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Effects of curcumin on oxidative stress, inflammation and apoptosis in L-arginine induced acute pancreatitis in mice

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ธิดารัตน์ จริงจิตร : ผลของเคอร์คูมินต่อภาวะออกซิเดทีฟสเตรส การอักเสบ และ การตายแบบอะโพโทซิสของเซลล์ในหนูไมซ์ที่ทำให้เป็นตับอ่อนอักเสบเฉียบพลันด้วยแอลอาร์จินีน (Effects of curcumin on oxidative stress, inflammation and apoptosis in L-arginine induced acute pancreatitis in mice) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดวงพร วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ. ประสงค์ ศิริวิริยะกุล, 61 หน้า.

แอลอาร์จินีนเป็นกรดอะมิโนที่ใช้ในการทำให้เกิดตับอ่อนอักเสบเฉียบพลันในหนู เคอร์คูมินเป็นสารออกฤทธิ์จากเหง้าของต้นขมิ้นชัน ได้ถูกนำมาใช้ในการรักษาอาการอักเสบ และมีฤทธิ์ทางชีวภาพที่หลากหลาย เช่น ฤทธิ์ต้านการอักเสบ และต่อต้านอนุมูลอิสระ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของเคอร์คูมินต่อภาวะออกซิเดทีฟสเตรส การอักเสบ และการตายแบบอะโพโทซิสของเซลล์ ในภาวะตับอ่อนอักเสบเฉียบพลัน โดยแบ่งหนูไมซ์เพศผู้ออกเป็น 4 กลุ่ม กลุ่มที่ 1 กลุ่มควบคุมหนูจะได้รับ DMSO 1% กลุ่มที่ 2 กลุ่มตับอ่อนอักเสบเฉียบพลัน ได้รับแอลอาร์จินีน 450 มิลลิกรัม/100กรัม ต่อน้ำหนักตัว ฉีดทางช่องท้อง 2 ครั้ง ห่างกัน 1 ชั่วโมง กลุ่มที่ 3 กลุ่มได้รับเคอร์คูมินขนาดต่ำ 50 มิลลิกรัม/กิโลกรัม ต่อน้ำหนักตัว โดยฉีดเข้าไปในช่องท้อง 1 ชั่วโมงก่อนการฉีดแอลอาร์จินีน วันละ 1 ครั้งต่อวันเป็นเวลา 3 วัน และกลุ่มที่ 4 กลุ่มได้รับเคอร์คูมินขนาดสูง 200 มิลลิกรัม/กิโลกรัม ต่อน้ำหนักตัว โดยฉีดเข้าไปในช่องท้อง 1 ชั่วโมงก่อนการฉีดแอลอาร์จินีน วันละ 1 ครั้งต่อวันเป็นเวลา 3 วัน หลังจากนั้นเก็บเนื้อเยื่อตับอ่อนเพื่อประเมินลักษณะทางพยาธิวิทยา และสำหรับย้อมพิเศษทางอิมมูโนฮีสโตเคมีของ NF-kB, การตายแบบอะโพโทซิสของเซลล์ และเอนไซม์ไมอีโลเพอร์ออกซิเดส เนื้อเยื่ออีกส่วนแช่แข็งจนกว่าจะใช้สกัดโปรตีนเพื่อการตรวจ 4-HNE และเก็บเลือดเพื่อวิเคราะห์ซีรั่มอะไมเลส ผลการทดลองในกลุ่มตับอ่อนอักเสบเฉียบพลัน พบว่าน้ำหนักตัวลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ในขณะที่กลุ่มเคอร์คูมินน้ำหนักตัวไม่เปลี่ยนแปลง ส่วนระดับซีรั่มอะไมเลส, เอนไซม์ไมอีโลเพอร์ออกซิเดส, NF-kB, การตายแบบอะพอพโทซิสของเซลล์ และการแสดงออกของ 4-HNE พบว่ามีค่าเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในกลุ่มตับอ่อนอักเสบเฉียบพลันเมื่อเปรียบเทียบกับกลุ่มควบคุม และลดลงอย่างมีนัยสำคัญในกลุ่มที่ให้เคอร์คูมินทั้งขนาดต่ำและขนาดสูง นอกจากนี้พบว่ากลุ่มตับอ่อนอักเสบเฉียบพลันเนื้อเยื่อมีการอักเสบบวม และพบการตายของเซลล์ไขมันในระดับรุนแรง ในขณะกลุ่มที่ให้เคอร์คูมินทั้งขนาดต่ำและขนาดสูงมีผลทางพยาธิวิทยาดีขึ้น อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างการให้เคอร์คูมินขนาดต่ำและขนาดสูง ดังนั้นสรุปได้ว่าเคอร์คูมินสามารถลดตับอ่อนอักเสบเฉียบพลันได้โดยผ่านคุณสมบัติต้านภาวะออกซิเดทีฟสเตรส ต้านการอักเสบ ต้านการตายแบบอะโพโทซิส และทำให้พยาธิสภาพของตับอ่อนดีขึ้น

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THIDARAT CHINGCHIT: Effects of curcumin on oxidative stress, inflammation and apoptosis in L-arginine induced acute pancreatitis in mice. ADVISOR: PROF. DUANGPORN WERAWATGANON, M.D., CO-ADVISOR: ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D., 61 pp.

L-arginine (L-arg) is an amino acid that has been used to induce acute pancreatitis (AP) in mice. Curcumin is an active ingredient constituent of rhizomes of the plant Curcuma longa Linn. It has been used for the treatment of inflammatory conditions and shown to exhibit a variety of biological activities such as anti-inflammatory and anti-oxidant. The present study aims to examine the effects of curcumin on oxidative stress, inflammation and apoptosis in AP. Male ICR mice were randomly divided into 4 groups. 1) Control group, mice will be received i.p. of 1% DMSO as a vehicle. 2) AP group, mice will be received two i.p. of L-arg 450 mg/100 g BW at an interval of 1 h apart. 3) AP + low dose curcumin group, mice will be received curcumin 50 mg/kg bw by i.p. 1 h before L-Arg injection and then once daily for 3 days. 4) AP + high dose curcumin group, mice will be received curcumin 200 mg/kg bw by i.p. 1 h before L-Arg injection and then once daily for 3 days. The mice were sacrificed at 72 h. The pancreatic tissue was fixed for histological evaluation and for NF-kB, apoptosis and MPO immunohistochemistry. The second part was frozen until using protein extraction for 4-HNE examination. Blood sample will be collected for amylase analysis. In AP group, body weight was significantly reduced than control group. While in curcumin group, body weight was maintained. The serum amylase, number of MPO positive cells, NF-kB positive cells, TUNEL positive cells, and 4-HNE expression were increased significantly in AP group when compared with control group, while decreased in low and high dose of curcumin group. Moreover, the AP group showed inflammation, edema and fat necrosis of severe activity of AP. While curcumin low and high dose group showed significantly attenuating histopathological score. However, there is no significant difference between low and high dose of curcumin. In conclusion, curcumin could attenuate AP via anti-oxidant, anti-inflammation and anti-apoptosis property leading to improve pancreatic damage.

Field of Study: Medical Science
Academic Year: 2017

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

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CHAPTER I

INTRODUCTION

1.1 Rationale

The pancreas is an organ committed to active production, storage and secretion of digestive enzymes as inactive zymogens. In severe acute pancreatitis, the tissue is affected by premature activation of these potentially harmful zymogens inside the acinar cells leading to pancreatic necrosis. Associated with pancreatic cell damage, extensive inflammation develops first locally in pancreas and later systemically. Most cases of, it works a mild pathway without complications. Whatever, approximately 25% patients develop severe acute pancreatitis (SAP), this can lead to systemic inflammatory response syndrome and consequent multiple organ dysfunction syndrome, and the mortality rate is up to 40% (1).

Acute pancreatitis (AP) is an acute inflammatory disorder of the pancreas with variable involvement of other native tissues. The main commonly enzymes of AP are increased concentration of serum lipase and amylase (2). AP is a reversible inflammatory disorder that changes in severity, edema and fat necrosis to widespread hemorrhagic parenchymal necrosis (3). In Western countries, AP is a common disease with increasing effect, the most common caused by consume too much alcohol gallstone, some medicines, and invasive techniques of the biliary and pancreatic ducts (4). The pathophysiology of AP is not clear, and its clinical course is uncertain and treatment of severe acute pancreatitis is supportive, as no specific therapy is currently available.

Curcumin is a main complement of rhizomes of the herb turmeric is a yellow pigment responsible for the *Curcuma longa*. It has been used for the treatment of inflammatory conditions (5) and shown to present a diversity of pharmacological activity. such as anti-inflammatory (6) and anti-oxidant (7). Previously, curcumin have demonstrated that inhibits the activation of transcriptional factors (for example, NF-kB, and the production of monocyte chemotactic protein (8), TNF-α (9) and IL-5

production (10)). However, the study about the effects of curcumin on tissue injury, oxidative stress, proinflammatory mediators and pancreatic enzymes in AP is still required. The present study aims to examine the effects of curcumin on oxidative stress, inflammation and apoptosis in AP mice.

1.2 Research questions

1. Can curcumin attenuate acute pancreatitis induced by L-arginine in mice model?
2. Does the protective effect of curcumin act by reducing inflammatory cytokines, anti-oxidant and decreasing apoptosis?

1.3 Research objectives

1. To investigate the effect of curcumin on apoptosis and histopathology in acute pancreatitis induced by L-arginine in mice.
2. To investigate the effect of curcumin on inflammatory cytokines in acute pancreatitis induced by L-arginine in mice.
3. To investigate the effect of curcumin on oxidative stress in acute pancreatitis induced by L-arginine in mice.

1.4 Hypothesis

Curcumin can attenuate acute pancreatitis in mice by decreasing oxidative stress, reducing inflammatory cytokines, decreasing apoptosis, and improving histopathology.

1.5 Assumption

All animals are not different.

1.6 Keywords

Curcumin, L-arginine, Oxidative stress, Inflammation, Apoptosis, Acute pancreatitis

1.7 Research design

Animal experimental design.

1.8 Expected benefit and application

This study will demonstrate the mechanism of curcumin against AP induced by L-arginine in mice on oxidative stress, anti-inflammation and apoptosis. These may be useful for the clinical application of AP treatment.

CHAPTER II

LITERATURE REVIEWS

Acute pancreatitis (AP) is an inflammatory situation with a wide spectrum of a severity disease with local and systemic complications (11). Severe acute pancreatitis (SAP) is related with a mortality rate of 10%–30% (12). The evidence of pathophysiology of AP has demonstrated that trypsin activation, inflammation, and microcirculatory disturbances (13). Trypsin activation appears in early and transient process, while inflammation in the pancreas continues for a long period and could help target treatment (14). Trypsin which is activated within the acinar cell is the major player to damage the acinar cell in AP. Although a large section of evidence reports that injury to the pancreas in each apoptosis or necrosis of acinar cells (15, 16). The makers of inflammation are neutrophil infiltrations, and compelling evidence has documented that neutrophils might have key roles in the induction of AP (17). Neutrophils release powerful substances, including free radical (18), competent of trypsin activation and resulting in tissue injury in inflammation of the pancreas. The two types of AP are mild pancreatitis, which is inflammation and edema of the pancreas and severe pancreatitis, which the necrosis of pancreas and nearby organs may become injured (19).

2.1 Pathology of acute pancreatitis

Acute pancreatitis is an inflammatory disease which occurs in the pancreatic acinar cells (20) when there is abnormal the pancreatic enzymes in the pancreas was activated. This occurs via the zymogens activation called a proenzyme, trypsinogen is an inactive precursor of an enzyme within the pancreas. During progression of AP, trypsinogen comes into communicate with lysosomal enzymes; cathepsin B, the lysosomal protease can activate trypsinogen into trypsin (21). The trypsin not only activates trypsinogen but also activates another enzyme. The activation of these pancreatic enzymes results in inflammation, edema, vascular injury, and cell death. Pancreatic cells death occurs through necrosis and leads to cell damage or

apoptosis. The mechanisms of acinar cell death are mediated by caspases that determine program cell death and anti-necrosis functions during pancreatitis such as prevented trypsinogen activation and by inhibited the inhibitors of apoptosis (IAPs). The initial injury is a comprehensive inflammatory response by reason of pancreatic cells synthesizing and secreting inflammatory cytokines: primarily TNF-α andIL-1. A hallmark of AP is an apparition of the inflammatory response, that is the recruitment of leucocytes such as neutrophils (22).

The pathogenesis of AP is not clear. Nevertheless necrosis, neutrophil infiltration, microvascular dysfunction and free radical are the main composition of pancreatitis. This is indicated by edema, vacuolization, inflammation, cell necrosis, leukocyte and digestive proteases (23). The AP releases reactive oxygen, nitrogen species and many kinds of inflammatory cytokines. Many factors are amenable for the pancreatitis such as alcohol consumption and gallstones. Repeated injures of AP have the potential to develop chronic pancreatitis or pancreatic cancer indicated by fibrosis and loss of pancreatic function (24).

2.2 Oxidative Stress

Free radical and inflammation have a play critical roles in the pathogenesis of AP and implicated in multiple disorders, oxidative stress is consist of a combination of increased production of reactive oxygen species (ROS) and impaired antioxidant potential (25). AP results in an oxidative stress which amplifies the inflammatory process via the enrollment and release of pro-inflammatory mediators cause a systemic inflammatory response (26). Reactive oxygen species contain a group of highly reactive oxygen metabolites are generated oxygen metabolism, produced by leukocytes and macrophages activation, and empowered weapons against invading microorganisms. Under normal circumstances, free radical are produced in quantities too numerous to be scavenged by the antioxidant defenses, which included the enzymes superoxide dismutase, catalases and glutathione peroxidase (27). However, the production of ROS can cause acinar cell injury, dysfunction by attacking biomolecules and transcription factors (28).

Whereas excessive amounts of ROS can trigger lipid peroxidation, it was indicated a major injurious mechanism in cells that influences cellular membrane integrity, signal transduction and compromise of cell viability. The measurements of lipid peroxidation in mitochondria, cells and tissue are necessary to understand scope of oxidative stress (29). Esterbauer *et al.* reported that the major products of lipid peroxidation are lipid hydroperoxides (LOOH). Moreover, there has been found the different aldehydes that can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (30, 31).

2.3 Apoptosis

Cellular apoptosis has been characterized by morphological changes, membrane blebbing, and protease activation. In contrast, cells undergoing necrosis show to be ruptured at plasma membrane and associate to apoptotic pathways. Intrinsic pathways include mitochondria targeting substances activate by environmental stress, ultraviolet radiation, and oxidative stress. TNFα, might also elevate the activation of NF-kB that lead to apoptosis. In contrast, overexpression of Bcl-2 lead to inhibition of extrinsic mediated apoptosis and stimulate anti-apoptotics are Bcl-2 family proteins. Oxidative stress is an intrinsic factor which can lead program cell death through direct interactions with mitochondria. It has been shown that mitochondrial membrane was depolarized H_2O_2 the mitochondrial membrane, resulting to cytochrome c release, caspase-3 activation, and DNA damage (32, 33). Presently, extrinsic pathways lead to apoptosis though stimulation of trans-membrane receptors by extracellular signals such as TNFR and Fas (34). These two pathways have a play key role in initiating apoptosis via mechanisms relate to alterations of mitochondria. However, recent studies showed that pancreatitis-associated protein (PAP)-1 is a key factor in redox-regulated programmed cell death during the pathogenesis of AP. Induction of oxidative stress triggers the binding of PAP-I promoter, indicating that reactive oxygen species can mediate its de novo synthesis (35). Overexpression of PAP-1 could inhibit reactive oxygen species induced programmed cell death. Their finding indicated that PAP-1 is related to the reactive

oxygen species induced apoptotic process during AP (36), and so forward verifying the role of PAP in redox regulation of apoptosis during a part of AP.

2.4 4-Hydroxynonenal (4-HNE)

4-HNE is cytotoxic product, which is produced from the pathway of bioactive marker of lipid peroxidation. This cause by ROS or chemicals which may exert a genotoxic effect in humans (37). 4-HNE is the most essential products because they are conducted in relatively large numbers. Accordingly, the 4-HNE and aldehyde act as second messengers of reactive oxygen species. They are one of physiological marker for lipid peroxidation and oxidative stress. Moreover, they are chemotactic aldehydic end-product of lipid peroxidation and essential lipid peroxidation product (38). Therefore, 4-HNE is considered as a signaling molecule relate to control of several transcription factors such as NF-kB, activating protein-1 (AP-1), and peroxisome-proliferator-activated receptors (PPAR), in cell differentiation, cell survival, degeneration, autophagy, programmed cell death, and necrosis (39).

2.5 Myeloperoxidase (MPO)

Myeloperoxidase is a peroxidase enzyme generated from primary granules in cells of the myeloid lineage of the promyelocyte and especially found in neutrophils, usually considered to be a marker of a marker for neutrophil infiltration in AP (40). Moreover, MPO activity can reflect the severity of inflammation (40). MPO activity is interestingly elevated with the induction of AP, and the elevated of pancreatic MPO activity demonstrated the progressive aggravation of pancreatic injury.

2.6 Amylase

Enzymes are produced from the exocrine acinar cells such as amylase, lipase, and trypsinogen inactive form, have been tested as biomarkers of AP. Serum amylase is the most widely used clinical practice.

Amylase is a glycoside hydrolase primarily synthesized in the pancreas and salivary glands. In acute pancreatitis, serum amylase rapidly increases in 3–6 hours of the initial attack of disease, exhibits a half-life of 10–12 hours, remains elevated for

3–5 days, so the concentration can normalise within 24 hours (41, 42). The reference range is normally 20–300 U/L, but does vary with age and gender. The sensitivity is also influenced by other factors, including the timing of the test and the because of the AP.

2.7 The role of nuclear factor kB (NF-kB) activation in acute pancreatitis

NF-kB is a transcription factor, commonly reserve in several cell types and initiating the expression of a large number of genes related to inflammation, tissue damage, and repair (43, 44). NF-kB is one of the groups of structurally related transcriptional proteins (45). These proteins have 2 subclasses. The first contains RelA (also known as p65), RelB, and c-Rel. These proteins are comprised of transcription-modulating domains that initiate gene transcription. The second class consist of NF-kB1 (p50 and its precursor p105) and NF-kB2 (also known as p100), which have no transcription-modulating domains. These subunits of NF-kB perform as either homodimers or heterodimers. In the pancreas, the predominant form of NF-kB is a p65/p50 heterodimer (46). Usually, NF-kB resides in the cytoplasm in an inactive form, which suppressed by kB (IkB). The major groups of IkB are mostly found in IkBα and IkBβ forms. While cells are stimulated by cytokines, lipopolysaccharide (LPS) or ROS, IkBs are immediately phosphorylated in particular serine residues by IkB kinase (IKK) (47). IKK activity depends on its phase of phosphorylation and work as a point of convergence for NF-kB signaling [NF-kB-inducing kinase (NIK) and protein kinase B (Akt)]. When IkB degradation, nuclear localization signals (NLS) induce NF-kB transcription factor translocate into the nucleus. To regulate the transcription of several genes involved inflammation and apoptotic responses. Moreover, inflammatory targets of NF-kB consist of cytokines, chemokines, adhesion molecules and programmed cell death. Multiple genes are linked to the development and progression of AP. In addition, NF-kB related with several anti-apoptotic molecules including BCL-XL and cellular inhibitors of programmed cell death (Figure 2.1) (45).

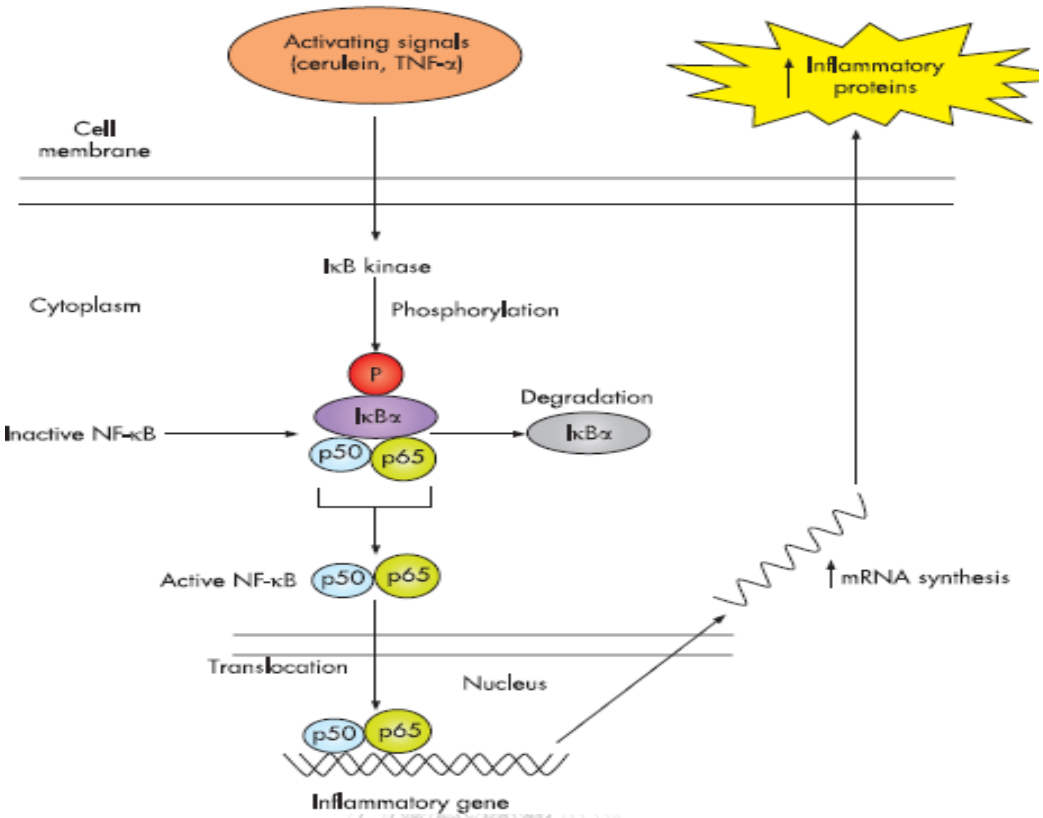


Figure2. 1 Schematic diagram illustrated NF-kB activation in pancreatic acinar cells in response to cerulein and inflammatory cytokines. Upon stimulation of cells, IkB kinase rapidly phosphorylates IkBα and IkBβ which leads to their degradation and release of active NF-kB. This allows NF-kB to translocate to the nucleus and bind to its cognate DNA and increase the transcription of several important proinflammatory genes (45).

2.8 L-arginine-induced acute pancreatitis

Animal models of AP which are same as the human condition and important to enhance understanding the complex mechanisms and in inventing therapeutic processes of AP. Multiple animal models of AP can be categorically divided into invasive models and non-invasive models. The example of invasive models are duct obstruction/ligation, closed duodenal loop, retrograde ductal infusion (e.g. bile acids, enterokinase, trypsin) into the pancreatic duct via the ampulla of Vater in the animal is known to induce pancreatic inflammation, and ischaemia/reperfusion model. Furthermore, the example of non-invasive model is hormone-induced model (e.g.

cerulein, a decapeptide analogue of cholecystokinin (CCK), which was used to completely evoke the AP in mice (48). Another non-invasive model is L-arginine model, which conduct acute necrotizing pancreatitis by administration of L-arginine high dose. L-arginine-induced model has high reproducibility and capability to produce selective dose-dependent pancreatic acinar cell necrosis. L-arginine induced AP is the most popular method because this technique is easily to perform, be inexpensive and generate histological changes similar to human pancreatitis.

L-arginine (L-arg) is an amino acid which was used to induce severe necrotizing AP in rats (49). The first study have been investigated the effect of an L-arg overdose on different tissues in rats by Mizinuma et al. (50). When using a single dose of L-arg 500 mg/100 g body weight (BW) by intraperitoneal (i.p.) injection adipose tissues around the pancreas found necrosis, without pathological change in other organs (liver, kidney, spleen, thymus, lung, heart, intestine, testis), except for the pancreas. Because of the weight of the pancreas was doubled within the first 24 hours by the effect of pancreatitis. Previous study showed that high dose i.p. injection of 500 mg/100 g BW L-arg can cause acute necrotizing pancreatitis in rats and mice (25, 51), and 70-80% of the pancreatic acinar cells were necrotized within 3 days (52). After that Kishino *et al.* observated the pancreas by electron microscopy, they found that distension of the endoplasmic reticulum changes in acinar cells after 24 hours and zymogen granules were degraded. After L-arg injection, isolation and acinar cells necrosis were occurs. The first pathological changes of the acinar cells might be linked to metabolic alterations participatory with the endoplasmic reticulum. The observation was that after injected i.p. L-arg overdose toxic to the rat pancreas (53). Tani *et al.* examined this work by investigating the effect of L-arg on the pancreas. They obviously demonstrated that high dose of L-arg cause a severe acute necrotizing pancreatitis (52). Many previous researches about the pathomechanisms of AP used 250 mg/100 g BW of L-arg 2 times at an interval of 1 hour (54-56). The dose- and time-dependent of the effects of L-arg can be used to study the different phases of AP.

Various doses of L-arg (250-500 mg/100g BW) are known to induce AP in experimental animals (50, 57). The pathomechanism of L-arg induce AP is still unclear, particularly regarding the firstly cases generation of the disease. There are two key enzymes which are related to the metabolism of L-arg; nitric oxide synthase (NOS) and arginase (Figure 2.2) (58). These enzymes catalyze the transformation of L-arginine to nitric oxide and L-citrulline. The iNOS activity was elevated after L-arg injection at 24 hours (59, 60). The mechanism underlying L-ornithine-induced pancreatitis is unknown. It is known that L-ornithine can serve as substrate for ornithine decarboxylase, the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. Polyamines are essential for normal cell growth and development. Pancreas appears to be the richest source of spermidine in the mammalian body. Polyamines thus play a role in major biological membrane functions (61). In pancreas, polyamines localize in zymogen granules and the levels alterations have been involved with apoptosis (62). L-arg large doses have been demonstrated that paradoxically decrease spermine and spermidine while increased putrescine levels resulting from increasing polyamine catabolism (63). Furthermore, reduction of spermine and spermidine are also participated in human acute necrotizing pancreatitis (63). Alternation of pancreatic polyamine levels tend to decrease an inhibition of DNA and protein synthesis, which lead to acinar cell death. In addition, the mechanisms of L-arg to induce AP are through excess production of nitric oxide, lipid peroxidation and inhibition of protein synthesis (64). The changes range between edema, inflammatory infiltration, acinar degranulation to necrosis (65).

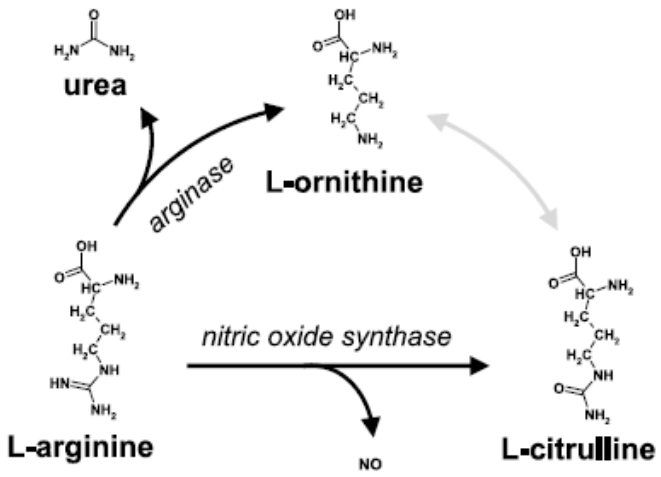


Figure2. 2 Two key enzymes involved in the metabolism of L-arginine. Nitric oxide synthase catalyzes the conversion of L-arginine to nitric oxide and L-citrulline. Arginase hydrolyzes L-arginine to L-ornithine and urea (58).

2.9 Curcumin

Curcumin or diferuloylmethane, the pigment was extracted from the rhizome of herb turmeric (*Curcuma Longa*) (Figure 2.3). The yellow principle bioactive component of turmeric is the biological source for curcuminoids, which are chemically related to its principal ingredient of curcumin. Curcuminoids are present in 3–5% of turmeric. The three main of curcuminoids that present in turmeric are curcumin (77%), demethoxy curcumin (17%), and bisdemethoxy curcumin (3%) (66). Curcumin is an active ingredient responsible for the biological acitivity, a yellow powder that insoluble in water due to its polyphenol structure, but soluble in organic solvents such as ethanol, methanol, dimethylsulfoxide (DMSO), and acetone (67). Curcumin exists in enol and β-diketone forms. Certainty, curcumin in solution contain primarily in its enolic form (68) has an important carrying on the radical-scavenging capacity of curcumin. Curcumin is stable at acidic pH but unstable at neutral and basic pH. Most curcumin (>90%) is rapidly degraded within 30 minutes of placement in phosphate buffer systems of pH 7.2 and found very slow at pH 1–6

(69), as commonly discovered in the stomach. There are several experimental studies shown that the protective of curcumin has been found anti-inflammatory, antioxidant, and anti-fibrosis effects. (70). In addition, it provides inhibitory effects on both acute and chronic inflammation by decreasing neutrophils infiltration, inhibiting lipid peroxidation, and downregulating NF- kB (71).

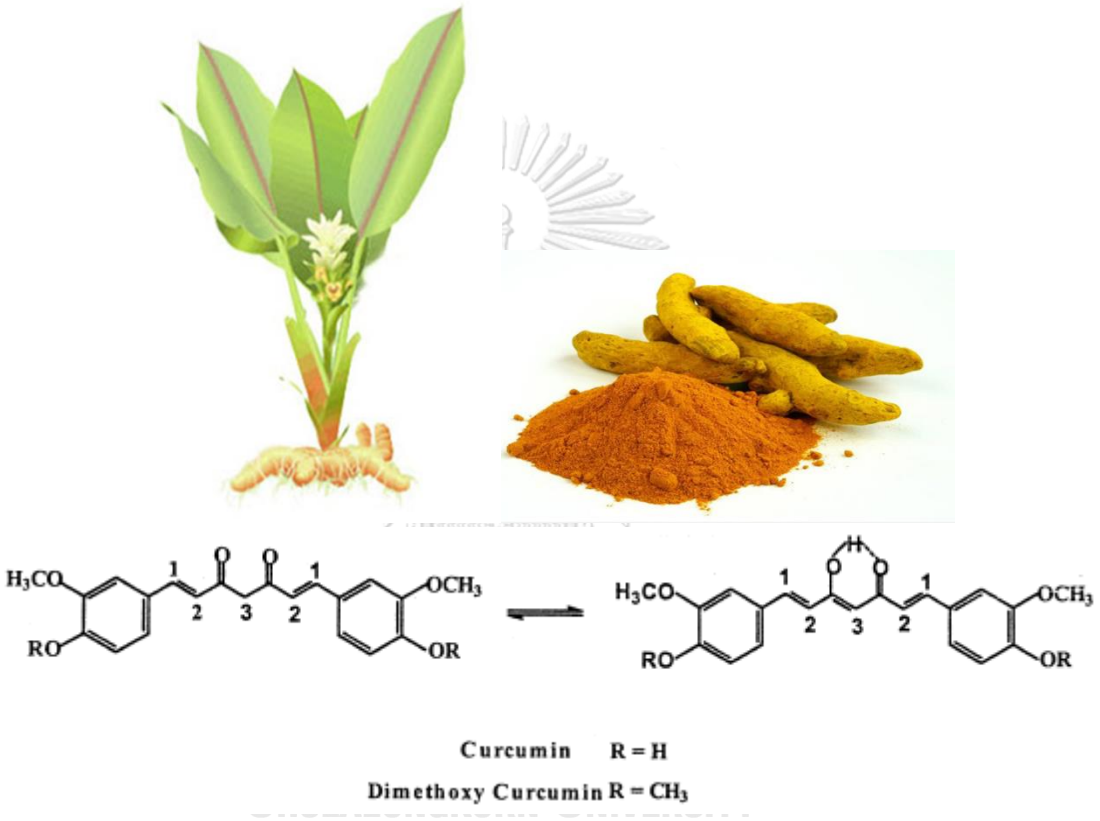


Figure2. 3 Structures of curcumin (66)

Pharmacokinetic Study and Safety

Curcumin has been reported various beneficial activities with less toxicity in high dose. Lao *et al.* (72) conducted a dose escalation study to determine the maximum tolerable dose and safety of a single oral dose of curcumin in 34 healthy volunteers. The volunteers were given amplifying doses of curcumin ranging from 500 to 12,000 mg, and safety was assessed for 72 hours after administration. Furthermore,

twenty-four participants completed the trial, seven of whom experienced minimal toxicity that did not appear to be dose-related. In another study, curcumin at doses ranging from 450 to 3600 mg/day, treated for 1 to 4 months was associated with nausea and diarrhea and caused an increase in serum alkaline phosphatase and lactate dehydrogenase contents in human participants (73).

Wahlstrom B and Blennow G were administrated curcumin orally in animal (74). They found that a dose of 1000 to 5000 mg/kg BW which provided to rats did not cause any adverse effects and it was excreted about 75% in the feces, while traces appeared in the urine. Moreover, measurements of blood plasma levels and biliary excretion demonstrated that curcumin was poorly absorbed by the gastrointestinal tract. Pan MH *et al*. reported the pharmacokinetic properties of curcumin in mice. After i.p. administration of curcumin 100 mg/kg in mice, about 2.25 microgram per millilitre (μg/mL) of curcumin presented in the plasma within the first 15 minutes (75).

Anti-inflammatory properties

Curcumin was reported that it inhibits the activation of NF-kB pathway in human myelomonoblastic leukemia cells and phorbol ester-induced c-Jun/AP-1 activation in mouse fibroblast cells (8). The mechanism of curcumin inhibits NF-kB activation was not understood, but related to inhibition of IkB degradation (76, 77). The authors examined the effect of curcumin on IEC gene expression and observed the modulatory potential of curcumin action on the IkB/NF-kB signaling pathway. Previous studies have shown that curcumin blocks IEC gene expression by inhibiting the signal leading to IKK activation without directly interfering with NIK or IKK. Blockade of IKK activation leaded to inhibition phosphorylation and degradation of IkBα and NF-kB activation (Figure 2.4). Understanding the mechanisms of action of many anti-inflammatory food products might induce to the production and acknowledgment of new improved therapies for inflammatory disorders.

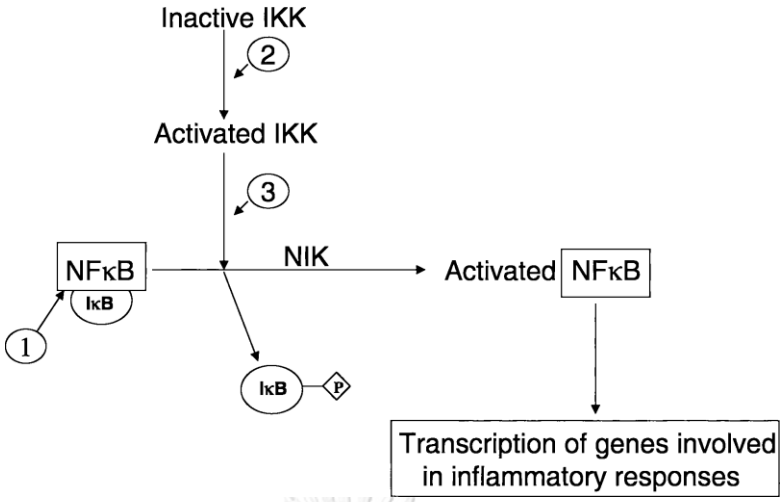


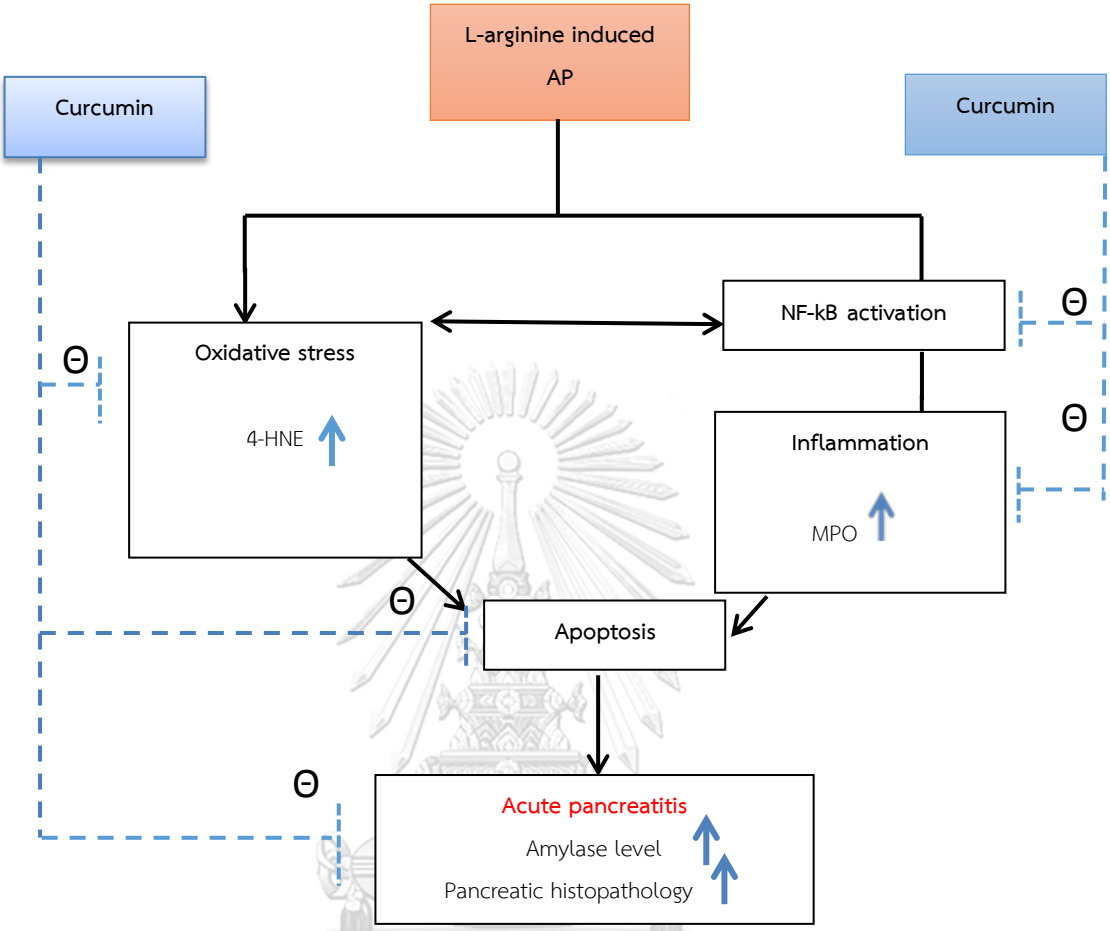
Figure2. 4 Curcumin interferes with inflammatory pathways by blocking the transcription factor NF-kB. The numbers 1, 2, and 3 represent the pathways that are described to be affected by curcumin as detailed in Brennan et al., 1998, Jobin et al., 1999 and Plummer et al., 1999, respectively. NF-kB: Nuclear transcription factor required for transcription of genes involved in the inflammatory responses; IkB: Cytosolic inhibitor of NF-kB; NIK: NF-kB inducing kinase; IKK: IkB kinases (77).

Numerous studies have documented for the anti-inflammatory effects of curcumin. Initial research by Srimal *et al*. demonstrated curcumin's anti-inflammatory action in a mouse and rat model of carrageenan induced paw edema (6). In mice, curcumin inhibited edema at doses between 50-200 mg/kg (71). A 50% reduction in edema was achieved with a dose of 48 mg/kg BW (73). In two rat models of experimentally-induced pancreatitis, curcumin decreased inflammation by markedly decreasing activation of NF-kB and AP-1 as well as inhibiting mRNA induction of IL-6, TNF-α, and iNOS in the pancreas. In both cerulean- and ethanol-induced pancreatitis, curcumin had inhibitory effects on inflammatory mediators resulted in improvement in disease severity as measured by histology, serum amylase, pancreatic trypsin, and neutrophil infiltration (78).

Antioxidant properties

The activity of curcumin is scavenges reactive oxygen, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) radical, and nitrogen free radicals from activated macrophages. (79, 80). It is a curcumin having two o-methoxy phenolic OH groups next to the α, β-unsaturated β-diketone (heptadiene-dione) moiety. The free radical scavenging activity of curcumin can occur each with the phenolic OH group or from the CH_2 group of the β-diketone moiety. ROS can go through electron transfer or inference H-atom from either of these two sites. Recently, there are some argument reports supporting the two different sites of attack for the ROS. Several reports based on pulse radiolysis and other biochemical processes associated the antioxidant activity of the phenolic OH group (80). But Jovanovic *et al*. demonstrated that hydrogen abstraction from the methylene CH_2 group is important for the remarkable antioxidant properties of curcumin (81). Afterwards, Barclay *et al* investigated the inhibition of styrene oxidation by a number of curcumin derivatives showed that the H atom from the phenolic OH is answerable for the capacity (82).

Conceptual framework



CHAPTER III

MATERIALS AND METHODS

3.1 Ethical consideration

The study protocols were submitted for approval by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. The study will be conducted in accordance to the guidelines for experiment animals as suggested by National Research Council of Thailand (1999).

3.2 Materials

3.2.1 Animal Preparation

Male ICR mice weighing 25-30 g (4 weeks) were purchased from the Nation Laboratory Animal Center, Salaya Campus, Mahidol University. The animals will be housed in a controlled temperature room at 25 – 30 ºC with a 12 hours light and dark cycle. All mice will be received proper care in accordance with the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

3.2.2 Chemicals

L-arginine (Sigma Aldrich Co Pvt Ltd, USA)

L-arginine powder prepared as a solution by dissolving in 0.9% saline and the pH was adjusted to 7 with 5 N HCl.

Curcumin (Cayman Chemical Company, USA)

Curcumin powder was prepared as a solution by dissolving in 1% dimethyl sulfoxide (DMSO).

NF-kB p65 (abcam, MA, USA; 1:800)

4-HNE (R&D Systems, Inc., USA)

MPO (Dako, CA, USA)

Thiopental (Abbott, Italy)

95% alcohol (Merck, Germany)

NaCl (Merck, Germany)

$NaHCO^-_3$ (Merck, Germany)

Formalin solution

Sterile normal saline solution

Distilled water

3.3 Experimental Protocols

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

Group 1 (Control group): Mice will be received intraperitoneal (i.p.) injection of 1% DMSO as a vehicle once daily for 3 days.

Group 2 (AP group): Mice will be received two i.p. injections of L-arg 450 mg/100 g BW at an interval of 1 hour apart to induce AP.

Group 3 (AP + Low dose cur group): Mice will be received curcumin 50 mg/kg BW by i.p. 1 hour before L-arg injection and then curcumin once daily for 3 days (83).

Group 4 (AP + High dose cur group): Mice will be received curcumin 200 mg/kg BW by i.p. 1 hour before L-arg injection and then curcumin once daily for 3 days (83).

The mice were sacrificed 72 hours after L-arg injections by using i.p. injection of an overdose thiopental sodium (50 mg/kg BW). The pancreas was rapidly removed, separated from the surrounding lymph nodes and fat. Pancreatic tissue sample was divided into two parts: one part was fixed in 10% formalin solution for paraffin embedded tissues and stained with hematoxylin and eosin (H&E) for histological evaluation and immunohistochemistry for analyzes NF-kB and apoptosis. The second part was frozen in liquid nitrogen and stored at -80°C until performed protein extraction for 4-HNE examination and MPO. At the end of experiment, blood samples were collected from cardiac puncture and allowed to clot for 30 minutes, at 25 °C. Then, clotted blood were centrifuged at 3,000 x g 4 °C for 10 minutes, and the serum was stored at -80°C for amylase level. The study protocols are shown in Figure 3.1.

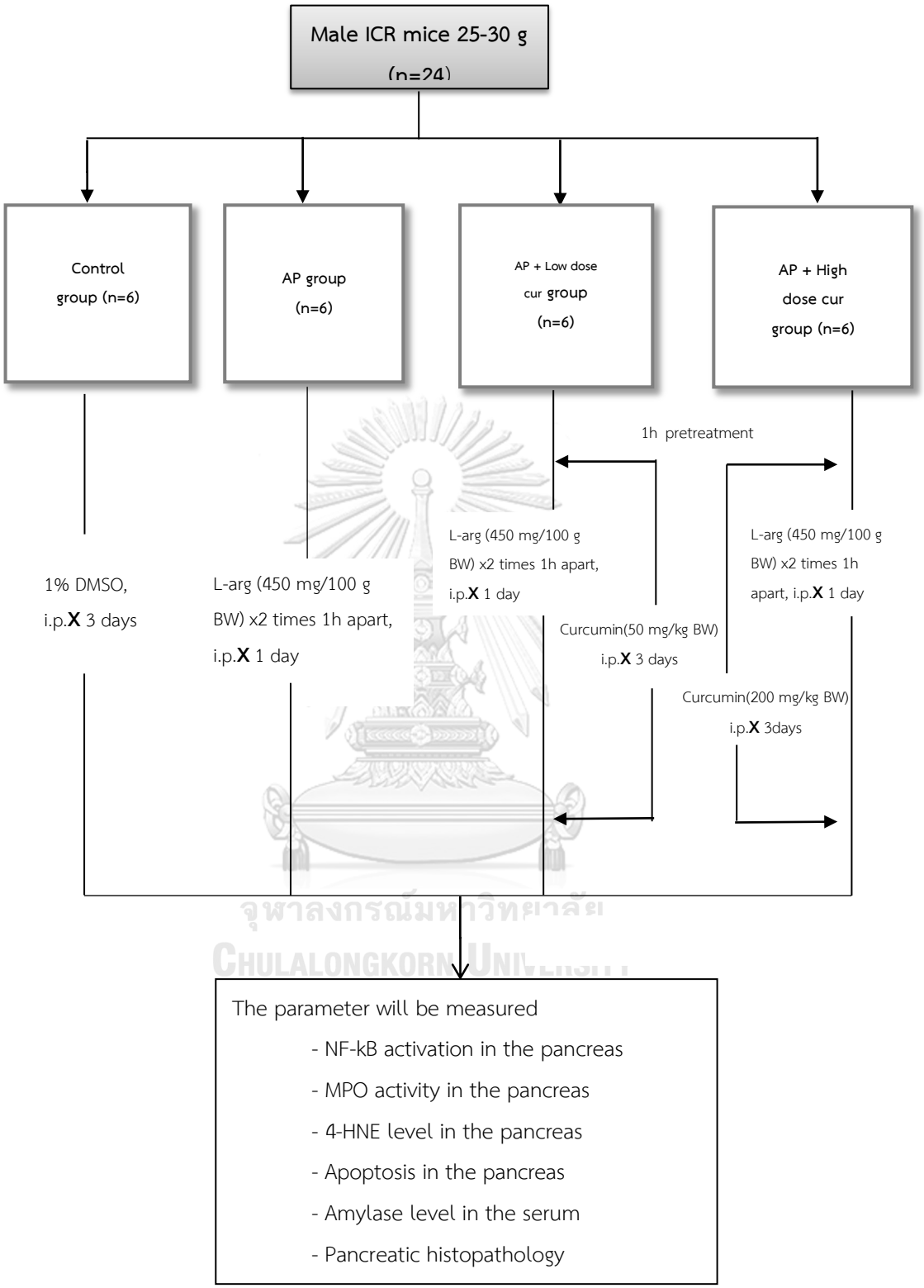


Figure3. 1 Schematic diagram of experimental protocol.

3.4 Methods

3.4.1 Measurement of serum amylase activity

Blood samples were collected from cardiac puncture. A 2 ml blood sample was extracted from each mouse and allowed to clot for 30 minutes at 25 ℃. Then, clotted blood were centrifuged at 3,000 x g 4 ℃ for 10 minutes, and stored at -80°C until amylase analysis. The levels of serum amylase were measured using a Hitachi 7600-020 automatic biochemical analyzer (Hitachi, Ltd., Tokyo, Japan).

3.4.2 Pathological examination of pancreas

- Tissue fixation: Immerse tissue in fixative (10% formalin solution) at room temperature for 24-48 hours.

- Washing: Wash in 50% ethanol for 15 minutes at room temperature.

- Dehydration: Immerse the tissue in grade series of ethanol from low to high percentage (70%, 80%, 95%, and 100%) for 15-30 minutes, twice each.

- Clearing and infiltration: Immerse the tissue in xylene for 15-30 minutes at room temperature, twice, immerse in soft paraffin (mixture of paraffin and xylene as 1:1 ratio), incubate in warm oven at 60°C for 15-30 minutes, twice, and immerse in melting paraffin at 60°C for 15-30 minutes, twice.

- Embedding: Embed the tissue in boat, leave for solidness.

- Sectioning: Cut the paraffin block as 4 micrometre (μm) thickness, then mount on glass slide and dry the tissue section at 45°C for 3-6 hours.

- Staining: Deparaffinization by placing the slide in xylene at room temperature, twice, hydration by immersing the slide in graded series of ethanol (high to low %), then immerse in distilled water before placing in Hematoxylin (H) for 5-10 minutes, washing in running tap water before double staining with Eosin (E) for 2-5 minutes, washing, and dehydration by dipping the slide in graded series of ethanol (low to high %), twice each.

- Clearing and mounting: Immerse the stained slide in xylene for 3-5 minutes, twice then mount the slide and cover glass with mounting media.

The histological slides were evaluated under light microscope (LM) by an experienced pathologist who is blinded to the experiment. Modified H. E. V. DE COCK criterion was employed for histopathological scoring in pathological evaluation (84). Details of the score assigned to the different degrees of infla*m*mation, edema and fat necrosis in AP are explained in Table 1. The sum of the points for each criterion in AP was calculated with a maximal score of 9 for AP. A score of 0 was considered normal pancreatic histopathology. A score of 1–3 was considered mild AP; score of 4–6, considered moderate AP; and score of 7–9 considered severe AP. (Table 3.1).

Table3.1 Scoring system used for characterizing inflammation in acute pancreatitis (84).

Score	Inflammation	Edema & 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 inflammatory infiltrate affecting 25-50% the pancreatic parenchyma	Moderate, present in 25-50% of the parenchyma
3	Severe neutrophilic inflammatory infiltrate affecting>50% of the pancreatic parenchyma	Severe, >50% of the parenchyma involved

3.4.3 Pancreatic Immunohistochemistry

Tissue microarrays (TMAs)

TMAs consist of paraffin blocks in which up to 1000 (85) separate tissue cores are assembled in array fashion to allow multiplex histological analysis. In the tissue microarray technique, drilled a hollow needle is used to remove tissue cores size 2.0 mm in diameter from regions of interest in paraffin-embedded tissues such as clinical biopsies or tumor samples. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut into 4 μm on a microtome, mounted on a microscope slide and then analyzed by any method of standard histological analysis. Each microarray block can be cut into 100 – 500 sections, which can be subjected to independent tests. Tests commonly employed in tissue microarray include immunohistochemistry, and fluorescent in situ hybridization.

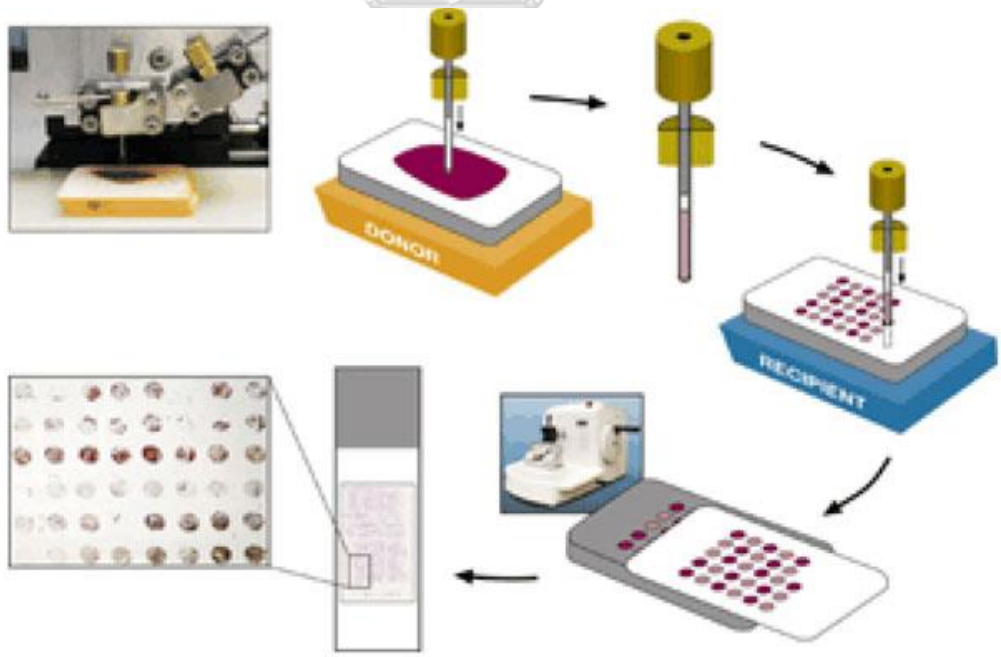


Figure3.2 TMA construction (From http://www.tissuearray.org/yale/tisarray.html).

Immunohistochemistry for expression of NF-kB

The pancreas were either frozen in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek, Inc, Torrance, CA) or fixed in 10% formalin (24–48 h) and embedded in paraffin. Immunohistochemical staining for cleaved p65 (1:50; Cell Signaling Technology) was performed in pancreatic paraffin sections. Briefly, frozen panceatic sections were deparaffinized with xylene and ethanol for 10 minutes. After water washing, sections retrieved the antigen (abcam, MA, USA; 1:800) with citrate buffer pH 6.0 in microwave for 13 minutes. Next, 3% H_2O_2 and 3% normal horse serum were performed on the slides to block endogenous peroxidase activity for 5 minutes and blocked nonspecific binding for 20 minutes, respectively. Then, the primary antibody used for NF-kB p65, a polyclonal antibody against the p65 subunit, was applied at a dilution of 1:150 for 4°C overnight. After washing, incubation with secondary antibody for 30 minutes and appropriate horseradish-peroxidase–labeled polymers (BioCare, Concord, CA) was performed. When the development of the color with diaminobenzidine (DAB) was detected, the slides were counterstained with hematoxylin. Under light microscopy, the positive stained cells showed dark brown in nucleus. The results were expressed as the number of positive stained cells per high-power field (86). The numbers of positive stained cells were counted by the Aperio ImageScope software (Leica Biosystems Imaging, Inc., MD, USA) and expressed as percentage of immunoreactive cells. The following formula for the NF-kB activation rate was utilized for statistical analysis.

NF-kB activation rate = (Total number of positive cells)/ (Total number of cells) × 100% (87).

$$\text{The percentage of immunoreactive cells (\%)} = \left(\frac{\text{number of nuclei stained cells}}{\text{number of examined cells}}\right) \times 100\%.$$

Immunohistochemistry for determination of apoptotic acinar cells

A hallmark of late apoptosis is extensive genomic DNA fragmentation that generates a multitude of DNA double-strand breaks (DSBs) with accessible 3'-hydroxyl (3'-OH) groups. This characteristic forms the basis for a well-established apoptosis detection method: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay (R&D Systems, USA) (88). TUNEL assays identify apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated addition of labeled (X) deoxyuridine triphosphate nucleotides (X-dUTPs) to the 3'-OH end of DNA strand breaks that are subsequently visualized depending on the introduced label, which can be visualized using immunohistochemical techniques. These are serving as parameter for the percentage of apoptotic cells within the analyzed cell population. The assay sensitivity strongly depends on the incorporation efficiency of the modified dUTP that is influenced by size/bulkiness of the attached label. A number of fluorescently labeled dUTPs, biotinylated dUTPs or digoxigenylated dUTPs are generally suitable substrates for TdT, but dUTP labeled with smaller labels such as bromine (BrdUTP) or an alkyne group (EdUTP) have been demonstrated to exhibit a higher incorporation efficiency and thus higher sensitivity in TUNEL assays probably due to minimal sterical hindrance (89, 90). TUNEL positive cells showed dark brown in nucleus and counted by the Aperio ImageScope software (Leica Biosystems Imaging, Inc., MD, USA) and expressed as percentage of immunoreactive cells.

$$\text{The percentage of immunoreactive cells (\%)} = \left(\frac{\text{number of nuclei stained cells}}{\text{number of examined cells}}\right) \times 100\%.$$

Immunohistochemistry for determination of Myeloperoxidase (MPO) activity

The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). In brief, formalin-fixed, paraffin-embedded samples were cut into 4 μm sections and each tissue section was deparaffinized and rehydrated with graded ethanol. The tissue was placed in EDTA antigen repairing buffer (PH 8.0) for antigen retrieval in the microwave oven. After natural cooling, tissues were put in phosphate-buffered saline (PBS) at pH 7.4 two times, each time for 5 minutes. Then tissues were incubated at room temperature (RT) for 25 minutes in the dark with 3% hydrogen peroxide solution. Slides were incubated overnight at 4°C in a humid chamber with an antibody against MPO (Dako, Denmark; 1:500). Then tissues were incubated by anti-goat secondary antibody (Dako, Denmark; 1:1000) for 30 minutes at 25°C. Finally, sections were counterstained with hematoxylin. The slides were observed under a light microscope. Immunohistochemistry and quantitative analysis of protein expression is according to previously described method (91, 92). The results were expressed as the number of positive stained cells per high-power field. All stained sections were counted in 10 randomly selected fields and values were obtained by counting the number of MPO positive cells in an average of 10 fields at magnification x400.

3.4.4 Western blotting for detection of 4-hydroxynonenal (4-HNE)

Tissue homogenate

- Homogenize the tissue in 5-10 mL of cold buffer per gram tissue.
- Centrifuge at 10000 g for 15 minutes at 40°C.
- Remove the supernatant and store on ice.
- The supernatant will have to be deproteinated before assayed for determine protein concentration by BCA method.

Total protein measurement with bicinchoninic acid (BCA) assay

The procedures of BCA assay wes performed following to BCA assay kit (Pierce®, Thermo scientifric, lnc., IL, USA).

1. Standard Preparation

Protein Standards: Dilute the contents of one Albumin Standard (BSA) ampule into several microcentrifuge tubes, preferably using the same buffer as the sample without the reducing agent. Use the following as a guide assay range (125-2,000 µg/mL) to prepare a set of standards.

2. Reagent Preparation

Working Reconstitution Buffer: Dilute the Reconstitution Buffer 1:1 with ultrapure water

Compatibility Reagent Solution: Add 100 µL of Working Reconstitution Buffer into the tube and dissolve by stirring at the bottom of the tube and pipetting up and down 15-20 times and protected from light.

BCA Working Reagent (WR): Mixed 50 parts BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B)

3. Assay Protocol

- Pipette 9 µL of each replicate of standard and unknown sample to the center of the microplate well and add 4 µL of Compatibility Reagent Solution to the sample in each well.

- Cover plate and mix on a plate shaker at medium speed for one minute. Incubate plate at 37°C for 15 minutes.

- Add 260 µL of the WR to each well. Cover plate and mix on a plate shaker for 1 minute. Incubate plate at 37°C for 30 minutes.

- Cool plate at room temperature for 5 minutes.

- Measure the absorbance of the standards and unknown samples at 562 nm on a plate reader.

- Prepare a standard curve by plotting the average blank-corrected 562 nm value for each BSA standard vs. its concentration (µg /mL). Use the standard curve to determine the protein concentration of each unknown sample.

Western blot analysis

This method was adopted from Levine *et al*. (93). For the detection of protein bound HNE 100 ml of the samples were solubilized in a solution containing 12% SDS, 6% 2-mercaptoethanol, 50 mM Tris pH 7.8, and 30% glycerol. Samples were allowed to aliquots of 10 µg for heating at 95 °C for 5 minutes protein were loaded to the gels. Electrophoresis and blotting was performed as described above. The primary antibody, raise against HNE-histidine (clone 1g4) (94), was used in a 1:500 dilution. Peroxidase-conjugated antimouse-IgG was diluted 1:3000. Chemiluminescence was detected on Polaroid-films using the ECL minicamera.

3.5 Statistical analysis

The data were expressed as mean ± standard deviation (SD). For comparison among all groups of animals, one-way analysis of variance (one-way ANOVA) and Tukey PostHoc comparisons were employed. Differences were considered statistically significant at $p < 0.05$. The data were analyzed using the SPSS software version 17.0 for windows.

CHAPTER IV

RESULTS

4.1 General appearance

General appearances of mice in each group were observed. Mice in the control group were very active whereas those in the AP group were downy with fluffy fur. After treatment with curcumin, mice became active. Curcumin improved mice physical appearance in low and high dose (Figure 4.1).

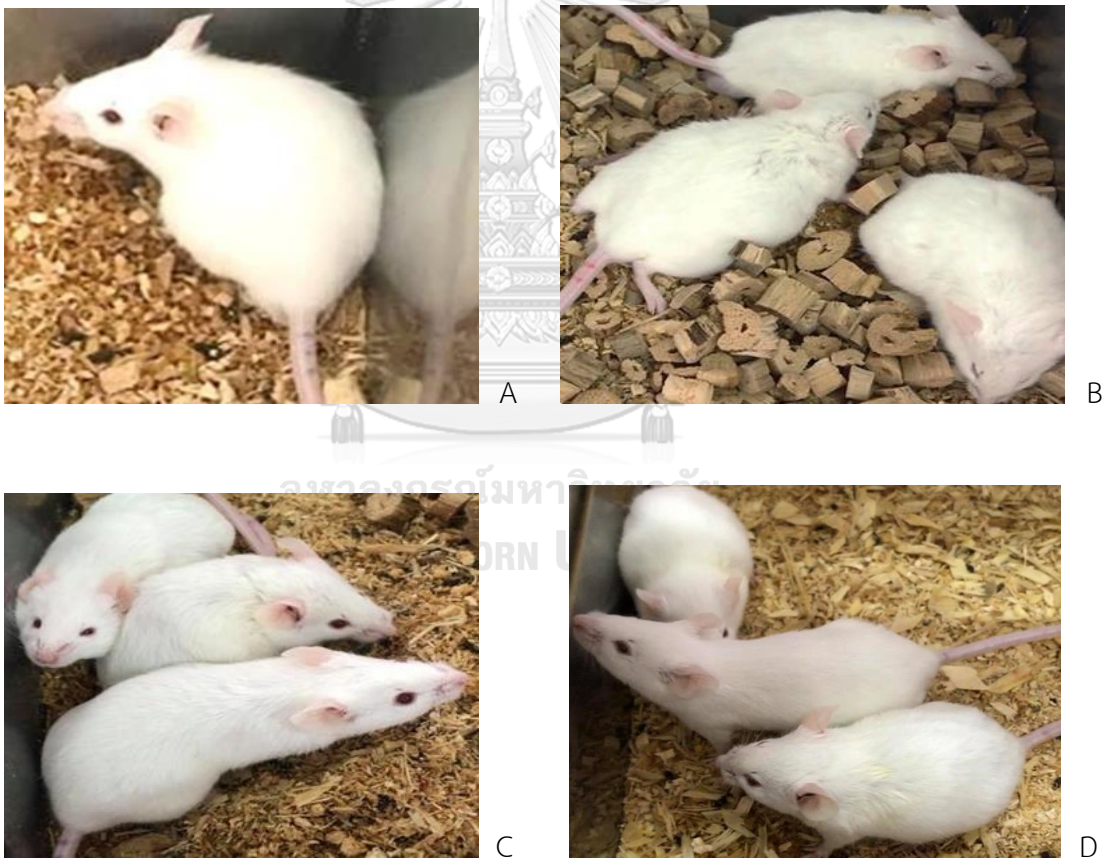


Figure4.1 The physical appearance of mice (A) Control group, (B) AP group, (C) AP+ Low dose cur group, and (D) AP+High dose cur group.

4.2 Change in body weight of mice

Male ICR mice weighing 25-30 g (4 weeks) were divided into 4 groups of experiment. At the beginning, the body weight of mice was not different among 4 groups. The changes of body weights before and after experimentation (ΔBW = Day 4 - Day 0) are summarized in Figure 4.1. The body weight of AP group was significantly decreased than control group (2.23 ± 1.52 vs -0.69 ± 0.68 g, $p<0.01$), When we treated with curcumin, in low dose cur group was significantly increased BW when compared with AP group (1.57 ± 0.43 vs -0.69 ± 0.68 g, $p<0.05$). There was no significant difference between low and high dose of cur group (1.57 ± 0.43 vs 1.15 ± 1.51g, $p>0.05$) (Figure 4.2).

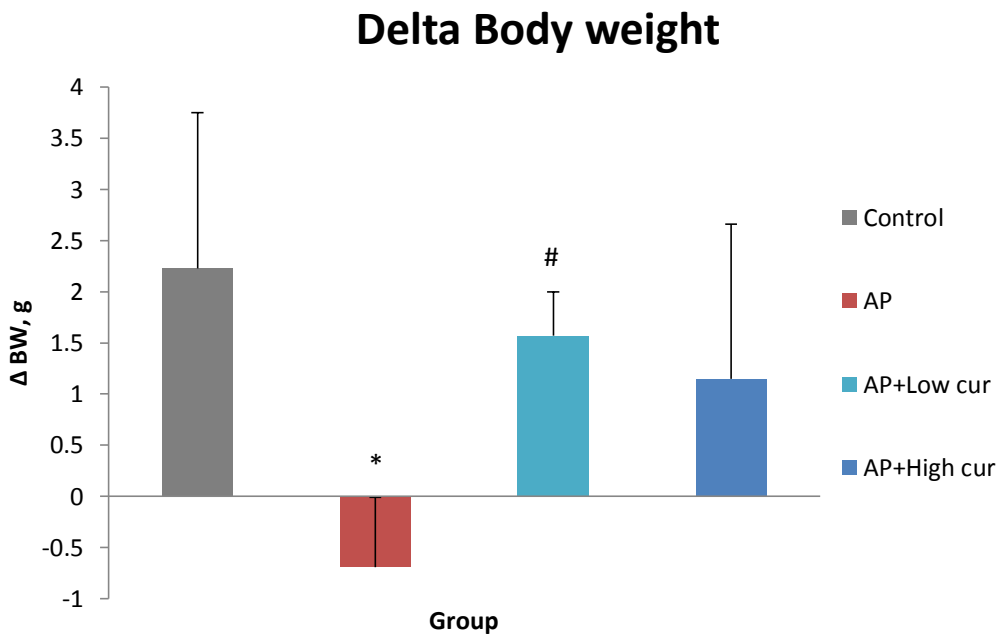


Figure4.2 Chart comparing the delta body weight (*P<0.05 versus control groups; #P<0.05 versus AP groups).

4.3 The effects of curcumin on serum amylase

The results of serum amylase levels were presented in Figure 4.3. The serum amylase in control group showed normal level (5880 ± 1561.98 U/L). These were significantly increased in AP group when compared with control group (8438.50 ± 2371.55 vs 5880 ± 1561.98 U/L, $p < 0.05$) and significantly decreased in AP + Low dose cur (8438.50 ± 2371.55 vs 4802 ± 486.85 U/L, $p < 0.01$) and AP + High dose cur groups (8438.50 ± 2371.55 vs 5069 ± 255.73 U/L, $p < 0.01$) when compared with AP group. In addition, there was no significant difference between low and high dose of cur group (4802 ± 486.85 vs 5069 ± 255.73 U/L, $p > 0.05$), respectively.

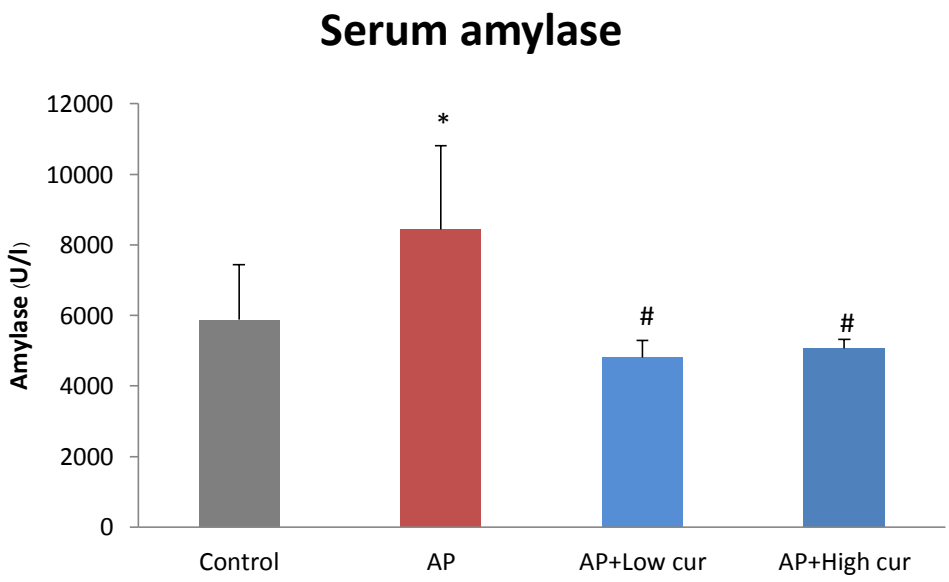


Figure4.3 Serum amylase level. Value represent mean ± SD (*P<0.05 versus control groups; #P<0.05 versus AP groups).

4.4 The effects of curcumin on pancreatic histopathology

The scores of inflammation, edema and fat necrosis were presented in Table 4.1 Values are number of animals. Each section were examined for scores of pancreatic pathology according to the criteria Modified by H. E. V. DE COCK *et al*. (84). The sum of the points for every criterion in AP was estimated with a maximal

score of 9 for acute pancreatitis. A total point score of 0 was considered normal pancreatic histopathology. A score of 1–3 was considered mild; 4–6, moderate; and 7–9, severe acute pancreatitis (Table4.2).

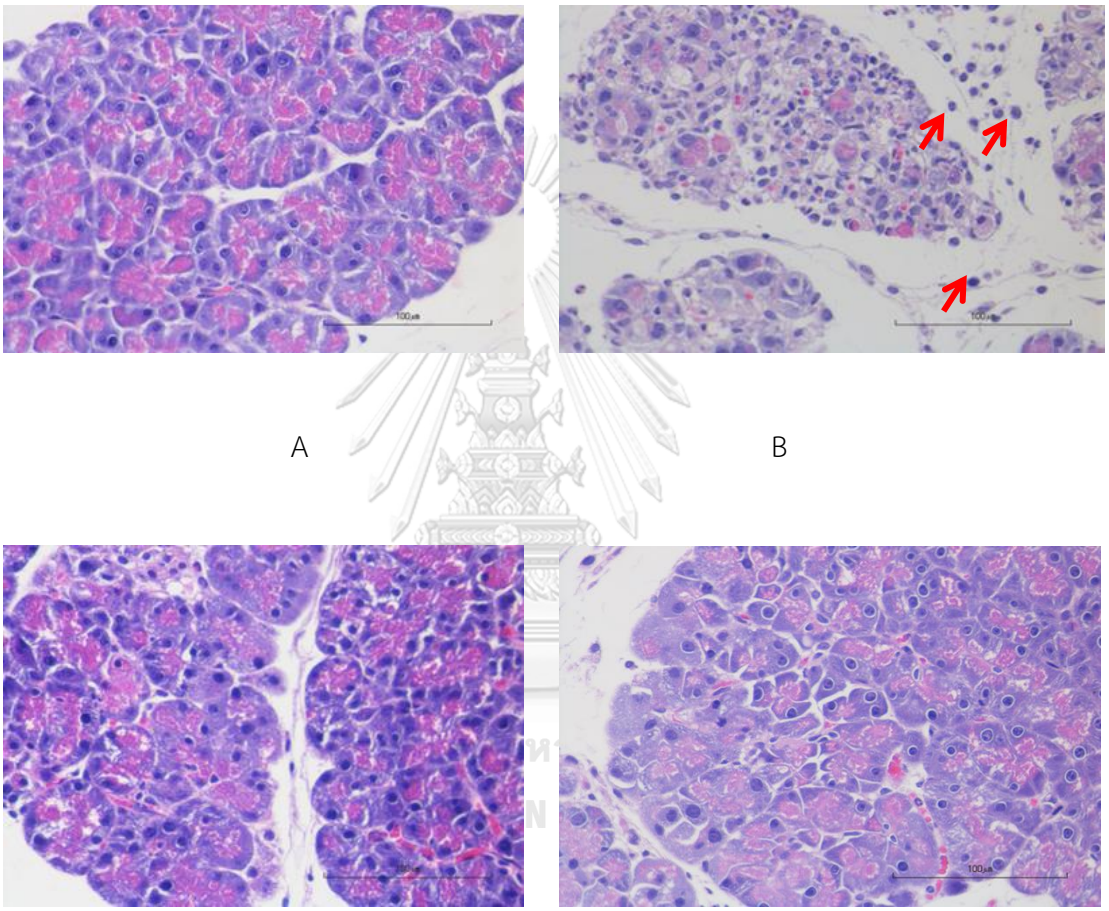
Table4.1 The scores of inflammation, edema and fat necrosis of pancreatic pathology.

Group	N	Inflamation				Edema				Necrosis			
		0	1	2	3	0	1	2	3	0	1	2	3
Control	6	6	-	-	-	5	1	-	-	6	-	-	-
AP	6	-	-	1	5	-	1	2	3	-	1	1	4
AP+Low cur	6	6	-	-	-	5	1	-	-	6	-	-	-
AP+High cur	6	6	-	-	-	6	-	-	-	5	1	-	-

Table4.2 Frequency of severity and summarized scores of pancreatic pathology.

Pathological scores						
Group	Number	Normal (0)	Mild (1-3)	Moderate (4-6)	Severe (7-9)	Mean±SD
Control	6	5	1	0	0	0.17 ± 0.41
AP	6	0	0	0	6	7.67 ± 0.82
AP+Low cur	6	5	1	0	0	0.17 ± 0.41
AP+High cur	6	5	1	0	0	0.17 ± 0.41

The histologic appearance of the pancreas in the control group was normal (Figure 4.4(A)). In AP group, the histopathologic features showed severe inflammation, edema and fat necrosis (Figure 4.4(B)). Mice treated with low and high dose of cur group attenuated the pancreas histopathology that showed only mild inflammation (Figure 4.4(C&D)).



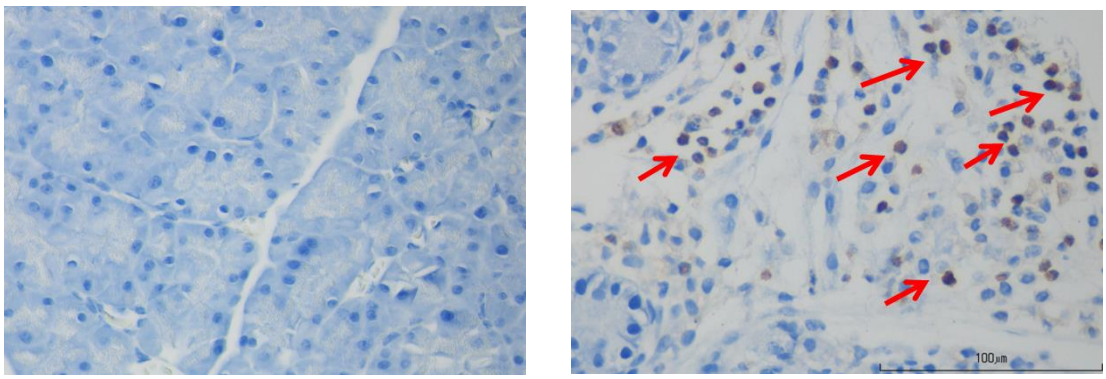
A B

C D

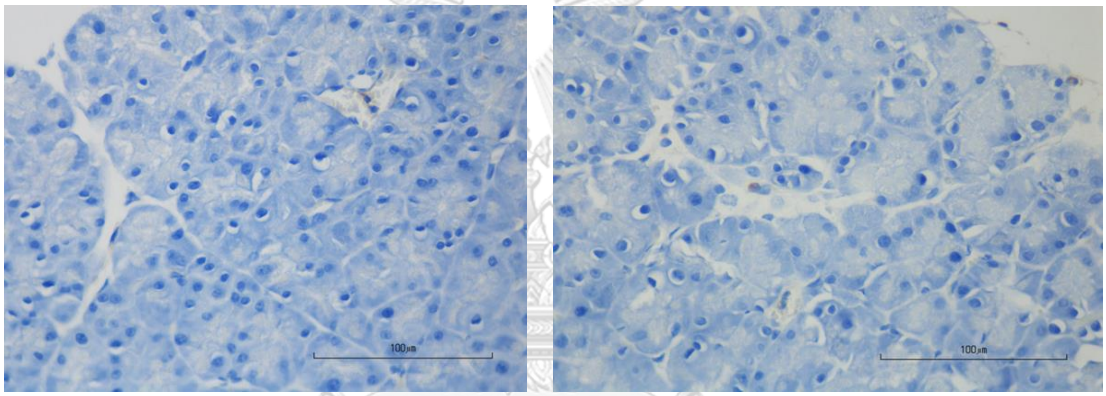
Figure4.4 The histological changes in the pancreas by H & E staining (40X) (A) Control group was normal, (B) AP group showed severe inflammation, edema and fat necrosis, (C) and (D) Low and high dose of curcumin group showed improved pancreatic damage. Arrows indicate the inflammatory monocyte infiltration.

4.5 Immunohistochemistry analysis of MPO activity

MPO activity in the pancreas was considered to evaluate the neutrophil infiltration to the damaged tissue using immunohistochemical staining (Figure 4.5). As shown in Figure 4.6, the number of MPO positive cells per fields in mice with AP showed increased MPO activity when compared with control group (29.57 ± 3.18 vs 0.08 ± 0.27 cells/high power field, $p < 0.001$), as well as curcumin significantly reduced the MPO activity in both low dose cur (1.25 ± 0.67 vs 29.57 ± 3.18 cells/high power field, $p < 0.001$) and high dose cur (0.75 ± 0.4 vs 29.57 ± 3.18 cells/high power field, $p < 0.001$) when compared with AP group.



A B



C D

Figure4.5 Pathological sections of immunohistochemistry for MPO in the pancreas was considered to evaluate the neutrophil infiltration into the damaged tissue(40X) (A) Control group, (B) AP group, the arrows indicated a MPO positive cells, (C) and (D) Low and high dose of curcumin group showed a decrease of MPO positive cells.

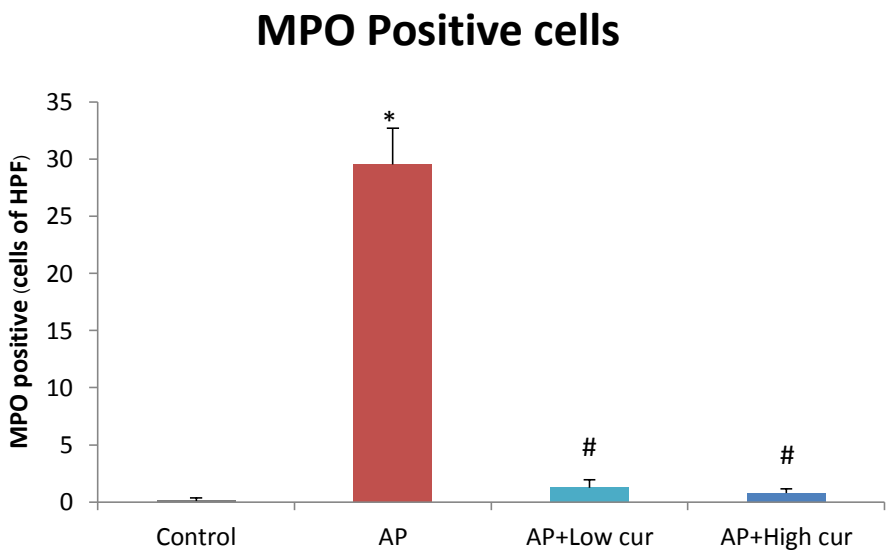


Figure4.6 The frequencies of MPO positive cells in pancreas. Value represent mean ± SD (*P<0.05 versus control groups; #P<0.05 versus AP groups).

4.6 Immunohistochemistry analysis of NF-kB

The expression of NF-kB in pancreas was determined by immunohistochemistry (Figure 4.7). The data of NF-kB expression in all groups were given in Figure 4.8. The percentage of positive cells in AP group was significantly higher than control group (47.95 ± 12.98 vs 6.27 ± 2.31%, P < 0.001). In contrast, the percentage of positive cells significantly decreased in both low dose cur (3.63 ± 1.61 vs 47.95 ± 12.98%, p < 0.001) and high dose cur (11.68 ± 6.44 vs 47.95 ± 12.98%, p < 0.001) when compared with AP group.

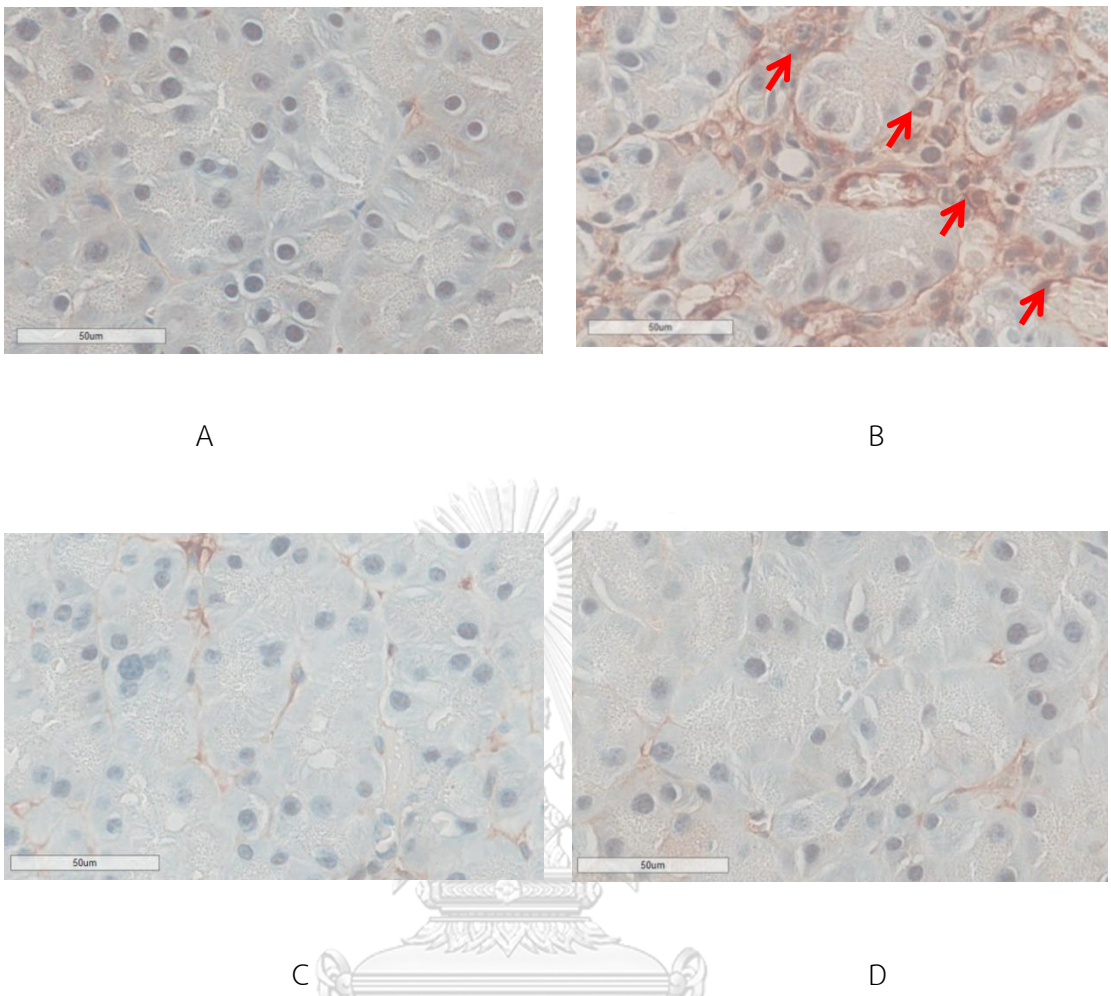


Figure4.7 Immunohistochemistry of NF-kB expression in mice pancreas (40X). (A) Control group. (B) AP group, the arrows indicated NF-kB positive cells in nuclei. (C) and (D) Low and high dose of curcumin group showed a diminishment of NF-kB expression.

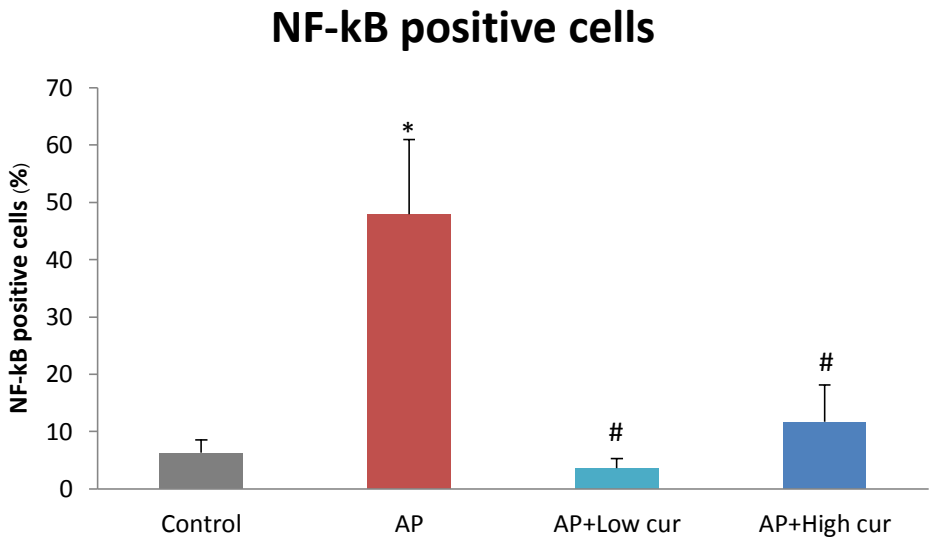
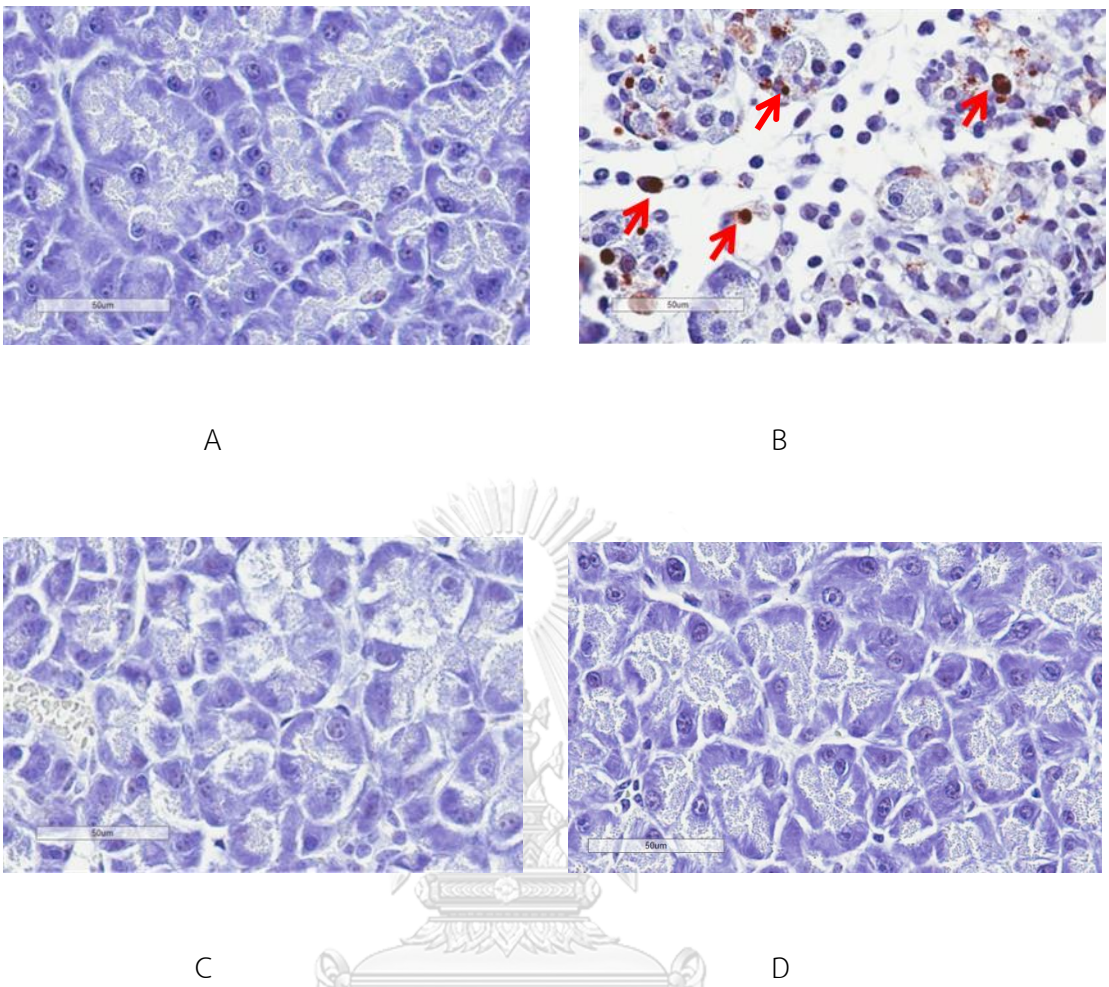


Figure4.8 The percentage of NF-kB positive cells in all groups. All data are expressed as mean ± SD (*P<0.05 versus control groups; #P<0.05 versus AP groups).

4.7 Immunohistochemistry analysis of apoptotic acinar cells

The apoptosis of acinar cells was analyzed by TUNEL staining (Figure 4.9). The data of TUNEL positive cells in all groups were given in Figure 4.10. The percentage of positive cells in AP group was significantly higher than control group (2.72 ± 2.72 vs 0.50 ± 0.33%, P < 0.05). In contrast, the percentage of positive cells significantly decreased in both low dose cur (0.46 ± 0.13 vs 2.72 ± 2.72%, p < 0.05) and high dose cur (0.37 ± 0.08 vs 2.72 ± 2.72%, p < 0.01) when compared with AP group.



A B

C D

Figure4.9 Effect of curcumin on apoptosis of acinar cells was analyzed by TUNEL staining (40X) (A) Control group, (B) AP group, the arrows indicated a TUNEL positive cells, (C) and (D) Low and high dose of curcumin group showed a decrease of TUNEL positive cells.

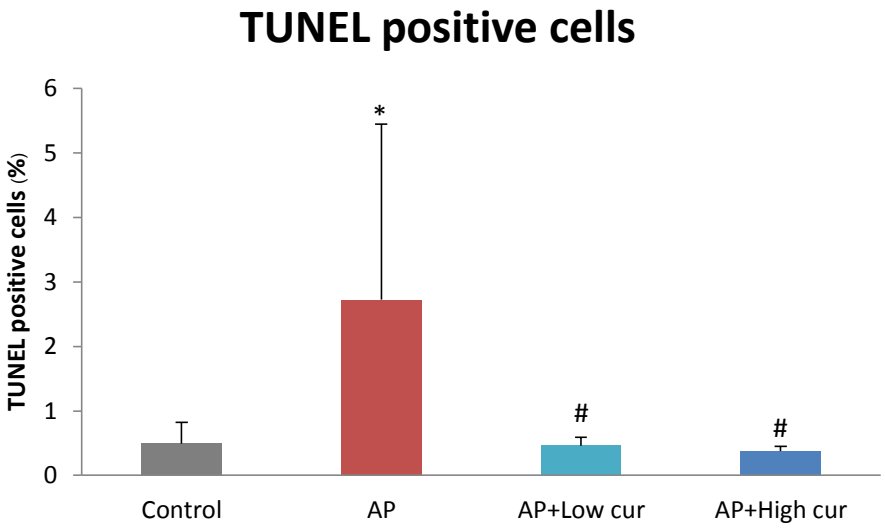
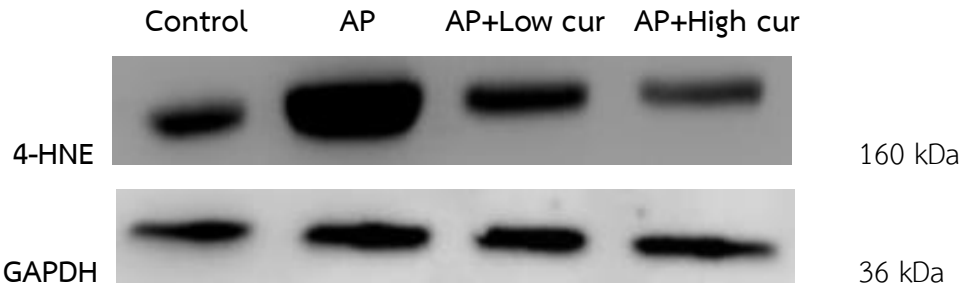


Figure4.10 The percentage of TUNEL positive cells in all groups. All data are expressed as mean ± SD (*P<0.05 versus control groups; #P<0.05 versus AP groups).

4.8 Western blot analysis of 4-HNE expression

4-HNE is an essential bioactive marker of lipid peroxidation. Western blotting shown as the relative ratio of 4-HNE expression was significantly increased in AP group compared to the control group (2.59 ± 0.46 vs 1.38 ± 0.70, P < 0.05). Curcumin treatment in both low dose cur (1.01 ± 0.73 vs 2.59 ± 0.46, p < 0.01) and high dose cur (1.46 ± 0.23 vs 2.59 ± 0.46, p < 0.05) inhibited the elevation of 4-HNE in AP mice (Figure 4.11).

A



B

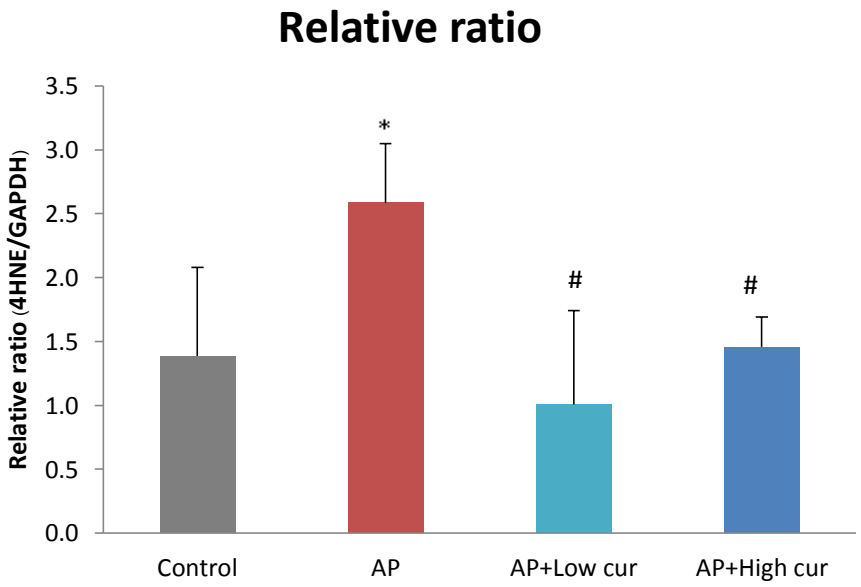


Figure4.11 Western blot analysis of 4-HNE expression in pancreas. (A) Western blot analysis of 4-HNE expression in pancreas. (B) The graph shows densitometric analysis of the 4-HNE relative to GAPDH. All data are expressed as mean ± SD (*P<0.05 versus control groups; $^{\#}$P<0.05 versus AP groups).

CHAPTER V

DISCUSSION AND CONCLUSIONS

The present study found that decreased body weight, reduced physical activities and look sick in mice with AP. When curcumin was applied, the body weight was maintained and active activity. Moreover there were increased in serum amylase, number of MPO positive cells, NF-kB positive cells, TUNEL positive cells, 4-HNE expression and the damage of pancreas pathology in mice with AP.

The effect of L-arginine induced AP in mice

This study showed that L-arg could induce AP by dose of L-arg 450 mg/100g BW i.p. injection 2 times for 1 day AP by elevated amylase. The histological alterations observed in response to 450 mg/100g L-arg were very similar to previous reports in male Wistar rats. Tashiro *et al* (95) report that, in L-arg induced AP, the severity of pancreatitis induced by 450 mg/100g arginine was accompanied by remarkable changes in the actin cytoskeleton, as imagined with rhodamine phallodin.

The effect of L-arginine on pancreatic enzyme (serum amylase) in mice

Serum amylase levels that indicated diagnostic markers for AP. They usually rise within 4–8 hours of the initial attack, peaks at 24 hours and converts to normal over the 72 hours (96). A number of experimental studies have showed that L-arg increased amylase enzymes (41). Identically, previous studies similar to the present study L-arg induction of AP significantly increased the serum amylase at 24 hours.

The effect of L-arginine on pancreas pathology in mice

Our study demonstrated that the pathological changes of pancreatic tissue damage in AP relate to the levels of inflammatory cytokines and ROS. In agreement with previous studies (96), evaluation of the histopathology indicated that, induction of pancreatitis caused pancreatic damage characterized by acinar cell necrosis, neutrophil infiltration and edema. Our study, the pancreas in AP group showed extensive infiltration of neutrophils, acinar cell necrosis involving all tissue, and edema.

The effect of L-arginine on inflammation (myeloperoxidases activity) in mice

Myeloperoxidases is a peroxidase enzyme produced by azurophilic granules in neutrophils and macrophages, which is used of biochemical markers for neutrophil infiltration in order to study about AP (97). Induction of pancreatitis, MPO activity was increased and this demonstrated the progressive aggravation of pancreatic injury. Our result has shown that induction of AP with L-arg increased the pancreatic MPO levels and can attenuate the pancreatic injury by inhibition of the neutrophil infiltration.

The effect of L-arginine on oxidative stress and apoptosis in mice

Acinar cell injury has been linked to oxidative stress that can initiate apoptosis by direct interactions with mitochondria. Apoptosis is important program in every cell for eliminate abnormally cells to minimize tissues or organs injury. This process result to cytochrome c release, caspase-3 activation, and DNA fragmentation (32, 33). Lipid peroxidation is the cellular process that indicates the existence of free radicals. The previous experimental studies have reported that L-arg induced AP elevated the formation of lipid peroxidation product, such as 4-HNE (98). 4-HNE can promote proliferation signaling of cell division and cell survival or prolonged arrest inhibition of cell division, cells die from apoptosis. 4-HNE can induce these processes by modulate the various transcription factors sensible to stress such AP-1 and NF-kB. According to NF-kB involving inflammation, 4-HNE involving in the activation of p53

might be one of the mechanisms function for inducing programmed cell death presented in various cell types. SH-SY5Y cells 4-HNE-induced oxidative stress was related with elevated expressions of Bax and p53; these events trigger other processes, ending in cell death. 4-HNE can induce programmed cell death via the death receptor Fas (CD95-) mediated extrinsic pathway as well as via the p53-dependent intrinsic pathway (99).

Using the TUNEL assay, our results showed that acinar cell apoptosis in L-arg induced AP has been related to oxidative stress and NF-kB activation. We found an increase in 4-HNE expression in AP group that agreed with the previous studies (98).

The effects of curcumin on L-arginine induced AP in mice

Curcumin is a potential antioxidant and anti-inflammatory properties it the ROS scavenging and inhibiting lipid peroxidation product and that inhibiting the expression of NF-kB. Oxidative stress can also initiate or amplify inflammation via the upregulation of many genes associated with the inflammatory response and are leading to lipid peroxidation. NF-kB gene is a key regulator of proinflammatory mediators, plays an important role in the systemic inflammatory response in AP.

The present study demonstrated that in AP group NF-kB activation was significantly increased in the nucleus of mice pancreas but when curcumin treatment was inhibited the elevation of NF-kB. Samuhasaneeto *et al* (83) recommend that curcumin treatment improved liver histopathology in liver injury induced by ethanol via decrease in oxidative stress and inhibition of NF-kB activation. Gukovsky *et al* (78) showed that curcumin decreased inflammation by decreasing NF-kB and AP-1 activation as well as inhibiting mRNA induction of IL-6, TNF-α, and inducible nitric oxide synthetase (iNOS) in the pancreas of experimentally-induced AP, evaluated by DNA binding and degradation of inhibitory IkB proteins. Normally, the inactive NF-kB resides in the cytoplasm, where it is excluded by IkB. When cells are stimulated by inflammatory factors, IkB kinase rapidly phosphorylates IkB proteins, which leading to their degradation and the release of active NF-kB. The NF-kB is activated and translocates to the nucleus and activates the transcription of several important pro-

inflammatory genes (44). Curcumin blocks NF-kB activation via the inhibition of phosphorylation of NF-kB, resulting to suppression of cell survival, proliferative and inflammatory gene products (100).

This study confirmed that elevated the NF-kB activation could be a main procedure in the pathogenesis of pancreatitis. NF-kB can initiate inflammation and induce free radicals which involve in lipid peroxidation, together with these processes, stimulate apoptosis. Our study demonstrated that treatment with both doses of curcumin (50 and 200 mg/kg BW) decreased the serum amylase which indicating the protective effect of curcumin on the disease progression. In addition, curcumin significantly decreased the inflammatory cells infiltration, 4-HNE expression and apoptotic cells in this pancreatitis model could probably due to its anti-inflammation and anti-apoptosis properties leading to improved pathology of AP through reduction of oxidative stress and inhibition of NF-kB activation. Treatment with curcumin protected the pancreas from L-arg induced pancreatitis.

Histopathology was graded into scores of inflammation (0-3), edema (0-3), and necrosis (0-3). All mice in control group were got score 0 for necrosis and inflammation, excepted edema score which only one mouse got score 1. This might due to morphological changes in pancreas itself. However, the other parameters that indicated the pancreatitis were normal.

The limitation of this study is the dosage of curcumin because there is no previous data about the doses of curcumin 50 mg/kg BW and 200 mg/kg BW to treatment pancreatitis. For future studies, we suggest to examine the effect of curcumin at doses 50 mg/kg BW for treatment because of this research demonstrated that curcumin can prevent L-arg induce pancreatitis.

In conclusion, L-arg can cause AP. This study showed that curcumin, a representative phenolic antioxidant and anti-inflammation, could attenuate histopathology of L-arg induced pancreatitis by decreasing the neutrophil infiltration, oxidative stress and inhibiting of NF-kB activation. Curcumin treatments resulted in decreasing the elevation of serum amylase, number of MPO positive cells, NF-kB positive cells, TUNEL positive cells, 4-HNE expression and attenuating pancreas pathology. Hence, curcumin might be a therapeutic strategy against AP. There is no significant difference between 200 mg/kg BW and 50 mg/kg BW doses of curcumin treatment.

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APPENDICES

LIST OF ABBREVIATIONS

AP	=	Acute pancreatitis
AP-1	=	Activating protein-1
BW	=	Body weight
BSA	=	Bicinchoninic acid
CUR	=	Curcumin
DAB	=	Diaminobenzidine
DMSO	=	Dimethylsulfoxide
g/kg	=	Gram per kilogram
H & E	=	Hematoxylin and eosin
H2O2	=	Hydrogen peroxide radical
HCl	=	Hydrochloric acid
i.p.	=	Intraperitoneal
IkB	=	Inhibitors of kappa B
IKK	=	IkB kinases
LM	=	Light microscope
L-Arg	=	L-arginine
mg/kg	=	Milligram per kilogram
mL	=	Millilitre
mg/day	=	Milligram per day
NaOH	=	Sodium hydroxide
NF-kB	=	Nuclear factor-kappa beta
NIK	=	NF-kB Inducing kinase
O2-	=	Superoxide radical
PBS	=	Phosphate buffer saline
ROS	=	Reactive oxygen species
RT	=	Room temperature
SDS	=	Sodium dodecyl sulfate
SD	=	Standard deviation

SOD	=	Superoxide dismutase
TMA	=	Tissue microarrays
TUNELS	=	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
TNF-α	=	Tumor necrosis factor-alpha
4-HNE	=	4-Hydroxynonenal
MPO	=	Myeloperoxidase
μm	=	micrometre
μg/mL	=	microgram per millilitre

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