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USE OF PROTEINASE INHIBITORS AND ANTIOXIDANTS FOR PHARMACOLOGICAL CORRECTION OF EXPERIMENTAL REPERFUSION SYNDROME

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Abstract

The problem of finding biochemical markers to be used in assessing the severity and prognosis of critical conditions, the degree of systemic inflammatory response syndrome involvement in their pathogenesis, as well as selection of the optimal target effects to achieve the best therapeutic effect assumes ever-greater importance. The aim of this work was to study the pathogenetic significance of determining components of the non-specific proteases and pro-inflammatory cytokines, as well as the effectiveness of the experimental pharmacological correction of reperfusion syndrome by affecting the basic links of pathogenesis using proteinase inhibitors and antioxidants.

The research results showed that reperfusion syndrome development is accompanied by increased proteolytic activity, decreased antiproteolytic activity, increased levels of the main proinflammatory cytokines (IL-1ß, IL-6 and TNF-α) in blood serum with the peak expression in 12 hours after reperfusion. Combined use of proteinase inhibitors and antioxidants for pharmacological correction of the experimental reperfusion syndrome is accompanied by a marked decrease in the total proteolytic activity, increased antiproteolytic activity, lower concentrations of the major proinflammatory cytokines of blood serum in comparison with monotherapy effects of mentioned preparations. The complex pharmacological influence on several key links of reperfusion syndrome pathogenesis using proteinase inhibitors and antioxidants is preferred to monotherapy.

Keywords: *Reperfusion, proteolysis, inhibition, proteinases.*

Introduction

Reperfusion syndrome, or ischemia-reperfusion syndrome, is a pathological process that develops as a result of blood flow restoration in the previously ischemic tissue. Most researchers rank reperfusion syndrome in a row with the entire group of critical conditions of infectious and non-infectious origin, such as acute pancreatitis, severe trauma, abdominal sepsis, in which the systemic inflammatory response syndrome (SIRS) develops [*Kubyshkin A., Fomochkina I., 2011; Gusev E., Chereshnev V., 2012*]. Given that the ischemia-reperfusion syndrome frequently occurs at interventions pertaining to angiosurgery, cardiology, traumatology, the mechanisms of this syndrome development – from the point of view of SIRS specific role – attract an increasing attention. It is indicated that after the operations of aortocoronary bypass the formation of SIRS clinical signs is observed in 100% of patients within the first 12 hours [*Michalopoulos A. et al., 1996*]. Similar changes were recorded during a number of other serious surgical interventions [*Bown M. et al., 2003; Takenaka K. et al., 2006*].

Among the mechanisms involved in the pathogenesis of SIRS the particular importance is attached to an excessive systemic activation of cytokines and proteolytic enzymes that occurs as a result of increased permeability of cell membranes and exocytosis of white blood cells and leads to dysfunction of the kallikrein-kinin system, hemostasis and complement systems [*Douvas S. et al., 2005; Rossi A. et al., 2007; Knight S., Johns E., 2008; Koseki K., 2011*]. It is assumed that cytokines – the main driving force of the pathological process – are regulatory factors, while proteolytic enzymes act as agents providing the basic damaging effects [*Kubyshkin A., Fomochkina I., 2011; Fedosov M. et al., 2012*]. In particular, an important damaging ef-

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fect of proteinases is shown under local pathological processes, such as pulmonary inflammation [*Kubyshkin A., Fomochkina I., 2008*]. Despite this, the study on SIRS development in critical conditions mainly focuses on cytokine homeostasis reactions [*Hayama T. et al., 2006; Mendonca-Filho H. et al., 2006*] without due attention to changes in the protease-inhibitory system.

The importance of proteases activation in the pathogenesis of critical conditions suggests that blocking the activation of proteolytic activity can prevent or significantly reduce the risk of developing SIRS. Moreover, during the treatment of experimental pneumonia the best effect is achieved with the use of protease inhibitors in combination with antioxidants [*Kubyshkin A., Fomochkina I., 2009*], whereas the use of this pharmacological combination in critical conditions has not been investigated.

In this regard, the purpose of this work was to study the character of changes in the proteinase-inhibitory system and in the system of proinflammatory cytokines in experimental ischemia-reperfusion syndrome and to assess the effectiveness of pharmacological correction of the ischemia-reperfusion syndrome using protease inhibitors and antioxidants.

Material and Methods

Experimental studies were carried out in 58 white male "Wistar" rats weighing 180-210 *g*. The study was approved by the Bioethics Committee of the University and conformed to the principles of the Guide for the Care and Use of Laboratory Animals published by the US NIH (No. 85-23, revised in 1985).

Ischemia-reperfusion syndrome simulation: Ischemia-reperfusion syndrome was modeled by applying rubber bands on both hind limbs at the level of the inguinal crease for a period of 6 hours. The width of clamping tissue was 2-3 *mm*. An indication of the tourniquet application correctness was the absence of limbs edema and their pale coloring. Revascularization was performed simultaneously by dissection of bands 6 hours after their imposition.

In 12 hours after reperfusion euthanasia of the animals was performed by decapitation under thiopental anesthesia, and blood samples were taken for research. Experimental animals were arranged into 5 groups: group of animals with untreated reperfusion syndrome (n=12); 3 groups with treatment (by 12 rats each); intact animals (n=10) served as control.

The use of proteolysis inhibitors and antioxidants for the treatment of ischemia-reperfusion syndrome: Treatment of experimental animals was performed in series: the first group was treated with proteolysis inhibitor "Gordox" ("Gedeon Richter", Hungary) at a dose of 20,000 *IU/kg* body weight, the second group was treated with the antioxidant (water-soluble form of quercetin "Corvitin" (Borshchahivskiy CPP, Ukraine) at a dose of 10 *mg/kg* body weight, and the third group was treated with a combination of proteolysis inhibitor and antioxidant (in appropriate dose levels). All treated experimental animals were exposed to single intraperitoneal administration of specified preparations in different syringes 30 minutes before revascularization of the limbs.

Determination of proteinases and their inhibitors activity: Determination of activity exerted by proteinase-inhibitor system components was carried out using enzymatic methods [*Kubyshkin A. et al., 2010*] on spectrophotometer "Biomat-5" (Great Britain). Trypsin-like activity (TLA) was measured by cleavage speed of N-benzoyl-L-arginine from the ethyl ester synthetic substrate N-α-Benzoil-L-arginine ethyl ester hydrochloride (BAEE) ("Sigma", USA). Determination of elastase-like activity (ELA) was based on the study for hydrolysis rate of the synthetic substrate Boc-L-alanine-4-nitrophenil ester (Boc-Ala-ONp) ("Sigma", USA). Determination of alpha-1-proteinase inhibitor (ATA) was performed through BAEE cleavage inhibition by trypsin. Similarly, the activity of acid-stable inhibitors (ASI) was determined after serum preparation upon heating in the acidic environment.

Determination of key proinflammatory cytokines concentrations: Determination of the major proinflammatory cytokines IL-1ß, IL-6 and TNF-α was performed using enzyme-linked immunosorbent assay (ELISA) reagents/kits ("RayBio", USA). Results were recorded using a microplate scanner at the wavelength of 450 *nm*.

Statistical analysis: Statistical data processing was carried out using the methods of variation statistics with the calculation of the average values (M), assessing the likelihood of discrepancies (m), as well as the reliability of changes using the Student's *t*-test. The difference between mean values was considered reliable at $p<0.05$.

Results

The studies showed that in 12 hours after the experimental reperfusion syndrome development there occurred marked changes in indicators of proteinase-inhibitory system activity and cytokines. As to proteinase activity, divergent changes were revealed: compared to control there was almost three times increase in proteases TLA alongside with decreased ELA.

The growth of TLA was accompanied by a parallel decrease in activity of ATA (1.5 times) and ASI (almost 2 times). In the system of pro-inflammatory cytokines a pronounced increase was observed in concentrations of all the studied proinflammatory cytokines. Thus, levels of IL-1β increased approximately 10 times, IL-6 was elevated 24 times, and TNF-α – 16 times, as compared with the control values.

The reperfusion syndrome development with pharmacological correction showed that the use of proteinase inhibitor and antioxidant reduced the severity of changes in the study parameters (Figure 1). Thus, TLA decreased 1.7 times at monotherapy by proteinase inhibitor, 1.5 times upon monotherapy by antioxidant, and 3.5 times with the combined use of mentioned substances, as compared with the untreated group. At monotherapy TLA remained above the reference (control) values, whereas the combined use of two preparations decreased TLA as related to control. The ATA reached a maximum value by the combined use of proteinase inhibitor and antioxidant, compared with monotherapy, 1.4 times exceeding the level in the untreated group, while at the same time becoming by 4% higher than the control (reference) value. A similar trend was observed in the dynamics of ASI activity. Furthermore, at the combined use of proteinase inhibitor and antioxidant the latter increased 2 times compared with the untreated group and, thereby, exceeded the control value by 1%.

Within the system of key proinflammatory cytokines the most effective decrease of IL-1β concentration (above 10 times as compared to the untreated group) was achieved in case of combined use of proteolysis inhibitor and the antioxidant: this component decreased below the reference value by 7% (Figure 2). The concentration of IL-6 both in case of monotherapy with the proteolysis inhibitor, and in case of treatment by its combination with the antioxidant effectively decreased more than 4 times compared with the untreated group. The concentration of TNF-α effectively reduced almost 4 times compared with the untreated group both at monotherapy and upon combined therapy with proteolysis inhibitor and the antioxidant.

Thus, development of reperfusion syndrome is accompanied by increased proteolytic activity of blood serum, inhibition of antiproteolytic activity, increased levels of key pro-inflammatory cytokines in the blood serum. These changes are most pronounced in the period of 12 hours after reperfusion.

In case of monotherapy by proteinase inhibitors or antioxidants the positive effect was obtained due to decreased activity of proteinase inhibitors, increased activity of their inhibitors and reduced concentration of key blood serum proinflamma-

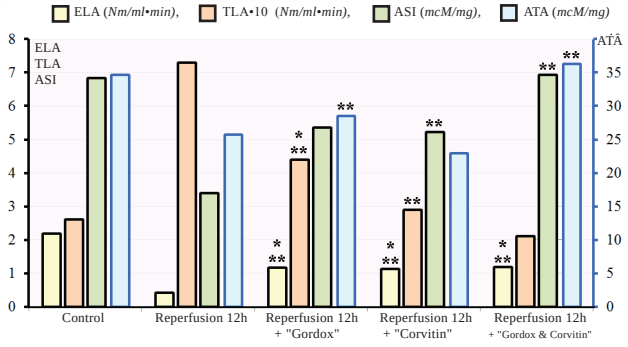

Figure 1. *Changes in the proteinase-inhibitory blood system of rats during the development of ischemia-reperfusion syndrome with treatment.* ***Note:*** *asterisks indicate significance of differences in indicators: * – with respect to the control group of animals; ** – with respect to the group of animals with reperfusion syndrome without treatment. * – p<0.001, ** – p<0.01.*

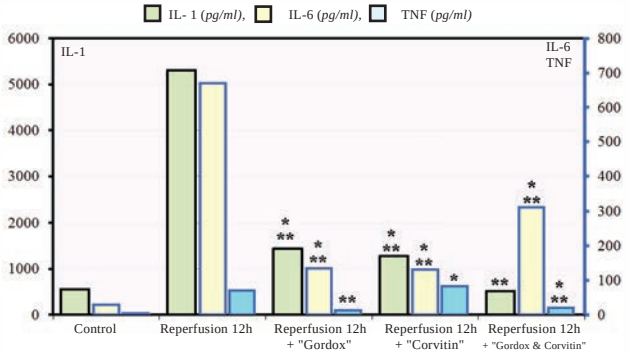

Figure 2. *Changes in blood cytokines system of rats at ischemia-reperfusion syndrome development with treatment.* ***Note:*** *asterisks indicate significance of differences indicators: * – with respect to the control group, ** – with respect to the group of animals with reperfusion syndrome without treatment.*

tory cytokines. However, the combined use of proteinase inhibitors and antioxidants for the experimental pharmacological correction of reperfusion syndrome led to a more pronounced decrease in the total proteolytic activity, increase in antiproteolytic activity, decrease in concentrations of blood serum key proinflammatory cytokines compared with the effects of monotherapy. Thus, the effect is simultaneously produced at several key links of pathogenesis specific for the given critical condition, according to mechanisms of action of the administered preparations.

Discussion

The research findings suggest that development of the experimental reperfusion syndrome is accompanied by pronounced activation of blood serum proteases and proinflammatory cytokines. Moreover, ten- to twentyfold increase in cytokines and three-fold increase of trypsin-like proteases might be considered as a predictor for development of SIRS. As known, SIRS is recently considered as one of the key pathogenetic mechanisms of critical conditions [*Kubyshkin A., Fomochkina I., 2011*], and its development is observed in the progress of acute pancreatitis, sepsis, hemorrhagic shock and other critical conditions [*Kibe S. et al., 2011; Pugin J., 2012*]. Given the high prevalence of SIRS in critical medicine, informative markers for diagnosis and prognosis of its development are searched. The possibility to use procalcitonin, cholinesterase, C-reactive protein, plasminogen activator as biochemical markers of SIRS is studied [*Eremenko A. et al., 2012; Gunjaca I. et al., 2012*], but most of the works are devoted to the study of cytokines: TNF-α, IL-6, IL-10, IL-1β, etc. [*Jin Qi-Hui et al., 2011; Bishehsari F. et al., 2012*].

The study thus performed revealed that development of severe reperfusion syndrome is really accompanied by avalanche-like activation of proinflammatory cytokines. However, our attention was drawn to the fact of significant activation of proteases, which occurred against the oppression of their inhibitors. At the same time, both labile proteinase inhibitors and acid-stable proteins were reduced in blood serum. These changes can be explained in terms of the excessive consumption of protease inhibitors; as a result, their synthesis in the liver has no time enough to provide sufficient levels in the blood. A similar situation was noted in the development of disseminated intravascular coagulation (DIC) syndrome when the synthesis of coagulation system proteins cannot keep up their consumption in the microvasculature. In this respect, DIC syndrome is also an important component of the severe pathological processes, and recently its development is considered as SIRS-associated coagulopathy [*Koseki K., 2011*].

Thus, formation of the systemic imbalance with activation of proteinases and decrease in levels of proteinase inhibitors in blood serum can be regarded as a sensitive test to determine development of SIRS, which characterizes the severity of the condition and its eventual outcome prediction. In addition, the important role of protease-inhibitor system in the pathogenesis of reperfusion syndrome might indicate the potential effectiveness of antiproteolytic preparations for their treatment.

The current study showed that administration of the polyvalent protease inhibitor and antioxidant had a strong protective effect. It should be noted that protease inhibitor "Gordox" due to its mechanism of action reduces the total proteolytic activity, blocks kallikrein-kinin system, inhibits the production of tissue thromboplastin, produces antifibrinolytic and hemostatic effects, while reducing the likelihood of DIC syndrome development. Application of antioxidant "Corvitin" promotes the protection of protease inhibitors against the oxidative damage and, in addition, antioxidant effect by reducing the generation of cytotoxic superoxide anion. The second effect of "Corvitin" is associated with lipoxygenase pathway of blocking leukotriene synthesis, which is accompanied by an inhibitory effect on the membrane-acting enzymes involved in degradation of cell membrane phospholipids (phospholipases, phosphogenases, cyclooxygenases), as well as inhibition of lymphocyte activation and production of proinflammatory cytokines. Moreover, the water-soluble form of quercetin ("Corvitin") contributes to the rapid achievement of the therapeutic effect, which is a prerequisite for the treatment of critical conditions.

Conclusion

The effectiveness of proteinase inhibitor and antioxidant application is confirmed by a pronounced effect associated with the normalization of the studied

parameters of proteinase-inhibitory system and cytokines. Moreover, the use of the proteinase inhibitor was accompanied by a more pronounced normalization of the protease-inhibitory system in comparison with the isolated use of an antioxidant. However, the best effect was observed in the group of animals with the combined administration of proteinase inhibitor and antioxidant. This finding indicates that development of both reperfusion syndrome and other critical conditions is the process with involvement of the variety of different pathogenic mechanisms, whereby the greatest effect is achieved by treatment upon block or correction of main pathogenic links. In any case, the use of proteinase inhibitors for treatment of critical conditions can be an effective means to improve the quality of treatment and prevention.

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