HG	6	2.9667	1.08382	.44247	1.8293	4.1041	1.20	4.00
Total	24	7.1083	9.88085	2.01692	2.9360	11.2807	.00	38.60

ANOVA: MPO

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1589.298	3	529.766	16.146	.000
Within Groups	656.220	20	32.811		
Total	2245.518	23			

Multiple Comparisons: MPO

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-20.66667*	3.30711	.000	-27.5652	-13.7681
	LG	-3.80000	3.30711	.264	-10.6985	3.0985
	HG	-2.63333	3.30711	.435	-9.5319	4.2652
AP	Con	20.66667*	3.30711	.000	13.7681	27.5652
	LG	16.86667*	3.30711	.000	9.9681	23.7652
	HG	18.03333*	3.30711	.000	11.1348	24.9319
LG	Con	3.80000	3.30711	.264	-3.0985	10.6985
	AP	-16.86667*	3.30711	.000	-23.7652	-9.9681
	HG	1.16667	3.30711	.728	-5.7319	8.0652
HG	Con	2.63333	3.30711	.435	-4.2652	9.5319
	AP	-18.03333*	3.30711	.000	-24.9319	-11.1348
	LG	-1.16667	3.30711	.728	-8.0652	5.7319

*. The mean difference is significant at the 0.05 level.

APPENDIX F

Descriptive: MDA

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	.06542	.007368	.003008	.05768	.07315	.054	.075
AP	6	.11942	.061739	.025205	.05463	.18421	.079	.242
LG	6	.06868	.003965	.001619	.06452	.07284	.063	.073
HG	6	.07023	.012596	.005142	.05701	.08345	.054	.087
Total	24	.08094	.037370	.007628	.06516	.09672	.054	.242

ANOVA: MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	3	.004	3.933	.023
Within Groups	.020	20	.001		
Total	.032	23			

Multiple Comparisons: MDA

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-.054000*	.018349	.008	-.09228	-.01572
	LG	-.003267	.018349	.860	-.04154	.03501
	HG	-.004817	.018349	.796	-.04309	.03346
AP	Con	.054000*	.018349	.008	.01572	.09228
	LG	.050733*	.018349	.012	.01246	.08901
	HG	.049183*	.018349	.014	.01091	.08746
LG	Con	.003267	.018349	.860	-.03501	.04154
	AP	-.050733*	.018349	.012	-.08901	-.01246
	HG	-.001550	.018349	.934	-.03983	.03673
HG	Con	.004817	.018349	.796	-.03346	.04309
	AP	-.049183*	.018349	.014	-.08746	-.01091
	LG	.001550	.018349	.934	-.03673	.03983

*. The mean difference is significant at the 0.05 level.

APPENDIX G

ANOVA: histopathology score

		Sum of Squares	df	Mean Square	F	Sig.
neutrophil	Between Groups	11.792	3	3.931	7.732	.001
	Within Groups	10.167	20	.508		
	Total	21.958	23			
edema	Between Groups	10.792	3	3.597	17.267	.000
	Within Groups	4.167	20	.208		
	Total	14.958	23			
necrosis	Between Groups	10.458	3	3.486	5.578	.006
	Within Groups	12.500	20	.625		
	Total	22.958	23			

Multiple Comparisons: histopathology score

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
neutrophil	Con	AP	-1.83333*	.41164	.000	-2.6920	-.9747
		LG	-1.00000*	.41164	.025	-1.8587	-.1413
		HG	-.33333	.41164	.428	-1.1920	.5253
	AP	Con	1.83333*	.41164	.000	.9747	2.6920
		LG	.83333	.41164	.056	-.0253	1.6920
		HG	1.50000*	.41164	.002	.6413	2.3587
	LG	Con	1.00000*	.41164	.025	.1413	1.8587
		AP	-.83333	.41164	.056	-1.6920	.0253
		HG	.66667	.41164	.121	-.1920	1.5253
	HG	Con	.33333	.41164	.428	-.5253	1.1920
		AP	-1.50000*	.41164	.002	-2.3587	-.6413
		LG	-.66667	.41164	.121	-1.5253	.1920
edema	Con	AP	-1.83333*	.26352	.000	-2.3830	-1.2836
		LG	-1.33333*	.26352	.000	-1.8830	-.7836
		HG	-1.00000*	.26352	.001	-1.5497	-.4503
	AP	Con	1.83333*	.26352	.000	1.2836	2.3830
		LG	.50000	.26352	.072	-.0497	1.0497
		HG	.83333*	.26352	.005	.2836	1.3830
	LG	Con	1.33333*	.26352	.000	.7836	1.8830
		AP	-.50000	.26352	.072	-1.0497	.0497
		HG	.33333	.26352	.220	-.2164	.8830
	HG	Con	1.00000*	.26352	.001	.4503	1.5497
		AP	-.83333*	.26352	.005	-1.3830	-.2836
		LG	-.33333	.26352	.220	-.8830	.2164

necrosis	Con	AP	-1.83333*	.45644	.001	-2.7854	-.8812
		LG	-1.16667*	.45644	.019	-2.1188	-.2146
		HG	-.83333	.45644	.083	-1.7854	.1188
	AP	Con	1.83333*	.45644	.001	.8812	2.7854
		LG	.66667	.45644	.160	-.2854	1.6188
		HG	1.00000*	.45644	.040	.0479	1.9521
	LG	Con	1.16667*	.45644	.019	.2146	2.1188
		AP	-.66667	.45644	.160	-1.6188	.2854
		HG	.33333	.45644	.474	-.6188	1.2854
	HG	Con	.83333	.45644	.083	-.1188	1.7854
		AP	-1.00000*	.45644	.040	-1.9521	-.0479
		LG	-.33333	.45644	.474	-1.2854	.6188

*. The mean difference is significant at the 0.05 level.

Descriptive: histopathology score

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Con	6	.0000	.00000	.00000	.0000	.0000	.00	.00
AP	6	5.5000	1.64317	.67082	3.7756	7.2244	4.00	8.00
LG	6	3.5000	1.76068	.71880	1.6523	5.3477	1.00	6.00
HG	6	2.1667	1.94079	.79232	.1299	4.2034	1.00	6.00
Total	24	2.7917	2.50181	.51068	1.7352	3.8481	.00	8.00

ANOVA: histopathology score

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	96.125	3	32.042	13.397	.000
Within Groups	47.833	20	2.392		
Total	143.958	23			

Multiple Comparisons: histopathology score

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	AP	-5.50000*	.89287	.000	-7.3625	-3.6375
	LG	-3.50000*	.89287	.001	-5.3625	-1.6375
	HG	-2.16667*	.89287	.025	-4.0292	-.3042
AP	Con	5.50000*	.89287	.000	3.6375	7.3625
	LG	2.00000*	.89287	.037	.1375	3.8625
	HG	3.33333*	.89287	.001	1.4708	5.1958
LG	Con	3.50000*	.89287	.001	1.6375	5.3625
	AP	-2.00000*	.89287	.037	-3.8625	-.1375
	HG	1.33333	.89287	.151	-.5292	3.1958
HG	Con	2.16667*	.89287	.025	.3042	4.0292
	AP	-3.33333*	.89287	.001	-5.1958	-1.4708
	LG	-1.33333	.89287	.151	-3.1958	.5292

*. The mean difference is significant at the 0.05 level.

VITA

Miss Jumlongluk Sriko was born on 3rd Mar, 1992, in Ubon ratchathani, Thailand. She graduated Bachelor degree of Science in Biology from Srinakharinwirot University, Thailand in 2014. She achieved the best award for outstanding oral presentation at 45th Annual Meeting of The Physiological Society of Thailand.