INFLUENCE OF NON-ENZYMATIC ANTIOXIDANTS ON ANTIOXIDATION STATUS IN ACUTE HAEMORRHAGIC NECROTIZING PANCREATITIS IN RAT

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Abstract

The aim of the work was to assess the influence of some non-enzymatic antioxidants (uric acid, blood urea, bilirubin) on the antioxidant status of rat’s organism during the experimental acute haemorrhagic necrotising pancreatitis. The experiment was carried out on male Wistar rats, body weight 280-320 g, which were divided randomly into control and experimental groups. The control group (n=8) underwent laparotomy. The experimental group (n=24) was injected into pancreatic duct with natrium taurocholate to induce acute pancreatitis – according to the Heinkel and Aho method. Total antioxidation status in plasma was assessed according to the Benzie method (FRAP); total bilirubin, urea, and uric acid in plasma were assessed as described elsewhere. These parameters were examined in each group 3 h, 24 h, and 48 h after the experiment started. Simultaneously α-amylase activity (indicator of pancreatitis severity) was assessed in plasma. The obtained results were analysed statistically. Pancreatitis achieved its maximum severity 24 h after the beginning of the experiment and then decreased. After 48 h α-amylase activity was only 2 times higher than that in the control group. FRAP maximum was noticed just after 3 h, then became lower and 48 h after the experiment onset its minimal value was 2 times lower than that of the control. Uric acid and bilirubin plasma concentration grew up 24 h after experiment beginning and next decreased. Only plasma urea concentration increased stably to the maximum value which was observed 24 h after the operation and after 48 h only slight decrease in its concentration was noticed. This phenomenon may be the result of parenchymatous organs damage occurring in acute haemorrhagic necrotising pancreatitis. It leads to interlobular and periacinar tissue necrosis as a consequence of lipid peroxidation. The obtained results showed the booster effect of non-enzymatic antioxidant agents on total antioxidation status during experimental pancreatitis. This effect correlated with pancreatitis severity which was evidenced by an increased α-amylase activity in plasma.

Key words: rats, haemorrhagic necrotizing pancreatitis, antioxidation status.

It is well recognised that oxidative stress is an important factor in the pathogenesis of acute pancreatitis (AP). However, it is still unclear whether oxygen free radicals (OFR) act only as mediators of tissue damage or represent the initiating event in acute pancreatitis in vivo. OFR – as a highly reactive species exert their pathophysiologic effects by directly attacking lipids and proteins in the biologic membranes in situ and indirectly act on the arachidonic acid cascade by two mechanisms: they increase the production of thromboxane which lowers tissue circulation by its potent platelet aggregation and vasoconstricting effects and they enhance the production of leukotriene B4 which promotes the activation of leukocytes and release of lysosomal enzymes. OFR influence pancreas by increasing Ca2+ in cytosol. The increase of cytosolic Ca2+ is also highly related to the activation of neutrophils, which contributes to neutrophil adhesion to the endothelium in the early phase of AP. The effect of fluid shear stress on Ca2+ may play a crucial role in pancreatic microcirculatory failure of AP. These changes contribute to further cell damage (24).

The development of destructive and purulent forms of acute pancreatitis was found out to be accompanied by activation of lipid peroxidation (LPO) processes, antioxidiant defence (AOD) breakdown, with profound disturbances having been disclosed in the metabolism of zinc, selenium, cuprum, manganum, that play an important part in LPO, AOD processes, and in those of immune defence. The results suggest that there is a need to include antioxidants and some trace elements (zinc, selenium) into a complex therapy of acute pancreatitis. The results suggest also that it is expedient to use indices for the LPO, AOD systems and for trace element status in the differential diagnosis of clinical forms of acute pancreatitis and prognostication of the development of purulent complications.

Blood flow in pancreatitis is altered by a number of endogenous and exogenous factors. Earliest changes involve trypsinogen activation and in this way induction of ischaemia. In the acute form, reduction in
blood flow and alterations in microvascular integrity resulting in impaired tissue oxygenation play a pivotal role in initiation and progression of the disease (23). Endothelin and nitric oxide are believed to be two of the most effective vasoactive mediators.

Acute necrotising pancreatitis is associated with an inflammatory explosion involving numerous pro-inflammatory mediator cascades and oxidative stress (21). Intraductal taurocholic acid and ischaemia-reperfusion provokes severe acute necrotising pancreatitis and leads to systemic inflammatory reaction which appears to be the consequence of the activation of the cytokine cascade and the production of cytokines such as tumour necrosis factor (TNF) and interleukin-6 (IL-6) (12). Acinar oxygen free radical production aggravates pancreatic tissue damage, and promotes cellular adhesion molecule upregulation resulting in leukocyte adherence and activation. The cerium capture of free radical histochemistry combined with reflectance confocal laser scanning microscopy allows the "in situ" histological demonstration of oxygen free radical formation in live tissues. This allows to investigate the association between oxidative stress, protease activation, and local production of proinflammatory cytokines and the severity and lethality of the disease. Although a reduction in peripheral lymphocytes has been reported in clinical cases of acute pancreatitis, the change in the thymus remains still unknown. To investigate impairment of cellular immunity in acute pancreatitis, alterations of the thymus in rats with acute pancreatitis were examined experimentally.

From experimental and numerous clinical observations it follows that acute pancreatitis, beside changes in the gland itself, causes a number of structural and functional disorders in the whole organism. Free radical reactions, occurring in the course of acute pancreatitis, initiate among others uncontrolled chain reactions, occurring in the course of acute pancreatitis, initiate among others uncontrolled chain peroxidation of lipid structures of cell membranes.

The aim of the work was to assess the influence of some non-enzymatic antioxidants (uric acid, blood urea, bilirubin) on the antioxidant status of rat organism during the experimental acute haemorrhagic necrotising pancreatitis.

**Material and Methods**

The studies were carried out on 32 Wistar male rats, with body weight of 280-320 g. Twenty-four hours before the experiment the animals were devoid of feed but had free access to drinking water, to which 1 h before the procedure dormicum (midazolam maleate) was added to evoke sedation. The animals were randomly divided into two groups: group I – control, without pancreatitis, and group II – animals with induced pancreatitis.

Group II (24 rats) was divided into 3 subgroups - A, B, C according to the time of acute pancreatitis progress. Blood was collected after 3 h in A subgroup, after 24 h in B subgroup, and after 48 h in C subgroup from the induction of pancreatitis.

Total antioxidative potential (FRAP), concentrations of uric acid, urea and bilirubin as well as α-amylase activity were determined in the plasma.

**Model of acute haemorrhagic necrotising pancreatitis** (18). All the animals were given general anaesthesia via intraperitoneal administration of 50 mg/kg b.w. of thiopental and 60 mg/kg b.w. of fentanyl. Then, in aseptic conditions laparotomy was performed by a small incision in the median line below the sternum. In group II animals, using a surgical technique, a polyethylene neoflon cannula (06/19 mm) was introduced via the duodenum to the common bile and pancreatic duct by Heinkel and Aho method (1). To avoid the reflux of duodenal contents a silk ligature was put on at the entrance of the duct to the duodenum and tightened around the cannula and duct wall. Hepatic duct was closed with small and soft haemostatic forceps near the hilus of the liver. Then, for 3 min 5% sodium taurocholate at the dose of 50 mg/kg was administered in the form of continuous infusion via the cannula. After the perfusion was completed, the cannula, forceps and ligature were removed and the duodenum and coverings were closed with two layers of single sutures.

Total antioxidant capacity in the plasma, FRAP assay (Ferric Reducing/Antioxidant Power assay), was determined according to Benzie et al. (6) by spectrophotometric method. The principle of the method is the ferric to ferrous iron reduction in the presence of antioxidants at a low pH and production of coloured ferrous-tripyridyltriazine complex. Quantity of the plasma antioxidative potential was expressed in µmol/l.

Uric acid, as well as total bilirubin concentrations were determined by using Cormay diagnostic sets. Urea concentration (20) was determined according to Bio Mérieux Urea - Kits S. Amylase (α-amylase activity) was determined according to Bertholf et al. (7) where CNP-G3 is a direct substrate for α-amylase, enabling the measurement of the enzyme activity without using auxiliary enzymes.

The results of these investigations were presented as mean values ± mean value standard error. Variance analysis was used in the single classification (ANOVA). Differences between groups were regarded as significant if variance between the groups was greater than the variances within the groups at P < 0.05 according to “F” Fisher test. Mean value comparisons in individual groups were made using “D” Duncan test of multiple gap, χ² test, and t-Student test for independent data were also applied.

**Results**

Pancreatitis achieved its maximum severity 24 h after the beginning of the experiment and then decreased. After 48 h α-amylase activity was only twice higher than in the control group (Fig. 1). FRAP maximum was noticed just after 3 h, then it became lower and 48 h after the beginning of the experiment its minimal value was twice lower than in the control (Fig. 2). Uric acid and bilirubin plasma
concentration grew up 24 h after the beginning of the experiment and then decreased (Figs 3 and 5). Only plasma urea concentration increased stably to the maximum value, which was observed 24 h after the operation and after 48 h only a slight decrease in its concentration was noticed (Fig. 4). This phenomenon may be the result of parenchymatous organs damage occurring in acute haemorrhagic necrotising pancreatitis. It leads to interlobular and periduodenal tissue necrosis as a consequence of lipid peroxidation.

In blood samples of A and B subgroup animals, collected 3 and 24 h after the pancreatitis induction, a significant growth of plasma antioxidative potential was found compared with that in the control group. After 48 h of pancreatitis (C subgroup) the oxidative potential value of plasma was almost half as much compared with the values in the control animals. (Fig. 3).

Uric acid concentration in the plasma of control animals was 2.10 ± 0.22 mg/dl, whereas in the animals with pancreatitis in A subgroup it was 3.70 ± 0.19 mg/dl and in B subgroup - 6.98 ± 1.06 mg/dl. In samples collected 48 h after the pancreatitis induction (B subgroup) the mean value of uric acid concentration in plasma was 1.21 ± 0.24 mg/dl and was significantly lower than in the control animals (Fig. 3).

Urea concentration in the plasma of control animals was on average 25.33 ± 3.35 mg/dl. After 3 h of the experiment, since sodium taurocholate was given (A subgroup), no statistically significant differences were found in the urea concentration compared with the results obtained from control group. Statistically significant increase in the urea concentration in plasma was found in the samples taken at 24 h and 48 h of the experiment. In B subgroup urea concentration in plasma increased on average by 64.8% compared with the value obtained in the control animals (41.75 ± 4.39 mg/dl) and in C subgroup it increased by 46.2% (37.05 ± 4.02 mg/dl) (Fig. 4).

Bilirubin level in the plasma of control animals was 0.26 ± 0.03 mg/dl on average. In A subgroup animals the increase of bilirubin contents was over twice higher (0.57 ± 0.09 mg/dl) compared with its contents in the control group. In subgroup B animals bilirubin concentration reached the value of 1.14 ± 0.34 mg/dl, which meant an over 4 times increase on average compared with the control. In the tests made at 48 h of the experiment (C subgroup) bilirubin level declined to 0.41 ± 0.21 mg/dl, however, in comparison with the results obtained in the control group that difference was not statistically significant (Fig. 5).

The analysis carried out by χ-square test between the antioxidative potential of plasma, values of uric acid, urea, and bilirubin in the blood of the examined animals showed a high correlation level, which points to the dependence of antioxidative potential on those antioxidants (Fig. 6).

Discussion

The role and importance of many pathological agents as well as pathogenesis and development of multiorgan insufficiency, mainly in the lung and kidneys, in the course of acute haemorrhagic necrotising pancreatitis have been the subject of numerous studies, discussions, and disputes for a long time. Since the reactive oxygen forms can have a great influence on the living organism - the role of free radical reactions in the pathogenesis of diseases and multiorgan complications has been a key issue as well as IL-1, TNF-α, and influx of Ca²⁺ in cytosol (12, 24). Oxidative stress is an important factor in the pathogenesis of acute pancreatitis, as shown in vivo by the beneficial effects of scavenger treatment. But it is still unclear whether oxygen free radicals (OFR) are only mediators of tissue damage or represent the initiating event in pancreatitis (15). These authors mentioned a significant decrease in GSH and elevated level of conjugated dienes (CD) in experimental acute pancreatitis. But in conclusion they reported that extracellular OFR alone do not induce the typical enzymatic and morphological changes in the course of pancreatitis (15).
Fig. 2. Oxidoreductive potential of plasma in rats 3, 24, and 48 h after the pancreatitis induction. Mean values ±SEM. *P<0.005; ** P<0.001 with respect to control group (Duncan test).

Fig. 3. Uric acid concentration in rat blood serum 3, 24, and 48 h after the pancreatitis induction. Mean values ±SEM. *P<0.05; ** P<0.001 with respect to control group (Duncan test).

Fig. 4. Urea concentration in rat blood serum 3, 24, and 48 h after the pancreatitis induction. Mean values ±SEM. *P<0.05; ** P<0.01 with respect to control group (Duncan test).
In our own experiments acute pancreatitis was induced in animals by Heinkel and Aho method consisting in sodium taurocholate administration into the pancreatic duct. It leads to sudden development of haemorrhagic necrotising pancreatitis with local alterations and those in the distant organs such as the heart, lung, and kidneys, similar to those observed clinically in humans. Intraductal protease activation and depletion of pancreatic protease inhibitors is the main pathogenetic factor in this model of acute pancreatitis (1).

In the initiation of inflammatory reaction in the pancreas, neutrophil polymuclear granulocytes are of great importance. Under the influence of chemotactic...
factors (TNF-alpha, interleukin-1, LTB-4) they are accumulated in the inflammatory focus where they become the source of oxygen free radicals (superoxide and hydroxyl radicals) and proteolytic enzymes, mainly elastase, catepsine and colagenase. Generating free oxygen radicals occurs as a result of NADPH oxidase activation on the neutrophil surface and proteases are released during the degranulation of azurophil granules (5, 10). Another source of free radicals are impaired mitochondria and reactions catalysed by xanthine oxidase in the ischaemia-reperfusion syndrome (9, 11). Besides, in acute pancreatitis reductions in blood flow and alterations of microvascular integrity resulting in impaired tissue oxygenation play an important part in the progression and initiation of this disease. Endothelin and nitric oxide are believed to be the two most effective mediators (23). Alhan et al. (2) reported that nitric oxide (NO) inhibitors increased the mortality and serum amylase activity in experimental acute necrotising pancreatitis and had no effect on urea and creatinin concentration, tissue damage, and liver transaminases. In conclusion – constitutive NO synthase inhibition worsens the course of acute necrotising pancreatitis and inducible NO inhibition has beneficial effects. Lubitski et al. (13) found the increased content of blood serum conjugated dienes and thiobarbituric acid reactive substances that also act as endotoxin. Blood serum antioxidant activity correlated with urate concentration. Oxygen free radicals cause a blockade of intracellular metabolic tracts, junction of lysosomic spaces of pancreatic vesicular cells with zymogenic ones, activation of proteo-and lipolytic pancreatic enzymes, and lipid oxidation with generating their peroxides. The after-effects are morphologic changes: interlobular and periglandular necrosis of fatty tissue and haemorrhagic coagulative necrosis of the pancreas (16, 17). The increase of peroxidation products was accompanied by hyperamylasaemia indicative of the development of acute pancreatitis.

The high value of total antioxidative potential in the plasma of animals with acute pancreatitis during the first 24 h of the experiment results from the increase in non enzymatic antioxidants i.e. uric acid, bilirubin, and urea. Bogusz (4) reported that uric acid makes up 33% to 58% in so-called plasmic antioxidant capacity and is the second essential (after thiol compounds) component of antioxidative defence. Reacting with a singlet oxygen and hydroxyl radical, it is an effective antioxidant. Ames et al. (3) proved that the level of that compound at about 300 μM in the plasma protects both haemoglobin from oxygen free radicals and lipid components of red blood cells from peroxidation. Dependence of plasmic antioxidative capacity on uric acid concentration and other non enzymatic antioxidants was also reported in the clinical studies of Tsai et al. (22). Bilirubin and urea also influence (though to a smaller extent) the level of total plasmic antioxidative potential. Bilirubin at micromolar concentrations in vitro efficiently scavenges peroxyl radicals and the antioxidiant activity of bilirubin is oxygen concentration dose dependent. Its maximum efficiency is achieved at liposomes (oxygen concentration under 2%) where bilirubin suppresses the oxidation more than α-tocopherol (19). Bilirubin as a possible physiological antioxidant is more effective than β-carotene but not α-tocopherol (14, 19). On the second day of the experiment the authors noted a significant decrease in the potential, which may be connected with intensive generation of oxygen free radicals and simultaneous depletion of non-enzymatic antioxidants. Similar clinical observations in humans were made by Curran et al. (8) who found that antioxidant concentration in serum is inversely proportional to the increase of CRP (acute-phase proteins) and that it was significantly higher in patients with the mild form of the disease than in those with the severe haemorrhagic necroting pancreatitis.

It follows from our studies that antioxidative defence systems are not able to efficiently protect the organism from the oxidative stress during acute haemorrhagic necroting pancreatitis, which entails the greater risk of developing multiorgan insufficiency syndrome. Precise explanation of the role of oxidative stress in the course of acute pancreatitis and its complications requires further integrated studies on molecular level, cellular cultures, and animal models. It also requires carrying out and estimating therapeutic trials to use preparations of antioxidative properties in the treatment and prophylaxis of organ complications in patients with acute pancreatitis. In conclusion, in acute haemorrhagic necroting pancreatitis occurs a temporary increase in plasmic antioxidative potential (FRAP) and main plasmic non-enzymatic antioxidants. In the course of severe form of haemorrhagic necroting pancreatitis, antioxidative defence systems are not able to efficiently protect the organism from oxidative stress, which may contribute to the higher risk of developing the multiorgan insufficiency syndrome.

References


