

BIOCHEMICAL CHANGES IN THE MOST IMPORTANT PATHOLOGIES OF PANCREATIC SECRETION AND THE RESULTS OF THEIR GENERAL ANALYSIS

Dalimova Manzuraxon Mukhtarovna

Senior Lecturer, Department of Biological Chemistry, Andijan State Medical Institute

Zagrutdinov Fanil Faridovich

Senior Lecturer, Head of the Department of Pharmacology, Andijan State Medical Institute

Ahmedov Azimjon Ilkhomovich

Andijan State Institute of Foreign Languages

Boltaboyeva Dilbarkhon Fayzirakhmanova

Senior Lecturer Department of Biological Chemistry Pharmacology, Andijan State Medical Institute

Tadjibayeva Mohinur Abduvohid qizi

Department of Biological Chemistry, Andijan State Medical Institute

Abstract. Despite advanced research and great progress in understanding the chronic pancreatitis (CP) pathogenesis, no current causal treatment for the condition is available. For preclinical studies, the existence of a well-characterized CP animal model is essential. The aim of the study was to assess the impact of chronic pancreatitis on the antioxidant enzymes activity in rat blood serum and on the level of glutathione (intracellular antioxidant) in rat pancreas. Methods: The experiments were carried out on the Wistar Kyoto rats in two groups: control and study group (CP), in which chemical induction of pancreatitis with dibutyl dichloride was performed. Serum enzyme activities of amylase, lipase, catalase and superoxide dismutase were analyzed. The levels of the following biochemical parameters were also investigated: total protein, albumin, calcium, magnesium, and triglycerides. Levels of low-molecular-weight thiols: reduced (GSH) and oxidized (GSSG) glutathione, were determined in pancreatic homogenates.

Keywords: chronic pancreatitis, amylase, lipase, catalase, superoxide dismutase, reduced and oxidized glutathione, dibutyltin dichloride.

INTRODUCTION

Chronic pancreatitis (CP) is a long-term inflammatory process occurring in the pancreatic parenchyma, characterized by progressive and irreversible morphological changes within this organ. In chronic pancreatitis, focal necrosis areas are followed by peri-intrapancreatic fibrosis and parenchymal calcification, as well as stone formation in the pancreatic duct and the emergence of pseudocysts [1]. Progressive loss of endocrine and exocrine functions is observed in the late stage of the disease. CP is the most common cause of exocrine pancreatic insufficiency in adults. The gastrointestinal (GI) hormones regulate the secretion and synthesis of all digestive enzymes in the pancreatic acini (enteroacinar axis) and the secretions of pancreatic islet hormone, particularly insulin (enteroinsular axis). The GI hormones that primarily stimulate motor activity are gastrin,

cholecystokinin (CCK), and motilin, while the peptides that inhibit motor activity are secretin, vasoactive intestinal polypeptide (VIP), glucagon, and enteroglucagon [2]. Alcohol and gallstones play a very important role in the pathogenesis of chronic pancreatitis. Recurrent oxidative stress or inflammation are the primary causes of the permanent damage to the pancreas. They give rise to irreversible changes in the structure and function of the pancreas and ultimately lead to chronic pancreatitis [3, 4]. Antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) play a crucial role in the body's defense against oxidative stress associated with inflammation caused by reactive oxygen species (ROS) [5]. Reduced glutathione (GSH) is a major non-protein mammalian cells' thiol. Similarly, to CAT and SOD it has a key function as an antioxidant. GSH is in equilibrium with oxidized glutathione (GSSG). The ratio of GSSG to GSH is a credible indicator of the oxidative stress as it reflects the balance between antioxidant status and cells' prooxidant reactions [6].

Despite advanced research and considerable progress in understanding the CP pathogenesis, currently no causal treatment is available for this condition. Thus, it is reasonable to search for a well-characterized animal model that would enable obtaining important information on the CP pathogenesis for future implementation in experimental therapy. It is equally relevant to search for diagnostic parameters that can determine the severity of the ongoing inflammatory process within the pancreas [7]. The recurring difficulty regarding new therapies is that they cannot be evaluated in clinical trials before prior conclusion that there are no contraindications in animal testing. There are many existing animal experimental models of acute pancreatitis (AP). These can be divided into noninvasive and invasive as well as extracorporeal models. However, there is no experimental animal model of chronic pancreatitis that completely corresponds to clinical conditions. Observations from standardized animal experiments are not always supported by studies in humans. The induced rat model of chronic pancreatitis certainly appears to be a suitable one to be evaluated despite some anatomical differences in the structure of the human and rat pancreas. The experimental pancreatitis model used in the study should, in each case, be selected for the specific medical problem [8].

MATERIALS AND METHODS

The 7–8-week-old male Rashidov Sobir (RS) rats were obtained from the Central Laboratory. All experiments were approved by the II Local Ethics Committee for Animal Experiments (Application No. WAW2/216/2023, September 17, 2023).

Rats were housed in cages in small groups (2–3 animals per cage) with unlimited access to water and food, as well as a varied environment introduced (e.g., cellulose tunnels). The space in which the animals were kept was controlled to maintain a temperature of 22°C ($\pm 2^\circ\text{C}$) and a humidity of 55% ($\pm 5^\circ\text{C}$). The light cycle was also controlled and set as follows: 12 h light/12 h darkness.

RESULTS AND DISCUSSION

The animals were divided into two groups. The first — control group — consisted of 11 RB rats that were injected intraperitoneally with an ethanol-glycerol solution. The second — study group (CP) — consisted of 11 RB rats in which chronic pancreatitis was induced by intraperitoneal injection of dibutyltin dichloride (DBTC) in an ethanol-glycerol solution (the ratio of DBTC to ethanol and

glycerol was 1:3). The dibutyltin dichloride solution was administered to animals from the study group in a volume of 0.3 ml at a DBTC dose of 8 mg/kg. Then, an abdominal massage was performed to better distribute the drug in the peritoneal cavity. The animals' condition was monitored within 48 h after administration of the compound by observing the following vital parameters: heart rate, respiratory rate and type, abdominal pain, food and water consumption. After injecting the DBTC solution, the animals received an opioid analgesic for at least 48 h. In the group of control rats, all stages were carried out analogously, with the difference that only the ethanol-glycerol solution was administered intraperitoneally in a volume of 0.3 ml.

Thirty days after dibutyltin dichloride (in study group) and ethanol-glycerol (in control group) solution administration, blood for biochemical analyses was drawn from animals that presented poor anesthesia (xylazine and ketamine). Blood was drawn from the right atrium of the heart. The procedure was performed using a needle with a syringe, from a left thoracic access in the axillary region in a puncture of the intercostal space. Collected blood was placed in plastic microtubes and left to develop a clot. The serum was separated by centrifugation and frozen in small volumes at -20°C for further analysis.

The animals were put into deep anesthesia by administering a lethal dose of xylazine and ketamine, followed by vertebral dislocation. Subsequently, a section was performed to collect the pancreas for biochemical and histopathological tests. Tissues collected during autopsy were washed out in cold saline and used for histopathological examination or were immediately frozen and stored at -20°C for other analyses.

Histopathological evaluation

Tissues sections of pancreas fixed in 10% buffered formalin were dehydrated using graded ethanol and xylene baths and embedded in paraffin wax. Sections of 3–4 μm were stained with hematoxylin and eosin (HE). The general histopathological examination was evaluated at magnification of 10 \times , 40 \times , and 100 \times (objective lens) and 10 \times (eyepiece) and photographic documentation was made. Micrographs were taken at the magnification of 10 \times or 40 \times (objective lens) and 10 \times (eyepiece), using a standard light microscope Olympus BX41 and CellSens software (Olympus Corporation, Tokyo, Japan). Tissue preparations were assessed by the experienced pathologist.

Determination of biochemical parameters and enzymes activity in rats' serum

The activity of amylase and lipase, and the level of total protein, albumin, calcium, magnesium, and triglycerides in serum were analyzed using Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN). The enzyme activity was expressed as units of enzyme per liter.

Catalase (CAT) and superoxide dismutase (SOD) activity was performed with a commercially available assay from Cayman Chemical (Michigan, USA) according to the manufacturer's protocol. Catalase Assay Kit (Item No. 707002) for CAT and Superoxide Dismutase Assay Kit (Item No. 706002) for SOD were selected for the experiments. Catalase activity was expressed in nmol/min/ml, which corresponded to the enzyme amount that converts 1 nmol of formaldehyde in 1 minute at 25°C . SOD activity was expressed in as units of enzyme per ml. One unit

was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical.

Tissue homogenates

To determine the levels of reduced (GSH) and oxidized (GSSG) glutathione by RP- HPLC (reversed-phase high-performance liquid chromatography method) and to evaluate the total protein concentration in rat pancreases, tissue homogenates were prepared. Detailed preparation procedures are described in our previous paper [2].

Determination of reduced glutathione, oxidized glutathione, and protein level in rats' tissues

The levels of reduced and oxidized glutathione were assessed using the RP-HPLC method of Dominick *et al.* [3] with modification by Bronowicka-Adamska *et al.* [4]. Standard curves were generated in the supernatant obtained from tissue homogenates in the range from 13 to 39 nmoles of each compound per ml. The GSH or GSSG level was expressed as nmoles per 1 mg of protein.

Protein content in tissues was determined by the method of Lowry *et al.* [5] using crystalline BSA as a standard.

Statistical analysis

All results were presented as arithmetic means with standard errors (SE). To verify the normal distribution of the data and homogeneity of variances, the Shapiro–Wilk test and Levene's test were used, respectively. Statistical analysis was performed using the Student's t-test or Mann–Whitney U test, when appropriate. Differences with a p value <0.05 were considered statistically significant. Analyses were performed using Statsoft Software Statistica 13 (Tibco, Palo Alto, CA, USA).

The experiments were carried out using biological material collected from animals divided into two experimental groups — control rats and rats with induced chronic pancreatitis. Histopathological imaging of the pancreatic parenchyma of rats with induced inflammation confirmed local lymphocytic interstitial chronic pancreatitis with features of fibrosis and minor parenchymal atrophy (Fig. 1A–B and Fig. 2A–B) as well as pancreatic islet degeneration features (Fig. 1C–F and Fig. 2C–F). Statistically significant ($p < 0.05$) reduced serum amylase and lipase activities of the study group (CP) were observed compared to the control group: 2838 ± 60 U/l and 9 ± 1 U/l for amylase and lipase, respectively.

CAT and SOD enzymes activities, both of which play a critical role in the body's defense against the oxidative stress induced by chronic pancreatic inflammation. A statistically significant elevated catalase activity was observed in the study group (CP) in comparison to the control. In contrast, superoxide dismutase activity did not significantly change and the values in the control and study groups were comparable (9.3 ± 1.0 U/ml for control and 7.9 ± 0.3 U/ml for CP).

The levels of reduced (GSH) and oxidized (GSSG) glutathione in the chosen experimental model. In the study group (CP), we observed a 15-fold decrease in the GSH compared to the control. The level of GSSG in both groups

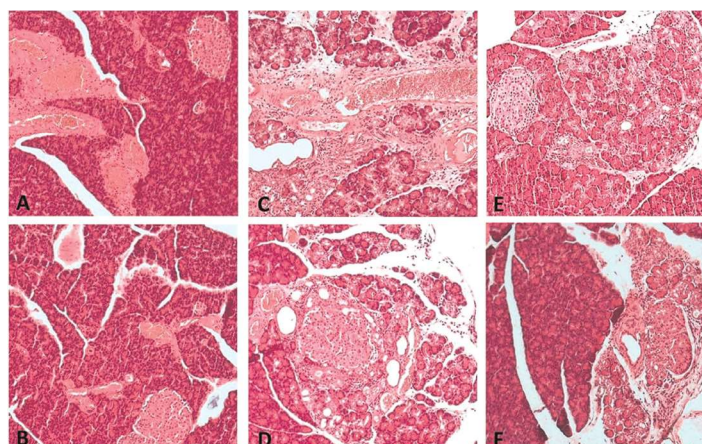


Fig. 1. Histopathological images of rat pancreatic parenchyma.

A, B — control rats; C–F — rats with inducible chronic pancreatitis; magnification/zoom 10×.

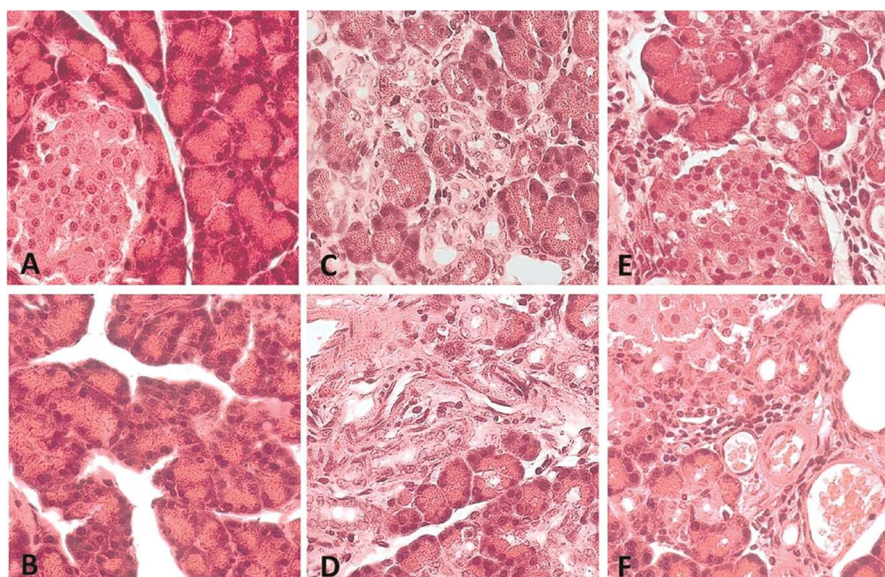


Fig. 2. Histopathological images of rat pancreatic parenchyma.

A, B — control rats; C–F — rats with inducible chronic pancreatitis; magnification/zoom 40×.

Table 1 displays the analysis of the biochemical parameters performed in the designed experiment. It confirmed statistically significant decrease in blood protein and albumin levels in the study group in comparison to the control group. Neither the triglycerides (TAG) level nor the calcium or magnesium ion levels exhibited statistically significant changes. Despite the lack of statistical significance upon the triglycerides comparison there is a slight decrease in TAG level in the CP group, which may be associated with a statistically significant decrease in lipase activity.

Table 1. Serum levels of proteins, ions and triglycerides in rats.

| | Control | Chronic pancreatitis |
|-------------------------------|------------|----------------------|
| Total protein, g/dl | 5.7 ± 0.1 | 5.2 ± 0.0* |
| Albumin, g/dl | 3.6 ± 0.1 | 3.3 ± 0.0* |
| Ca²⁺, mg/dl | 10.2 ± 0.1 | 10.3 ± 0.1 |
| Mg²⁺, mg/dl | 2.9 ± 0.2 | 2.9 ± 0.1 |
| Triglycerides, mg/dl | 111 ± 16 | 81 ± 7 |

Data represent an arithmetic mean ± SE (n = 6–7 animals per group). *p <0.05 chronic pancreatitis vs. control.

Our studies proved that chemical induction of the pathological state with DBTC leads to the development of chronic pancreatitis in rats. The experimental data proves that the inflammatory process occurring in the pancreas is advanced. DBTC-induced pancreatic fibrosis differs from other experimental models of chronic pancreatitis. The fibrotic areas are characterized by the extensive infiltration of mononuclear cells, without pancreatic atrophy. The presence of chronic inflammatory changes with the destruction of the exocrine parenchyma and fibrosis, and in later stages of the endocrine parenchyma, indicates chronic pancreatitis. Experimental model of DBTC-induced acute interstitial pancreatitis in rats presented by Merkord *et al.* [3] can also be used as an experimental model of chronic pancreatitis. Depending on time, acute lesions are prone to develop into a chronic course of the disease when duct obstruction and cholestasis are persistent. Repeated administration of DBTC to rats at a dose of 4 mg/kg intravenously at 3-week intervals induced acute pancreatitis and changes in hepatopancreatic duct after 6 weeks. Pancreatic fibrosis and liver changes (portal tract inflammation, intrahepatic bile duct hypertrophy and necrosis) developed after 9–12 weeks [4]. Our findings show that the serum total protein and albumin levels in the CP group are reduced which may indicate a progressive liver failure [6]. This is due to dibutyltin dichloride which may induce severe liver damage including biliary hypertrophy, inflammatory cell invasion and liver parenchyma necrosis [8].

Oh *et al.* [7] confirmed that in humans, serum levels of pancreatic enzymes decrease with the progression of chronic pancreatitis. During chronic pancreatitis, the secretory pancreas capacity decreases and serum amylase and lipase values may decrease depending on the residual functional pancreas capacity [2]. Patients with established calcific CP had significantly lower levels of serum pancreatic enzymes (amylase and catalase) than the healthy control group [7]. Studies conducted by our team also confirmed statistically significant decreases in amylase and lipase activity in a rat experimental model. We can discuss it may be an indication of a disruption of the pancreas exocrine function.

Fukui *et al.* [4] determined the activity of the antioxidant enzyme, catalase, in patients diagnosed with acute pancreatitis, chronic pancreatitis and pancreatic cancer. Statistical analysis showed that CAT activity was significantly higher only in the group with acute pancreatitis compared to the control group. Reactive oxygen species are involved in the acceleration of the acute pancreatitis development. In our studied rat model of chronic pancreatitis, we were also able to confirm an elevated catalase activity.

Such an increase in catalase activity may suggest the activation of the anti-oxidant mechanisms in pancreas affected by the ongoing inflammation. In contrast, the activity of superoxide dismutase in the control and study groups was relatively comparable. We propose that, the role of catalase is of the most importance for assessing the inflammatory process severity in the rat model, and it deserves further consideration. Catalase in addition to superoxide dismutase, could be worth investigating more closely in terms of its role in the treatment of chronic pancreatitis.

Various experimental pancreatitis models are used to evaluate the pathophysiological mechanisms occurring during the pancreatitis development and treatment. Ghrelin administration protects the liver, pancreas and remote organs against direct damage and oxidative injury induced by obstruction of the bile duct or common pancreaticobiliary duct ligation as demonstrated in animal model studies by Kasımay *et al.* [6]. Ghrelin and obestatin (encoded by the same gene as ghrelin) protective and therapeutic effects were described in the pancreas based research. Pretreatment with ghrelin and obestatin exhibited a protective and healing effect in AP [7].

Papazachariou *et al.* [5] reported that chronic pancreatitis patients exhibited lower average serum concentration levels of creatinine, urea, albumin, sodium, total calcium and magnesium when compared to the control group. These findings are consistent with the research on malabsorption syndrome which occurs in severe chronic pancreatitis and may result in decreased concentration levels of serum albumin and electrolytes [4]. In our conducted study, we recorded no changes in serum calcium and magnesium levels. However, in rats with CP we did observe decreased levels of the total protein/albumin.

A paper by Schoenberg *et al.* [5] reported that, as a result of oxidative stress, reduced glutathione is significantly lower in tissue samples taken from chronic pancreatitis patients as compared to samples taken from the healthy ones. The decrease in GSH is accompanied by an increase in its oxidized form in these patients. Our study confirms a shift in balance towards GSSG formation in the pancreases of rats with CP relative to control animals. However, similar changes were reported in our previous work [6] conducted in an experimental rat model of acute pancreatitis. There is no evidence that the oxidative stress is the only pathogenic factor in chronic pancreatitis. ROS induced oxidative stress has a proinflammatory effect and contributes to acute and chronic pancreatitis through direct cell damage as well as through activation of inflammatory cells and pancreatic stellate cells [7].

Although many aspects still remain unknown, it was observed that the interplay between inflammation, coagulation, and endothelial activation is involved in the earliest local events in acute pancreatitis and is associated with the early phase of systemic disease in severe acute pancreatitis. Taken together systemic inflammation as seen in severe acute pancreatitis is rarely associated with thrombotic disorders, and activation of coagulation can further aggravate inflammation. Laboratory tests in severe acute pancreatitis often reveal abnormalities of coagulation, while clinically relevant disorders of coagulation in acute pancreatitis are associated with a significantly worse prognosis [8].

Our study undoubtedly brings out several strengths. We investigated a rare model that is not well described in the literature as most of the pancreatic-theme research is concentrated on diabetics and cancer. However, we are aware that there are existing limitations to this study that should be taken into consideration. The pancreas anatomy as well as molecular ductal rat physiology differs from that

in the human, thus the pancreatitis experimental models differ as well. In rats, the pancreas exists as groups of lobules spread across the mesentery supplying the initial part of the small intestine. In addition, rats have a less developed pancreatic duct system [8]. Observations undertaken in standardized experimental conditions on an animal model do not always support the studies in humans. Despite the above limitations our study research provided new information on chronic pancreatitis in terms of changes in selected biochemical parameters and antioxidant enzyme activity in rat serum and provides a rare insight into the problem of pancreatic diseases undertaken in the literature.

CONCLUSION

Histopathological examination of tissues taken from DBTC-treated rats confirmed the following: focal lymphocytic interstitial CP with features of fibrosis and mild parenchymal atrophy along with features of pancreatic island degeneration.

There were statistically significant decreases in protein and albumin concentration levels, as well as in rat serum amylase and lipase activities. The changes are an indication, most probably, of pancreas exocrine function disorders. Elevated catalase activity and decreased GSH levels were observed in the study group (CP), suggesting the activation of antioxidant mechanisms in the pancreas affected by ongoing inflammation.

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