Genistein Attenuated Severity of Acute Pancreatitis Induced by L-Arginine in Mice

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Abstract

The roles of genistein on acute pancreatitis are not yet clear. This study was conducted to determine the effects of genistein on anti-inflammatory and improved histopathology of acute pancreatitis in mice. ICR mice were divided into 3 groups (n = 6, each). *Control group (Con)*: Mice received intraperitoneal (i.p.) injection of dimethyl sulfoxide (DMSO) once daily for 4 days; *Acute pancreatitis group (AP)*: mice received two i.p. injection of 350 mg/100 g BW of L-arginine dissolved in normal saline, at an interval of 1 hour. *Genistein group (Gen)*: Mice received 100 mg/kg genistein in 2% DMSO by i.p. injection 2 hours before induction with L-arginine and once daily for 3 days. Mice were sacrificed 72 hours after L-arginine-induced acute pancreatitis. Body weight change, serum amylase, serum interleukin-6 (IL-6), serum C-reactive protein (CRP) and histopathology score were collected. *Results:* Levels of body weight significantly decreased in AP when compared with Con (AP *vs.* Con: -1.46 ± 0.37 *vs.* 1.75 ± 0.19 g). Furthermore, serum amylase, serum IL-6, and serum CRP significantly increased in AP when compared with Con group (AP *vs.* Con: 13,860.00 ± 5,918.26 *vs.* 5,714.00 ± 201.11 U/l, 124.68 ± 106.27 *vs.* 18.59 ± 18.90 pg/ml, and 11,687.07 ± 3,691.95 *vs.* 8,068.63 ± 3,065.24 ng/ml, respectively; $P < 0.05$). Genistein resulted in significantly increased levels of body weight compared with AP (Gen *vs.* AP: 0.41 ± 0.54 *vs.* -1.46 ± 0.37g). In addition, Gen group had significantly decreased serum amylase, serum IL-6, and serum CRP compared with AP group (Gen *vs.* AP: 8,728.33 ± 3,213.61 *vs.* 13,860.00 ± 5,918.26 U/l, 52.58 ± 42.70 *vs.*124.68 ± 106.27 pg/ml, and 7,607.77 ± 2,757.94 ng/ml *vs.* 11,687.07 ± 3,691.95 ng/ml, respectively; $P < 0.05$). Moreover, Gen group showed improved inflammation and histopathology scores. *Conclusion:* Genistein can attenuate severity of acute pancreatitis via the mechanism of reduced inflammatory cytokines (IL-6 and CRP) and improved histopathology.

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Keywords: *Genistein, L-arginine-induced acute pancreatitis, amylase, IL-6, C-reactive protein*

Introduction

Acute pancreatitis (AP) is a sudden inflammatory disorder of the pancreas, a potentially life-threatening disease, and a cause of suffering. 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 pathogenetic process, found in 15-25% of case.[1,2] Two most common causes of AP in human are gallstones and alcohol consumption. Various studies believe that the pathology of acute pancreatitis arises from an early intra-acinar cell conversion of inactive enzymes into active forms, leading to autodigestive pancreas. This encourages the synthesis and release of many proinflammatory cytokines and chemokines, including oxidative stress, causing local inflammation.[1-3] Acute pancreatitis is characterized by interstitial edema, acinar cell necrosis, hemorrhages, and neutrophil infiltration. Moreover, inflammatory mediators trigger the development from local to systemic inflammation, potentially resulting in multiple organ dysfunction syndrome, arising from the excess secretion of proinflammatory mediators into the circulation.[4,5] Over the past three decades considerable progress has been made, but the treatment of acute pancreatitis 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 acute pancreatitis.[2]

Phytoestrogens are natural chemical compounds derived from plants, which have structures and functions similar to estrogens generated within the endocrine system. Genistein (4',5,7- trihydroxyl-isoflavone) is a phytoestrogen that belongs to the category of isoflavones. It has remarkably similar pharmacological activity and structure to 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

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phenolic hydroxyl groups to deleterious free radical molecules.[7,8] It can also increase the activity of anti-oxidant enzymes, including superoxide dismutase, glutathione reductase, and glutathione peroxidase.[9] Furthermore, several studies have shown the anti-inflammatory properties of genistein. It can effect-tively decrease many inflammatory mediators related with inflammation, for instance, C-reactive protein (CRP),[10] interleukin 1-β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin (IL-6), and nuclear factor kappa B (NF-kB).[11,12]

The anti-inflammatory properties of genistein have been shown by several studies. As shown in an *in vitro* study with lipopolysaccharide (LPS)-stimulated BV2 microglia, genistein inhibited the increased release and expression of inflammatory cytokines, including IL-1β and TNF-α, prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2), and was able to suppress the production of nitric oxide (NO) by inhibiting inducible NO synthase (iNOS).[13] Experimenting with LPS-treated RAW 264.7 macrophages, Liang *et al*. (1999) demonstrated that the anti-inflammatory activity of genistein inhibited NO and PGE2 production by modulating COX-2 and iNOS expression,[14] as well as decreasing the production of tumor TNF-α, IL-6, and NF-kB. In addition, this compound administration has a significant anti-inflammatory effect on high-fat diet-induced nonalcoholic steatohepatitis (NASH) rats.[15] Furthermore, Gupta *et al*. study showed that genistein has the ability to protect streptozotocin (STZ)-induced diabetic cardiomyopathy in rats, showing reduced inflammation, decreased expression of CRP, and TNF-α, and a slight increase in antioxidant enzymes.[10]

The roles of genistein on acute pancreatitis are not definite. This study was conducted to determine the effects of genistein on anti-inflammatory and improved histopathology of L-arginine-induced acute pancreatitis in mice.

Materials and Methods

L-arginine preparation

L-arginine (L-arg), powder (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline and the pH was adjusted to 7 with 5*N* HCl. L-arg was administered at a dose of 350 mg/100g BW, in two intraperitoneal injections with an interval of 1 hour.

Genistein preparation

Genistein (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 2% dimethyl sulfoxide (DMSO) that was freshly prepared for the experiment.

Animal Protocols

Male ICR mice, weighing about 30-40 g, were purchased from the Nation Laboratory Animal Center, Salaya Campus, Mahidol University, Nakhon Pathom, Thailand. The animals were housed in a controlled room temperature at 25 ± 1ºC with 12:12 hour light-dark cycle and were fed *ad libitum*. All rats received proper care in accordance with the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

ICR mice were divided into 3 groups (n=6, each). **Control group (Con):** Mice were given intraperi-toneal (i.p.) injection of DMSO once daily for 4 days. **Acute pancreatitis group (AP)**: Mice were given two i.p. injection of 350 mg/100 g BW of L-arg dissolved in normal saline, at an interval of 1 hour. **Genistein group (Gen):** Mice were given 100 mg/kg genistein in 2% DMSO by i.p. injection 2 hours before induction with L-arg and once daily for 3 days. Body weight change, serum amylase, serum IL-6, serum CRP, and histopathology score were collected. In the experiment, body weight was recorded in all of animals. At the end of the study, all mice were sacrificed 72 hours after L-arg induction. The abdomen was opened and whole pancreas was rapidly removed and washed with cold normal saline. Pancreas was fixed in a 10% formalin solution for histological examination. Subsequently, blood samples were drawn by cardiac puncture and allowed to clot by being kept at room temperature for 2 hours. Afterwards, it was centrifuged at 1000 g for 20 minutes, after which serum was collected for determination of amylase enzyme, IL-6, and CRP.

Body weight of all mice was measured. Levels of body weight change (g) were compared between first and last day of each group. Amylase activity was measured via the colorimetric method, using the biochemical analyzer Reflotron® Plus. The results were expressed as enzyme concentration (in U/l). Serum concentrations of IL-6 and CRP were measured using the commercial mouse IL-6 assay and mouse CRP assay kits, respectively (R&D Systems, Minneapolis, MN, USA).

For histopathological examination, the pancreas tissue was transferred into a fixative of 10% formalin solution immediately after collection at room temp-erature, they were processed using hematoxylin and eosin (H & E) staining method. Briefly, pancreatic tissue was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and then picked up on glass slides for light microscopy. An experienced pathologist evaluated all samples while being blinded to the experiment. All fields in each section were examined for grading of neutrophil infiltration, edema, and fat necrosis. According to the criteria described by De Cock *et al*., 2007,[16] The histological grading, with scales ranging from 0-3, was as follow: 0 = Not Present, 1 = Mild or < 25% of the pancreatic parenchyma, 2 = Moderate or present in 25-50% of the parenchyma, 3 = Severe or > 50% of the of the parenchyma.

Statistical 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 comparisons test were employed. Descriptive statistics was used for the histological examination of the pancreas. Differences were considered statically significant at $P < 0.05$.

Results

The effect of genistein on body weight change

The results of weight change were shown in Figure 1A. The levels of body weight in the control group showed a normal increase (Con: 1.66 ± 0.28 g). These were significantly decreased in AP group when compared with control group (AP *vs.* Con: -1.46 ± 0.37 *vs.* 1.66 ± 0.28 g). The body weight of Gen group was significantly increased when compared with AP group (Gen *vs.* AP: 0.41 ± 0.54 *vs.* -1.46 ± 0.37 g; $P < 0.05$).

Serum Amylase (AMY) levels

The results of serum AMY were shown in Figure 1B. Serum levels of AMY in AP group were significantly higher than Con group (AP *vs.* Con: 13,860.00 ± 5,918.26 *vs.* 5,714.00 ± 201.11 U/l). Nevertheless, AMY levels in serum were significantly decreased by genistein administration when compared with AP group (Gen *vs.* AP: 8,728.33 ± 3,213.61 *vs.* 13,860.00 ± 5,918.26 U/l; $P < 0.05$).

Serum IL-6 and CRP levels

The results of serum IL-6 were shown in Figure 1C. The levels of IL-6 in AP group were significantly higher than Con group (AP *vs.* Con: 124.68 ± 106.27 *vs.* 18.59 ± 18.90 pg/ml). In contrast, Gen group had decreased elevation of IL-6 levels when compared AP group (Gen *vs.* AP: 52.58 ± 42.70 *vs.* 124.68 ± 106.27 pg/ml; $P < 0.05$).

The results of serum CRP were showed in Figure 1D. The levels of CRP in AP group were significantly higher than Con group (AP *vs.* Con: 11,687.07 ± 3,691.95 *vs.* 8,068.63 ± 3,065.24 ng/ml). In contrast, Gen group had decreased elevation of CRP levels when compared AP group (Gen vs. AP: 7,607.77 ± 2,757.94 *vs.*11,687.07 ± 3691.95 ng/ml; $P < 0.05$).

Histopathological examination

The results of histopathology score were shown in Table 1 and Figure 2. Histological examination of Con group showed normal architecture and absence of neutrophil infiltration, edema, and acinar cell necrosis. Administration of L-arg in AP group induced extensive tissue damages characterized by neutrophil infiltration, edema, and acinar cell necrosis, and thus received significantly higher scores than Con group (AP *vs.* Con: 5.32 ± 1.86 *vs.* 0.00 ± 0.00). Nevertheless, treatment with genistein significantly improved pancreatic damage in L-arg-induced acute pancreatitis (Gen *vs.* AP: 2.16 ± 1.96 *vs.* 5.32 ± 1.86).

Discussion

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. The pathology of acute pancreatitis is believed to stem from an early conversion of

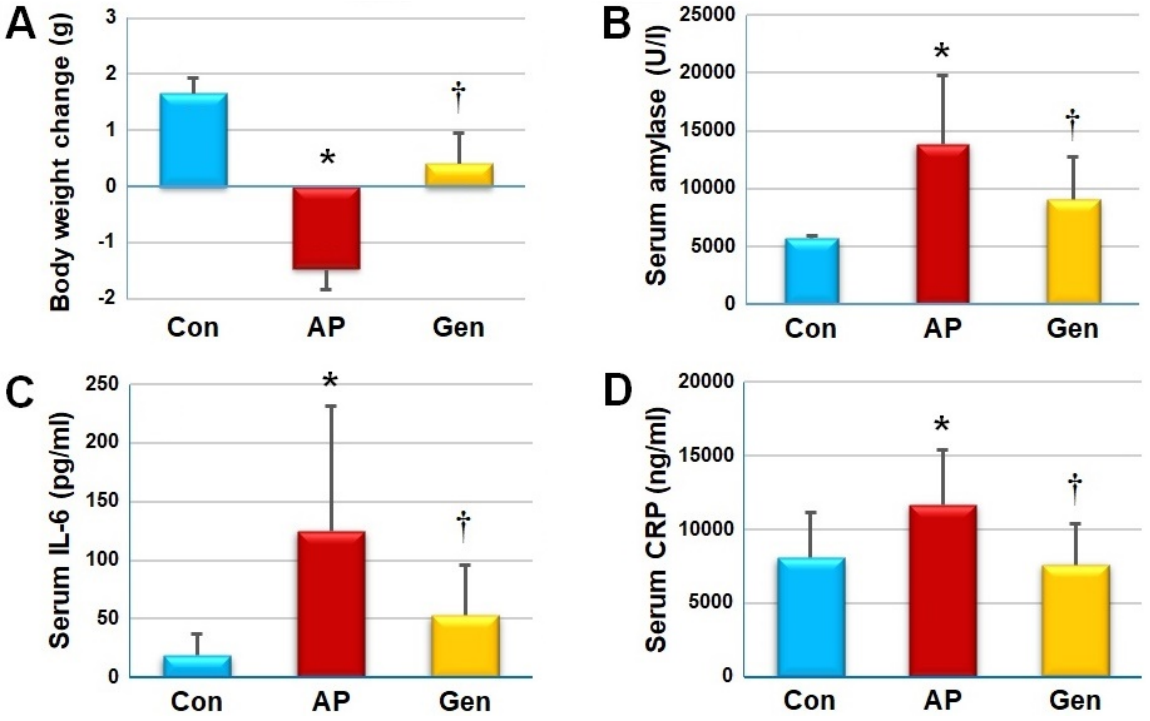

Figure 1 Effects of genistein treatment on (***A***) body weight change, (***B***) serum amylase levels, (***C***) interleukin-6, and (***D***) C-reactive protein, in L-arginine-induced acute pancreatitis. Mean ± SD of 6 animals in each group are shown. **P* < 0.05, compared with Con group; † *P* < 0.05, compared with AP group.

Table 1 Effect of genistein on the histopathological parameters in L-arginine-induced acute pancreatitis

Parameters /groups	Groups		
	Con	AP	Gen
Neutrophil infiltration	0.00 ± 0.00	1.83 ± 0.98*	0.67 ± 0.82†
Edema	0.00 ± 0.00	1.67 ± 0.82*	1.00 ± 0.00†
Acinar cell necrosis	0.00 ± 0.00	1.83 ± 0.75*	0.83 ± 1.67†
Total	0.00 ± 0.00	5.32 ± 1.86*	2.16 ± 1.96†

Data are histological grading according to De Cock, *et al*.[16].The scale ranges from 0-3: 0 = Not Present, 1 = Mild or < 25% of the pancreatic parenchyma, 2 = Moderate or 25-50%, and 3 = Severe or > 50%. Con, Control group; AP, Acute pancreatitis group; Gen, Genistein group. Values are mean ± SD from 6 animals in each group; *P < 0.05, compared with Con group; †P < 0.05, compared with AP group.

enzymes in intra-acinar cells, from inactive to active forms, leading to autodigestion within the pancreatic tissue. This encourages the synthesis and release of many proinflammatory cytokines and chemokines, including those causing oxidative stress, leading to local inflammation.[1-3] Pathological characteristics of acute pancreatitis include 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 inducing acinar cell injury was first discovered by Mizunuma in 1984, after i.p. injection of L-arg in rats. The result showed selective damages on pancreatic acinar cells without any morphological change in other tissues. L-arg is a precursor of polyamines putrescine, spermidine, and spermine, which have important roles in stabilizing ribosomes, nucleic acids, and cell functions. Acinar cells in pancreas are the structure that has the highest concentration of spermidine for protein metabolism. Therefore, acinar cells are the first target of L-arg overdose that results in degeneration or necrosis. This model is very popular because the technique is easily implemented, inexpensive and similar in its course, including histological changes, to human pancreatitis.[17]

To diagnose acute pancreatitis, the investigation of AMY is the most commonly performed laboratory tests, as AMY are secreted by the acinar cells of the pancreas. The blood levels of AMY rapidly rise within 3-6 hours of the symptom onset, with a half-life of 10-12 hours, and remain elevated for 3-5 days. The cut-off AMY levels for diagnosis of AP is 3 times above the upper limit of normal.[18] In accordance with previously reported, L-arg induction of acute pancreatitis in the present study significantly increased serum AMY at the end of the study.[19] Administration of genistein decreased serum AMY levels, indicating a protective effect of genistein against pancreatic damage.

Cytokines are inflammatory markers, produced and released by a number of cell types, though

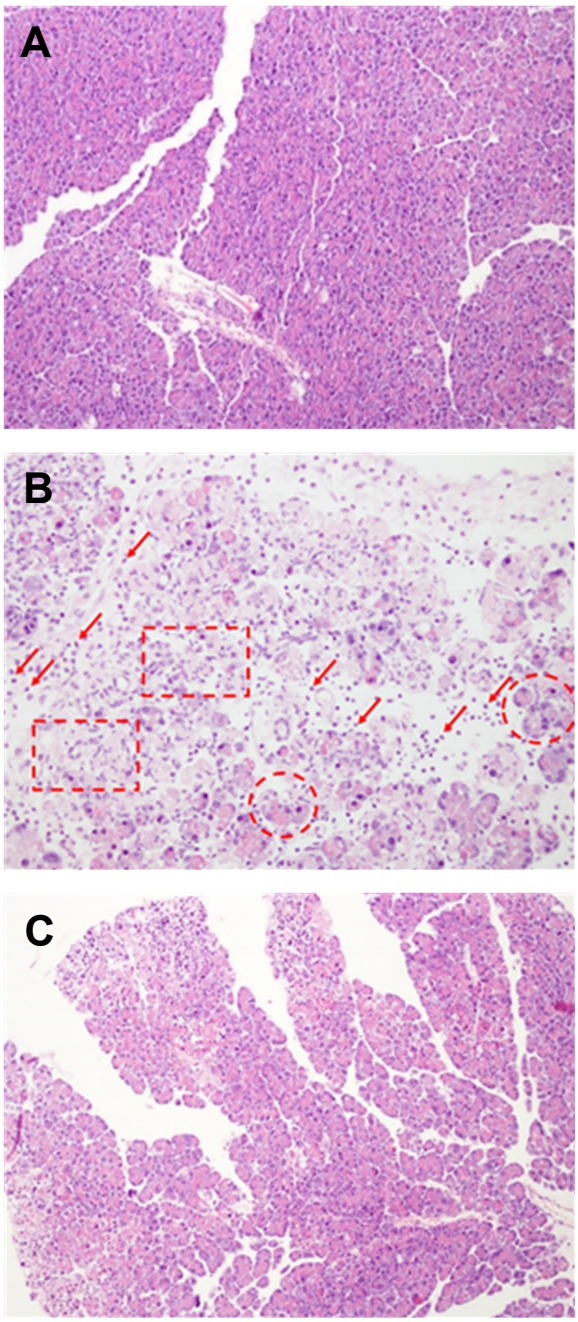

Figure 2 Pancreatic histopathology in L-arginine-induced acute pancreatitis (H&E, x 200). (***A***) Control group (Con) showed normal histology, (***B***) Acute pancreatitis group (AP) showed extensive tissue damage characterized by neutrophil infiltration (arrows), edema (circles), and acinar cell necrosis (rectangles), and (***C***) Genistein group (Gen) showed improved pancreatic damage.

predominantly by leukocytes. Cytokines appear to play a pivotal role related to the pathogenesis of pancreatitis. One of the important inflammatory cytokines in L-arg -induced acute pancreatitis is IL-6. IL-6 is a principal mediator which has a role in the regulation of immune response and inflammatory process.[20,21] In addition, C-reactive protein (CRP), an acute phase reactant synthesized by the hepatocytes, is usually elevated in inflammatory conditions. The

production of CRP, which is stimulated by cytokines in blood and considered as a marker of neutrophil activities, significantly increased in acute pancreatitis.[22] In the present study, levels of serum IL-6 and CRP proved to be increased in L-arg-treated group. However, treatment with genistein significantly decreased the levels of serum IL-6 and CRP, indicating that the genistein protective effect may probably be due to its antiinflammatory action (Figure 1).

Finally, the histopathology of pancreas was determined in each treatment group. According to many previous studies, it is well known that the extent of pancreatic tissue damage in acute pancreatitis correlates with the levels of inflammatory mediators.[23,24] The current study revealed that genistein pretreatment significantly attenuated the histological parameters, i.e., neutrophil infiltration, edema, and acinar cells necrosis (Table 2, Figure 2), probably due to its antiinflammatory effect which can reduce inflammatory mediators. In addition, administration of L-arg showed significantly decreased levels of body weight from poor ingestion of food because of sickness and the enzymes autoactivation released by acinar gland during progression of AP. However, this symptom was improved by genistein.

To our knowledge, our present study is the first report that administration of genistein inhibits the development of acute pancreatitis in mice. The administration of L-arg significantly induced acute pancreatitis, characterized by decreasing levels of body weight, and increasing serum AMY, serum IL-6, serum CRP, and histopathology score. Genistein is able to counteract L-arg-induced change in laboratory parameters of acute pancreatitis. Injection of genistein can beneficially decrease serum AMY, inflammatory cytokines (IL-6 and CPR), and prevent histological damage to the pancreas. These effects may be due to antiinflammatory action of genistein. Taken together, our study showed that genistein could reduce the severity of acute pancreatitis in mice. It is possible that genistein may be a promising clinical therapeutic strategy for the alternative treatment of acute pancreatitis in the future.

Conclusion

Genistein can alleviate the severity of acute pancreatitis by reducing inflammatory cytokines (IL-6 and CRP) and improving histopathology.

Acknowledgments

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Conflict of Interest

None to declare.

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