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The effects of glycyrrhizin on experimental acute pancreatitis in rats

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Abstract. – INTRODUCTION: Although physiopathology of acute pancreatitis (AP) is not fully understood, the roles of reactive oxygen species (ROS) and changes of cytokines have been determined.

AIM: To investigate anti-inflammatory and anti-oxidant effects of glycyrrhizin (GL) on taurocholate-induced AP in rats.

MATERIALS AND METHODS: Thirty six rats were randomly divided into three groups as sham, AP and AP+GL (n=12 per group). AP was induced by 1 ml/kg body weight using 5% taurocholate injection into the biliopancreatic duct in groups II and III after clamping the hepatic duct. In groups III, GL (20 mg/kg) was given by oral gavage twice daily for 4 days. Group I and II did not receive any treatment. After the rats were killed; blood samples were taken to measure amylase, lipase, calcium, albumin, urea, glucose, AST and LDH assays before killing. Pancreatic tissue samples were also taken for biochemical analyses and histopathology.

RESULTS: Amylase, lipase, AST and urea levels were significantly lower in the AP+GL group than in the AP group. Cytokines including IL-6, TNF-α and MPO levels were significantly lower in the AP+GL group than in the AP group. Even so there is no statistically difference between in the AP+GL group and the AP group in terms of pancreatic tissue IL-1β, IL-6 and TNF-α levels.

DISCUSSION: GL treatment significantly decreased pancreatic tissue MPO activities and MDA levels in the AP+GL group compared with the other groups ($p = 0.001$ and $p = 0.05$, respectively). Acinar cell necrosis, hemorrhage, and edema determined that were significantly lower in the AP+GL group than in the AP group ($p < 0.001$).

CONCLUSIONS: GL treatment for acute necrotizing pancreatitis in rats suppressed the levels of pro-inflammatory cytokines, and caused a clear recovery of histological changes.

Key Words:
Glycyrrhizin, Acute Pancreatitis, Pro-inflammatory cytokines.

Introduction

Acute pancreatitis (AP) is a disease with high morbidity and mortality. It has a relative frequency ranging 10 to 80 cases per 100 000 population in the world. AP is caused by gallstones or alcohol abuse in 80 percent of patients. AP resulting systemic effects is local inflammation in pancreatic tissue. Although physiopathology of AP is not fully understood, the roles of reactive oxygen species (ROS) and changes of cytokines have been determined. Increased levels of pro-inflammatory cytokines such as tumor necrosis factor-a (TNF-a), interleukin (IL)-1 and IL-6 are responsible to local tissue damage in pancreas and multiple organs' failure. Productions of ROS cause the changes in the fundamental components of the cytoplasm, membrane lipid peroxidation, and protein damage in pancreas activating digestive enzymes[1-6].

Glycyrrhizinic acid or glycyrrhizin (GL) is a natural sweetener derived from the roots a Licorice (Glycyrrhyza glabra L. Leguminosae) plant which is dried in the sun. It has been using as herbal medicine since ancient times throughout the world[7]. A

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number of components including triterpenes, saponins, flavonoids, isoflavonoids and chalcones have been isolated from licorice with glycyrrhizic acid being considered the main biologically active component. Licorice root, a traditional drug, has been used for centuries in treatment of diseases of lung, peptic ulcer, hepatitis C and skin[8].

Background

Nowadays, clinical and experimental studies have revealed that GL has antiviral, antimicrobial, anti-inflammatory, anti-oxidant as well as many pharmacological properties. Some studies have showed that it has anticancer activities, immunomodulatory, hepatoprotective effects and the protective effects on the heart[9-11].

The anti-inflammatory properties of β-Glycyhrritinic acid and GL were shown in several studies[12-14]. Recently, some glycyrrihetinic acid derivatives have shown their inhibitory activity against IL-1b-induced prostaglandin E2 production in normal human dermal fibroblasts[10].

There are many studies to create an experimental model of acute pancreatitis using invasive and non-invasive models such as sodium taurocholate, ethanol, caerulein, L-Arginine induction of choline-deficient, diet containing DL-ethionine and ischaemia/reperfusion. Taurocholate-induced pancreatitis model was chosen for the study. This model is appropriate for studies of systemic issues. The retrograde injection of salts into the pancreatic duct of animals is not an easy, effective and reproducible model for creating a severe, rapidly evolving variety of acute haemorrhagic pancreatitis and lethal[15-17].

Aim

The aim of this study is to investigate anti-inflammatory and anti-oxidant effects of GL on taurocholate-induced AP in rats.

Materials and Methods

This experimental study protocol was approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy, Turkey and was performed in accordance with the standard guidelines for the care and handling of animals.

Animals

Thirty-six male Sprague-Dawley rats weighing from 200 to 250 g were obtained from Gulhane School of Medicine Research Center, Ankara, Turkey. Animals were kept at constant room temperature in a 12-h light-dark cycle with free access to water and standard rat chow at least 1 week before the experiments. Animals were randomly divided into three groups such as Group I (sham), Groups II (AP) and III (AP+GL).

Induction of Pancreatitis

Animals were anaesthetized by intraperitoneal administration of 50 mg/kg Ketamine (Ketalar® Parke Davis, Eczacıba ı, Istanbul, Turkey) and 5 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany), and then laparotomy was performed to all groups through a midline incision. The common biliopancreatic ducts of animals were cannulated with a 28 gauge ½-inch, microfine catheter, except group I. Then, 1 ml/kg of 5 percent sodium taurocholate (Sigma, St. Louis, MO, USA)[18] was slowly infused into the common biliopancreatic duct, and the infusion pressure was kept below 30 mmHg, as measured with a mercury manometer calibrated system[16], and monitored with a monitoring kit (Transpac IV Safeset, Abbott, Dublin, Ireland) attached to the infusion line with a three-way stopcock. When the infusion was finished for group and II and III, abdomen was closed in two layers for all groups.

Study Protocol

Group I (sham) underwent laparotomy with manipulation of the pancreas without induction of pancreatitis and received 10 ml/kg saline intravenously (single dose). Groups II (AP) and III (AP+GL) were underwent laparotomy with induction of pancreatitis. Group II did not receive any treatment. In Group III, after 6 hour the induction of AP, GL (20 mg/kg) was given by oral gavage twice daily for 4 days. On the fifth day of induction, all animals were killed with intracardiac pentobarbital (200 mg/kg) injection. Blood samples were taken from the heart before killing. Pancreatic tissue samples were also taken for biochemical analyses and histopathology.

Tissue Preparation

The frozen pancreatic tissues were homogenized in 50 mM phosphate buffer (pH 7.4) by means of a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany) on an ice cube. Homogenates were centrifuged at 7530 × g in 4°C for 10 min. The protein content of pancreatic homogenates was measured according to the method described by Lowry et al[19].

Biochemical Analyses

A Hitachi 917 autoanalyzer (Boehringer Mannheim, Mannheim, Germany) was used for amylase, lipase, calcium, albumin, urea, glucose, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) assays.

TNF-α, IL-1b and IL-6 Assays

Blood samples were collected and centrifuged (at 3000 rpm for 5 min). The sera were stored at -40°C. TNF-α, IL-1beta and IL-6 levels were measured in serum samples using quantitative sandwich enzyme linked immunosorbent assay kits (R&D Systems Inc., Minneapolis, MN, USA).

Histopathologic Analysis

A part of the pancreatic tissue from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. Two pathologists who were blinded to the treatment protocol scored the tissue sections stained with haematoxylin and eosin (H&E) for acinar necrosis, inflammation and perivascular inflammation, hemorrhage and edema in 20 fields. The scores of each histological examination were summed up, with a maximum score of 24 as defined by Schmidt et al[20].

Evaluation of Oxidative Stress

Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice, centrifuged and supernatant was used for analyses. Tissue malondialdehyde (MDA) concentration was measured by method as described by Ohkawa et al[21]. MDA level was expressed as nmol/mg protein. Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine[22] and was expressed as U/mg protein. Myeloperoxidase (MPO) levels were measured by ELISA kit (Cusabio Biotech Co., Ltd. Wuhan, Hubei Province, China) and were expressed as ng/ml and ng/mg protein.

Statistical Analysis

All statistical measurements were made by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All results were expressed as median (25%-75%). The Kruskal-Wallis test is used in all groups' comparisons and then Mann-Whitney U test was used to compare the groups in pairs, which had significant results. $p < 0.05$ values were considered significant.

Results

Serum biochemical parameters are compared in Table I. The serum amylase, lipase, AST and urea levels were significantly lower in the AP+GL group than in the AP group. However, in the AP+GL group serum albumin and calcium levels were higher than in the AP group ($p = 0.001$ and $p < 0.001$).

The serum cytokines including IL-6, TNF-α and MPO levels were compared among the groups (Table II). All of them were significantly lower in the AP+GL group than in the AP group. Even so there was no significant difference between the AP+GL and sham groups for all of these parameters. There was no statistically difference between in the AP+GL group and the AP group in terms of pancreatic tissue IL-1β, IL-6 and TNF-α level (Figure 1). GL treatment significantly decreased pancreatic tissue MPO activities and MDA levels in the AP+GL group compared with the AP group ($p = 0.001$ and $p = 0.05$, respectively) (Table III).

Table I. Biochemical parameters in the all experimental groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
Amylase (U/L)	1271.5 (1256-1876)	2047 (1763-2372)	1711 (1410-2144)	0.015
Lipase (U/L)	8.5 (7-13)	13.55 (9-18)	11.55 (7-13)	0.015
Albumin (g/dL)	2.89 (2.73-3.91)	2.01 (1.78-3.27)	3.19 (2.16-3.24)	0.001
AST (U/L)	154.5 (118-444)	268.5 (108-680)	167 (89-884)	0.043
Calcium (mg/dL)	10.45 (8.1-10.65)	7.89 (6.78-9.87)	9.92 (8.1-10.65)	< 0.001
LDH (U/L)	1005 (217-3929)	1551 (474-6008)	1480 (661-4078)	0.686
Urea (mg/dL)	43 (38-50)	49 (39-58)	45 (41-54)	0.043
Glucose (mg/dL)	193 (145-273)	225.5 (157-261)	196.5 (136-282)	0.386

AP: acute pancreatitis; GL: glycyrrhizin; AST: aspartate aminotransferase; LDH: lactate dehydrogenase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

Table II. Serum cytokines' levels in all groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
IL-1β (pg/mL)	62.4 (31.8-143)	125.5 (49-431)	77.1 (15.15-388)	0.083
IL-6 (pg/mL)	45.4 (14.3-71.6)	70.73 (3.5-112.8)	51.9 (30.4-116.4)	0.018
TNF-α (pg/mL)	10.35 (8.13-12.55)	21.3 (10.51-26.7)	10.9 (9.8-14.35)	0.001
MPO (ng/mL)	0.19 (0.56-1.80)	1.30 (0-1.35)	0.42 (0.02-2.01)	0.001

AP: acute pancreatitis; GL: glycyrrhizin; TNF: tumor necrosis factor; IL: interleukin; MPO: myeloperoxidase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

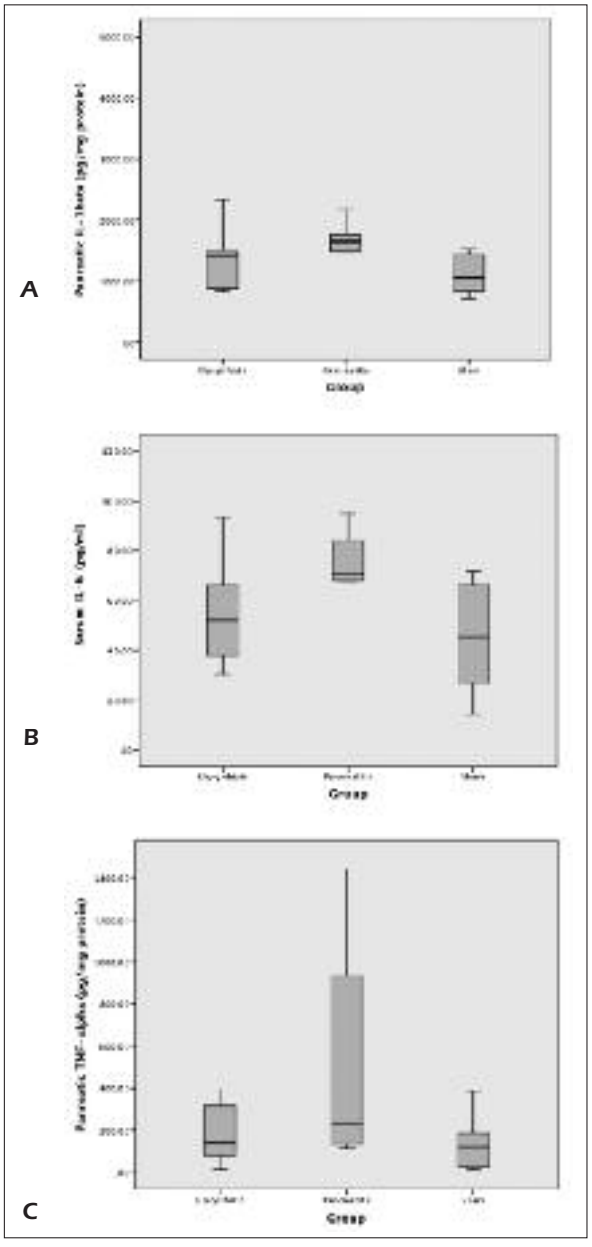

Figure 1. Pancreatic tissue cytokines' levels. ***A,*** IL-1β levels, $p = 0.100$ (AP vs AP+GL). ***B,*** IL-6 levels, $p = 0.800$ (AP vs AP+GL). ***C,*** TNF-α levels, $p = 0.13$ (AP vs AP+GL).

Histopathological scores are also shown in Table IV. The histopathology evaluations of the groups determined that acinar cell necrosis, hemorrhage, and edema were significantly lower in the AP+GL group than in the AP group ($p < 0.001$). The level of inflammation-perivascular inflammatory cell infiltration was significantly higher in the AP+GL group than in the sham group ($p < 0.001$). Figure 2 shows histologic images of all groups. Histological examination of pancreatic tissues confirmed amelioration by treatment GL on AP.

Discussion

AP is a disease with high morbidity and mortality. However, the standard treatment for AP is still mainly based on supportive treatments including vital signs are followed, intravascular volume is maintained, electrolyte balance is achieved, analgesics are provided and treatment for possible complications. No clear benefit from the administration of medications, such as aprotinin, gabexate mesylate, glucagon and calcitonin, to treat AP has been demonstrated[20,23].

Following the extravasations of pancreatic secretions through the tissue spaces, proteolytic enzymes such as activated trypsinogen start the process of auto digestion of pancreatic tissue[23,24]. Tissue edema develops and it is along with microcirculation failure and ischemia at the cellular level. Circulatory failure leads to increased severity of the inflammation and accumulation of toxic mediators within pancreas and monocytes-macrophages releases some cytokines[25-28].

Substances such as activated protein C[3], cyclooxygenase inhibitors[29], melatonin[30,31], allopurinol[32] and octreotide[33] were used to prevent the pancreatic damage caused by released ROS and cytokines in experimental studies.

Table III. Pancreatic tissue oxidative stress parameters in all groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
MPO (ng/mg protein)	5.48 (1.03-13.94)	23.11 (1.4-74)	9.12 (1.7-16.6)	0.001
MDA (nmol/ mg protein)	2.46 (0.13-9.97)	8.86 (2.5-43.6)	4.78 (2.43-12.9)	0.050
GPx (U/mg protein)	1.28 (0.8-2.84)	1.47 (1.07-3.52)	1.21 (0.8-2.84)	0.225

AP: acute pancreatitis; GL: glycyrrhizin; MPO: myeloperoxidase; MDA: malondialdehyde; GPx: glutathione peroxidase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

Table IV. Histological injury scores in pancreatic tissues in all experimental groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
Acinar cell necrosis	0 (0-1)	3.5 (1-4)	2 (1-3)	< 0.001
Hemorrhage	0 (0-1)	2 (1-4)	1 (0-4)	< 0.001
Inflammation and perivascular infiltration	0 (0-1)	3 (1-4)	1 (0-2)	0.060
Edema	0 (0-1)	3 (1-4)	2 (1-3)	< 0.001

AP: acute pancreatitis; GL: glycyrrhizin. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

In this study, acute necrotizing pancreatitis was induced by retrograde infusion of sodium taurocholic acid (5%) into the biliopancreatic duct, as Aho et al[18,34] described. The application route and dose for glycyrrhizin was selected according to the data presented in the literature[35,36]. GL is believed to be partly responsible for its anti-inflammatory effect[37]. Akamatsu et al[38] showed that GL is not a scavenger of ROS but it exerts an anti-inflammatory action by inhibiting the generation of ROS by neutrophils. In experimental studies, it was determined that glycyrrhizin inhibe phospholipase A2 of the arachidonic acid cascade and reduce cytokines in rats, secondary to AP[3,39].

The sensitivity and specificity of the diagnosis of AP are higher than 90% in patients with appropriate clinical signs. Serum amylase and lipase levels increase within 24 h following the acute attack and decrease gradually to the normal values[40]. In our work, serum amylase, lipase, AST, LDH and urea levels were significantly lower in the AP+GL group than in the AP group. However, in the AP+GL group serum albumin and calcium levels were higher than in the AP group. After pancreatitis induction, GL treatment significantly increased serum albumin and calcium levels. Nagai et al[41] showed that GL suppressed the elevation lipid peroxides, AST, LDH and decreased morphological damage in the liver. In another study, Shiki et al[42] also showed that GL deceased release of AST and inhibit phospholipase A in the liver.

Failure in the microcirculation led to a histopathologically significant increase in acinar cell necrosis, hemorrhage, inflammation and perivascular inflammatory cell infiltration and edema level in AP. Glycyrrhizin might protect the organ function by regulating the excessive inflammatory reactions and attenuated organ injury

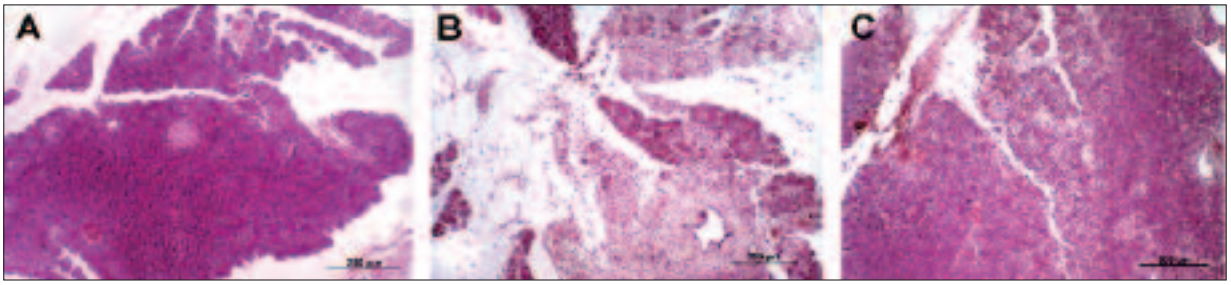

Figure 2. Histological images of pancreatic tissues in the *(A)* Sham, *(B)* Acute pancreatitis and *(C)* Glycyrrhizin groups. (H&E, Scale bars 200 μm).

induced by lipopolysaccharide[43]. After administration of GL in the treatment group showed that a significant decrease in these parameters except level of inflammation and perivascular inflammatory cell infiltration compared with the AP group.

Stimulation of production of either acute phase proteins and adhesion molecules or several inflammatory cytokines including TNF-α, IL-1β and IL-6 occurs after taurocholic acid activation in acute pancreatitis[34]. In this study, the concentrations of cytokines including TNF-α, IL-1β and IL-6 were increased in the AP group rats. Our results have shown that GL inhibited the release of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in serum and pancreatic tissue. In addition to these parameters, we have found high MPO levels in the AP group. MPO activity has been used to determine quantitatively the extent of neutrophils infiltration. Our findings show that GL also reduces MPO levels after AP such as caffeic acid phenethyl ester[44]. The anti-inflammatory and anti-oxidant effects of GL may explain our results. Akamatsu et al[38] and Wang and Nixon[45] found that glycyrrhizin inhibited the generation of ROS by neutrophils which were the potent mediator of tissue inflammation in the in vitro study. Wang et al[43] showed that GL decreased the inflammatory cytokines through inhibiting their gene and protein expression.

As a result, retrograde infusion of 5% taurocholate into the biliopancreatic duct in rats induces AP biochemically and histologically. GL treatment following this procedure suppressed the levels of pro-inflammatory cytokines, and caused a clear recovery of histological changes.

Conclusions

GL might be beneficial in decreasing the severity of AP. However, further investigations are needed to demonstrate the potential roles of glycyrrhizin in taurocholate-induced acute necrotizing pancreatitis.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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