MORPHOLOGICAL CHANGES OF THE PANCREAS IN COURSE OF ACUTE PANCREATITIS DURING TREATMENT WITH ULINASTATIN

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Acute pancreatitis is a severe clinical condition that causes significant mortality in patients. Since we do not have at the moment effective causal treatment research on the use of protease inhibitors can produce tangible benefits. In view of the growing number of cases and high mortality in severe AP with one hand, and the lack of a usual treatment research efforts undertaken to search for effective drugs for this disease seem to have deep reasons.

Aim of the study was to determine the histopathological changes in the pancreas in the treatment of acute pancreatitis with Ulinastatin.

Material and methods. The study was conducted in male Wistar rats weighing 250-300 grams. 150 individuals were used for the experiment, 60 of them were treated with Ulinastatin. Experimental acute pancreatitis was induced by the model proposed by Aho and Henckel using sodium taurocholate. Ulinastatin dose numer depended on the duration of the experiment. For histopathological examination pancreatic fragments weighing approximately 1 g each were taken. Assessment and documentation of histopathological preparations were made by light microscopy.

Results. Evaluation of the histological preparations of various time groups showed significantly improved results after application of Ulinastatin, depending on the duration of the inflammation and the number of doses of the drug.

Conclusions. Application for the treatment of UTI leads to inhibition of the inflammatory process at the stage of pancreatic edema and in cases of severe necrotizing course limits the progression of the disease which gives grounds for its clinical use in humans.

Key words: acute pancreatitis, Ulinastatin

Acute pancreatitis (AP) is an acute inflammatory process of the gland itself with involvement, to a different degree, of adjacent tissues and distant organs (1). The observed morphological picture may vary from edema of pancreatic parenchyma to its necrosis. In about 85% of patients, AP is of edematous form with the mild clinical course (2). Patients in whom symptoms of necrosis of the pancreas or surrounding tissues or cisterns with liquid of the acute phase are found suffer from the severe form of AP which pertains to about 15% of patients. Severe form of AP may be accompanied by shock, sepsis, metabolic disturbances, bleeding into the gastrointestinal tract, disseminated intravascular clotting and multi-organ insufficiency. Mortality in this group of patients reaches 50%.

In recent years, AP morbidity still increases. Frequency of this disease in Europe is rated as 54/million of people to 115/million in the USA. The incidence increases with age,
thus in the USA indexes corresponding to 100 000 for respective groups are: up to 15 year of age – 2.7, between 15 and 44 year of age it is nearly 100 times higher and past 65 year of age it increases 200 times (3). The reason of about 80% of all cases of AP is alcohol and diseases of biliary ducts (4). Acute inflammation develops in the pancreas in case of insufficiency of natural immune systems and activation of proteolytic enzymes leading to SIRS and MOF (5). In a view of constantly increasing number of cases and high mortality in severe cases of AP on one hand and lack of causative treatment on the other, research efforts undertaken for the search of effective drugs for this disease seem to be very justified.

A hope for the improvement of therapy was related to the discovery of protease inhibitors. Ulinastatin, earlier called Urinastatin, is a protease inhibitor obtained from the urine of healthy people (6). It is found in the English language literature under the name of Urinary Trypsin Inhibitor-UTI (7).

Polish translation („moczowy inhibitor trypsyny”) has not been used up to now /maybe due to the lack of Polish literature on this subject /therefore further in this paper, the following equivalent terms will be used interchangeably: Ulinastatin, Urinastatin, UTI.

Ulinastatin is manufactured by the Japanese company Mochida Pharmaceutical Co. Ltd. Tokyo Japan and distributed as Miracilid, in the form of a dry, freeze-dried substance in sterile vials of 300.000 units of enzyme or the preparation is produced in Europe by the company KRAEBER GmbH & C.O. ELLERBEK Germany. It is distributed as Ulinastatin UTI in vials of 300.000 active units of the enzyme.

Using of experimental model of haemorrhagic, severe form of AP developed by Heinkel and Aho (8, 9), while giving at the same time the protease inhibitor Ulinastatin to the studied group of animals, determination of histopathologic changes of the pancreas was aimed, depending on the use of protease inhibitor and time of its administration.

**MATERIAL AND METHODS**

Procedures of the experimental model were carried out based on a consent of Local Ethics Committee in the Medical University in Lublin for Research on Living Vertebrates.

**Experimental groups**

Studies were carried out on male Wistar rats weighing 250-300 grams. The animals were housed in cages for a week, they were fed with a standard diet. They were given only water a day before experiment. The animals were divided into 4 groups:

- Z – a group of healthy animals used for determination of normal standard pictures of the pancreas in histopathological image – 20 animals
- K-1 – a group of operated animals in which acute pancreatitis was induced by an injection of 5% sodium taurocholate in a bile duct at a dose of 0.1 ml/100 g body weight – 35 animals
- D – a group of operated and treated animals in which AP was induced by the injection of 5% sodium taurocholate into the bile-pancreatic duct. In 1 hour after induction of acute pancreatitis, administration of the protease inhibitor Ulinastatin was started. It was given intraperitoneally at a dose of 10 000 units per kg of body weight, that is 3000 units per one animal per one dose – 60 animals
- K-2 – a group of operated animals in which only 0.9% NaCl was injected into the bile-pancreatic duct – 25 animals.

The protease inhibitor Ulinastatin UTI used in the experiment was produced by KRAEBER GmbH & C.O. ELLERBEK Germany. For the present experiment, the manufacturer agreed to produce 300.000 IU vials for economical reasons. UTI as a dry substance was dissolved in normal saline directly before intraperitoneal administration to the experimental animals of group D. Additionally, depending on time of the conducted studies, the animals were assigned randomly to 5 time groups for monitoring of histopathological lesions. Number of animals was as follows:

- after 2 hours (K1-7; D-12; K2-5),
- after 6 hours (K1-7; D-12; K2-5),
- after 12 hours (K1-7; D-12; K2-5),
- after 24 hours (K1-7; D-12; K2-5),
- after 48 hours (K1-7; D-12; K2-5),
- after 72 hours (K1-7; D-12; K2-5),

Altogether, 150 experimental animals were used in the experiment, divided additionally
into 18 subgroups according to the above principle. Treatment with Ulinastatin was introduced in 60 animals. The last 2 subgroups (after 72 hours of disease) were used only for evaluation of a long-term survival rate. After 2, 6, 12, 24 and 48 hours, the animals were sacrificed, laparotomy was conducted and internal organs were taken for histopathological examinations. In a group of treated animals, attention was paid to the possible complications of intraperitoneal administration of the drug, but such cases were not observed.

Experimental, acute pancreatitis (eAP) was induced according to the model proposed by Aho and Henckel with the use of sodium taurocholate (8-12). The animals were sacrificed under sterile conditions by administration of ketamine intramuscularly at a dose of 5 mg/kg body weight. Peritoneal cavity was opened by a cut. After exposing of proximal part of duodenum, pancreas and bile-pancreatic duct were identified. Sodium taurocholate was administered to the duct through the duodenum by 0.5 x 16 mm needle. In animals of K-1 and D groups, sodium taurocholate solution was given in a concentration of 5 g/100 ml of normal saline at a single dose of 0.1 ml per 100 g body weight. Animals of group K-2 were given only the solvent by this method, i.e. normal saline. After surgery, integuments of abdominal cavity were sutured by a monolayer continuous suture. In 3 cases of single individuals from different groups, due to gnawing of the suture by the animals, initial symptoms of exenteration were observed. In these animals, the wound was additionally sutured. In group D animals, one hour after the injection, administration of Ulinastatin was started. The drug was given intraperitoneally. The preparation was given in amount of 10,000 units per kg body weight per hour, on average at the dose of 3,000 unit per animal per hour. Administration of UTI was continued until the end of the first day. In the last two subgroups, there was a night break of 8 hours. The number of UTI doses depended upon duration of the experiment and was for individual time groups 1, 5, 11, 15, 15, respectively.

Histopathological examinations

For histopathological examinations, fragments of the pancreas and liver weighing about 1 g each were taken. Tissues were fixed for 3 days in 10% buffered formalin solution, and then rinsed for 4 hours. Sections were dehydrated with series of alcohols of increasing concentrations and exposed to xylene for 10-15 minutes. The received sections were embeded in paraffin and cut with toboggan microtome into sections up to 1 micrometer. Preparation obtained in such way were stained with hematoxyline and eosine according to Bagiński method (11). Evaluation of histopathologic preparations and documentation was done under light microscope Jenalumar in Histopathology Laboratory of Independent Public Hospital No. 1 in Lublin. A degree of development of pancreatic lesions was evaluated according to point Spormann scale (tab. 1) (13, 14).

Histopathological evaluation in a course of eAP in respective groups of animals

Group Z

Microscopic image of histopathological preparations stained with hematoxyline and eosin in this group of animals complied with normal descriptions.

K-1 group

After 2 hours, in interlobular spaces, symptoms of oedema was observed due to gathering

Table 1. Evaluation of intensification of histological lesions according to Spormann et al.

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Intensification of lesion</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oedema</td>
<td>moderate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>3</td>
</tr>
<tr>
<td>Inflammatory infiltration</td>
<td>moderate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>3</td>
</tr>
<tr>
<td>Fat necrosis</td>
<td>&lt;2/section</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3-5/section</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>&gt;5/section</td>
<td>7</td>
</tr>
<tr>
<td>Glandular necrosis</td>
<td>focal (&lt;5%)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>and/or sublobular (&lt;20%)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>and/or lobular (&gt;20%)</td>
<td>7</td>
</tr>
<tr>
<td>Extravasations of blood</td>
<td>moderate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>high</td>
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of amorphous protein liquid. In this liquid, extravased erythrocytes and sometimes infiltrations of neutrophils were found. These lesions pertained only to extraglandular part and did not include Langerhans islets. The sum of points according to Spormann scale was from 2 to 7 for a single animal.

After 6 hours, microscopic lesions were of similar character, but were more common. There were more extensive extravasations, coagulant necrosis and more neutrophils. The process of the disease include also peripancreatic tissue. Intraglandular part was without lesions. The sum of points according to Spormann scale was from 5 to 12 (fig. 1).

After 12 hours, in this group of animals, microscopic pictures showed edematous changes in the interlobular spaces and in the peripancreatic tissue with presence of lesions as in a group after 6 hours. There were also focuses of enzymatic fat necrosis in the pancreas. Intraglandular part was normal. The sum of points according to Spormann scale for single animals was from 8 to 15 (fig. 2).

After 24 hours, fields of extensive fat necrosis appeared including both extra- and intraglandular part of the pancreas. They were accompanied by large focuses of hemorrhagic necrosis of the gland. Extensive fields of necrosis included also peripancreatic tissue, where also macro- and micro-abscesses appeared with extensive infiltration of granulocytes. The sum of points according to Spormann scale was from 8 to 18 for a single animal (fig. 3).

After 48 hours, further intensification of lesions was observed. There were extensive interstitial infiltrations of the pancreas composed of neutrophils, lymphocytes and macrophages. Both in the pancreas and the peripancreatic tissue, extensive fields of hemorrhagic necrosis was found and formation of abscesses. The sum of points according to Spormann scale was from 12 to 20 for a single animal (fig. 4).

**Group D**

Total description of morphological changes given by a pathologist for all treated animals was put in order according to an increase of acute pancreatitis severity with assignment of the number of studied group without knowing a principle for identification of respective groups. Lesions of the smallest intensity consisted in the presence of amorphous protein mass in interlobular spaces, extravased erythrocytes and sometimes infiltrations of granulocytes and lymphocytes with passive hyperemia and small foci of Balser necrosis. Group K-1 – 6 hours of the experiment. Staining H + E. Magnification 200x

Fig. 1. Inflammatory infiltrations of granulocytes and lymphocytes with passive hyperemia and small foci of Balser necrosis. Group K-1 – 6 hours of the experiment. Staining H + E. Magnification 200x

Fig. 2. Extravasation of blood, purulent focus, Balser necrosis in peripancreatic tissue. Group K-1 – 12 hours of experiment. Staining H + E. Magnification 200x

Fig. 3. Infiltration of inflammatory cells in interstitial tissue of the pancreas. Group K-1 – 24 hours of experiment. Staining H + E. Magnification 200x
rocytes and sometimes neutrophils – treated groups up to 6 and 24 hours, number of Ulinastatin doses – 5 and 15. The sum of points according to Spormann scale for a single animal was from 4 to 5 (fig. 5).

In more enhanced, apart from the described lesions involving larger interstitial areas, foci of small extravasations and presence of mononuclear cells of lymphocyte type, plasmatic cells or single macrophages was observed – a group of treated animals selected in 2 hours after induction of acute pancreatitis and administration of 1 dose of Ulinastatin. The sum of points according to Spormann scale for a single animal was from 5 to 7 (fig. 6). In groups of animals treated with UTI after 2 and 12 hours of the experiment, small focuses of early phase of parenchymal necrosis of the pancreas appeared in few lobuli. Number of Ulinastatin doses 1 and 11. The sum of points according to Spormann scale for a single animals was from 6 to 8 (fig. 7).

Exudative lesions, more intensely enhanced, included also peripancreatic tissue, and small and clear focuses of fat necrosis were more common.

Groups of animals treated and selected after 12 and 48 hours. Number of Ulinastatin doses 11 and 15. The sum of points according to Spormann scale for a single animal was from 7 to 8 (fig. 8).

Most enhanced lesions consisted in extensive fields of necrosis including both extra- and intraglandular part. Extensive fields of fat necrosis involved also peripancreatic tissue, sometimes with the tendency to form micro-
Morphological changes of the pancreas in course of acute pancreatitis during treatment with Ulinastatin

abscesses. A group of animals treated for up to 48 hours after induction of AP. The number of Ulinastatin doses 15 (fig. 9).

The analysis of treated group D showed non-uniform images in respective time groups. Among treated animals, there were subgroups of milder course and better histological image and groups of more severe course and worse histological images. Therefore in group D they were classified additionally into subgroups: a – of better histological image and smaller number of points, b – of worse histological image and higher number of points.

Observations of researchers indicate that all animals in certain time group responded to treatment in the same way. This difference is most visible in group D of animals after 48 hours of treatment. Some animals responded very positively to treatment, and in histopathological image, the changes were rated as 7-8 in Spormann scale. Second part of the animals of much more severe course with glandular necrosis of the pancreas was rated as 10-16 points in Spormann scale. In order to determine possible differences in histopathological images of the pancreas of the treated and untreated animals, comparative analysis of these images was carried out. Group K-1 after 2 hours showed only small oedema and very small infiltration giving images at the limit of normal. Number of points according to Spormann 2-7.

In group D after 2 hours and administration of only one dose of Ulinastatin, histopathologic images were rated as 5-7 points according to Spormann scale. Histopathological lesions in this group were in some cases more advanced than in untreated group of animals, so the conclusion was drawn that a single UTI dose did not have a therapeutic importance.

Groups K-1 and D after 6 hours of the experiment showed already definite differences between untreated and treated animals. In a group K-1, animals showed much more intense symptoms of oedema, cellular infiltrations and extravasations of erythrocytes with involvement also of peripancreatic tissue with the sum of points 5-12 according to Spormann scale. In a group of treated animals D, lesions were rated as 4-5 points according to Spormann scale, so the same or even slightly less than it was in group D after 2 hours. Comparison of histopathological images of the pancreas of animals untreated and treated with UTI showed that 5 doses of the drug definitely inhibited development of acute pancreatitis at the level of oedematous form.

Groups K-1 and D after 12 hours of the experiment showed also very significant differences in histopathological images of untreated and treated animals. In animals K-1, fat necrosis of the pancreas and peripancreatic tissue with further quantitative enhancement of lesions found in 6-hour group. Enhancement of these lesions was evaluated as 8-15 points according to Spormann scale. In group D of 12-hour treated animals, which received 11 doses of UTI, the lesions are rated as 6-8 points according to Spormann scale with division into subgroup „a” 6-8 points and subgroup „b” 7-8 points according to Spormann scale. However, both of these subgroups showed much better histopathological image...
of all treated animals not exceeding 8 points in a Spormann scale, when changes in untreated animals K-1 exceeded most commonly 8 points reaching 15 points according to Spormann scale. Comparison of these values clearly indicates protective influence of Ulinastatin on the pancreas of the experimental animals after administration of 11 doses of the drug.

In groups after 48 hours, further development of pathologies in the pancreas was found. This phenomenon concerned all animals in group K-1, whereas in treated group D only some animals, but of much smaller intensity compared to K-1. In group D 2 clear subgroups were observed: a – in which lesions were rated as 7-8 points according to Spormann scale, b – in which symptoms of necrosis of the gland occurred with rating of 10-16 points.

However, it should be noted that in group „b”, despite histological exponents of necrosis, quantitative development of lesions of this type was significantly smaller than that in group K, what probably determined much higher survival rate of 48-hour animals of group D. In group K-2 – animals which were given 0.9% NaCl solution into the bile-pancreatic duct, only hypertension itself in bile-hepatic ducts itself was the reason of induction of acute pancreatitis, but even most severe forms in this group did not exceed 10 points in Spormann scale.

**DISCUSSION**

Ulinastatin was used in the experimental models first of all for the studies on many aspects of acute pancreatitis (15-24). Lack of causative treatment which could definitely stop development of acute pancreatitis in its edematous stage, and protect patients from life-threatening complications justifies further studies in this field. It seems that improvement of treatment results of the patients with most severe forms of acute pancreatitis is rather a result of an advance in intensive therapy and multi-disciplinary attitude towards the disease than new possibilities of causative therapy (25). Treatment of most severe forms of acute pancreatitis in at least 30% is not successful (2). Advance in knowledge of pathological mechanisms of acute pancreatitis at the cellular and molecular level raise hope for getting to know factors which may significantly limit percentage of severe forms of AP. Well-known and still relevant, with some modifications, proteolytic theory indicates that AP is induced by an activation of non-active trypsynogene to trypsin (1). It is also known that development and unfavourable evolution of AP depends also on balance of equilibrium of natural proteases and antiproteases in patient’s organism (1). Hence, theoretical premises rise for use of antiproteases in the form of synthetic or naturally obtained protease inhibitors given from outside. Unfortunately, substances blocking proteases known for years had not explicitly positive effects after the clinical use (26, 27).

The results of laboratory and clinical studies carried out and published in Japan with the use of natural antiprotease named Ulinastatin seemed encouraging for further experiments (28, 29, 30). Aho et al. proved that histopathological lesions in the experimental AP appear already after few minutes and are a results of a direct dissolving action of taurocholate on the pancreas (8, 9, 10). Extravasations appeared just after 2-3 hours, and in 6 hours after induction, fat tissue necrosis was diagnosed. Similarly as with others, in authors’ experiment severe, necrotic-haemorrhagic forms of AP were obtained with time in all animals of K-1 group, in whom only sodium taurocholate was given (31, 32, 33).

In our own experiment, in group K-1, clear lesions in the pancreas were seen as soon as after 2 hours. They had a form of edema and haemorrhagic extravasations, initially small, developing with time, with an increase of neutrophil infiltrations and later, of lymphocytes. In consecutive time intervals, symptoms of necrosis developed in the pancreas itself as well as in peripancreatic tissue. Thus, from 6 to 48 hours of experiment duration, in the preparation of the pancreas of untreated animals, definite development of exponents of severe pancreatitis with its necrosis was seen. Analyzing then a group of experimental animals – group D treated with Ulinastatin, different results were obtained. In some animals, the process of disease progression was inhibited at the stage of oedema. From analysis of histopathological images, it also results that groups of animals on which autopsy was performed after 2 hours of disease and after administration of 1 dose of the drug have greater lesions in the pancreas than those after 6
and 24 hours of treatment. Ulinastatin inhibited progression of the disease. Numerical data explicitly indicate protective influence of Ulinastatin on the pancreas. Similarly, Tani proved the protective influence of UTI on the pancreas manifesting by a decrease of edema, degree and size of vacuole and a decrease of development of cellular infiltrations (7) in his studies. It results from comparative analysis of histopathological images of the pancreas that Ulinastatin exerts protective effect on the pancreas decreasing the number of AP forms with necrosis and increases probability of survival in severe forms pancreatitis.

CONCLUSIONS

The use of UTI in treatment of eAP leads to an inhibition of inflammatory process at the stage of pancreatic oedema and in case of necrotic inflammation of severe course, reduces disease progress what is a premise for its clinical use in humans.

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