УДК 616.151.5-02:616-005.4:616.155.37
https://doi.org: 10.20538/1682-0363-2020-3-67-75

Changes in the blood coagulation system and non-specific plasma proteinases in ischemia-reperfusion injury

Pisarev A.A.[1], Petrenko V.I.[1], Kubyshkin A.V.[1], Kharchenko V.Z.[1], Fomochkina I.I.[1], Kuzichkin D.S.[2]

[1] *Medical Academy named after S.I. Georgievsky, V.I. Vernadsky Crimean Federal University*
5/7, Lenin Boul., Simferopol, Republic of Crimea, Russian Federation

[2] *State Scientific Center of the Russian Federation, Institute of Medical and Biological Problems of Russian Academy of Sciences*
76a, Khoroshevskoe Highway, Moscow, Russian Federation

ABSTRACT

The aim of this study was to determine the general patterns of pathogenetic changes in the blood coagulation system and in non-specific proteinases and their inhibitors during the development of experimental ischemia-reperfusion injury.

Materials and methods. The study was conducted on 48 male Wistar rats (180–200 g). We used a model of ischemia-reperfusion injury achieved by applying rubber tourniquets to both hind limbs at the inguinal fold level for 6 hours. Revascularization was performed for 6, 12, or 24 hours following the application of tourniquets, after which we examined the state of the internal and external blood coagulation pathways and the activity of non-specific proteinases and their inhibitors.

Results. Indicators of blood coagulation system change show the development of blood hypocoagulation changes as the reperfusion time increases. By the 6th hour of reperfusion, the prothrombin time (PT) was lengthened by 112.0% ($p = 0.0142$) and the activated partial thromboplastin time (APTT) by 170.0% ($p = 0.0147$) compared with values in the control group. By the 12th reperfusion hour, the PT was lengthened by 174.2% ($p = 0.0389$), and the APTT increased 4.9-fold ($p = 0.0002$). When the reperfusion period was increased to 24 hours, it was characterized by lengthened PT and APTT, accompanied by an increase in antithrombin III by 11.5% ($p = 0.0371$) and a decrease in protein C by 71.4% ($p = 0.0071$). Changes in the non-specific proteinases and their inhibitors were characterized by a 2.8-fold increase in the trypsin-like proteinase activity ($p < 0.001$) relative to the control, as well as a 2.2-fold decrease in antitrypsin activity and acid-stable inhibitors ($p < 0.001$), which reached a maximum after 24 hours of reperfusion. A direct correlation was found between indicators characterizing the deficiency of coagulation system factors and a decrease in antiproteinase potential.

Conclusion. Hemostatic system disorders are characterized by the development of hypocoagulation during ischemia-reperfusion injury as the result of an increase in the trypsin-like proteinase activity and a decrease in the levels of inhibitors. The established changes may be associated with the deficiency of coagulation factors and proteinase inhibitors and share common pathogenic mechanisms.

Key words: blood coagulation, ischemia-reperfusion injury, non-specific proteinases.

Conflict of interest. The authors declare no obvious or potential conflict of interest related to publication of this manuscript.

Source of financing. The study was conducted with support of 2015-2024 V.I. Vernadsky Crimean Federal University Development program in line with the realization of “Network of Academic Mobility” project “The Development of Scientific Research in Experimental Medicine” and with the support of Russian Foundation for Basic Research (project № 17-415-92010p_a).

✉ *Kubyshkin Anatoly V.,* e-mail: kubyshkin_av@mail.ru.

Conformity with the principles of ethics. The study was approved by the Ethics Committee at V.I. Vernadsky Crimean Federal University (Protocol No. 2 of 11.09.2015).

For citation: Pisarev A.A., Petrenko V.I., Kubyshkin A.V., Kharchenko V.Z., Fomochkina I.I., Kuzichkin D.S. Changes in the blood coagulation system and non-specific plasma proteinases in ischemia-reperfusion injury. *Bulletin of Siberian Medicine.* 2020; 19 (3): 67–75. https://doi.org: 10.20538/1682-0363-2020-3-67-75.

Изменение показателей свертывающей системы крови и неспецифических плазменных протеиназ при развитии синдрома ишемии-реперфузии

Писарев А.А.[1], Петренко В.И.[1], Кубышкин А.В.[1], Харченко В.З.[1], Фомочкина И.И.[1], Кузичкин Д.С.[2]

[1] Медицинская академия им. С.И. Георгиевского, Крымский федеральный университет (КФУ) им. В.И. Вернадского
Россия, Республика Крым, 295051, г. Симферополь, бул. Ленина, 5/7

[2] Государственный научный центр Российской Федерации (ГНЦ РФ), Институт медико-биологических проблем (ИМБП) Российской академии наук (РАН)
Россия, 123007, г. Москва, Хорошевское шоссе, 76а

РЕЗЮМЕ

Цель. Определить общие закономерности патогенетических изменений в свертывающей системе крови, неспецифических протеиназ и их ингибиторов при развитии экспериментального синдрома ишемии-реперфузии.

Материалы и методы. Исследование проведено на 48 половозрелых самцах крыс линии Вистар массой 180–200 г. Модель синдрома ишемии-реперфузии создавали наложением резиновых жгутов на обе задние конечности на уровне паховой складки сроком на 6 ч. Реваскуляризацию производили через 6, 12 и 24 ч после наложения жгутов. Оценивали состояние внутреннего и внешнего путей свертывания крови, активность неспецифических протеиназ и их ингибиторов.

Результаты. Показатели свертывающей системы крови свидетельствуют о развитии гипокоагуляционных изменений по мере удлинения времени реперфузии. Выявлено повышение значения протромбинового времени (ПВ) на 112,0% ($p = 0,0142$) и увеличение активированного частичного тромбопластинового времени (АЧТВ) на 170,0% ($p = 0,0147$) к 6-му ч реперфузии по сравнению с группой контроля. К 12-м ч реперфузии протромбиновое время возрастало до 174,2% ($p = 0,0389$), АЧТВ – в 4,95 раз ($p = 0,0002$), а растворимых фибрин-мономерных комплексов (РФМК) – на 121,3% ($p = 0,0300$). Длительность реперфузионного периода до 24 ч характеризовалась сохранением высоких значений ПВ и АЧТВ, РФМК с повышением содержания антитромбина III – на 11,4% ($p = 0,0371$) и снижением протеина С на 71,4% ($p = 0,0071$). Изменение показателей неспецифических протеиназ и их ингибиторов характеризовалось ростом активности трипсиноподобных протеназ в 2,8 раза ($p < 0,001$) по отношению к контролю, а также снижением антитриптической активности и уровня кислотостабильных ингибиторов в 2,2 раза ($p < 0,001$) с максимумом через 24 ч реперфузии. Выявлена прямая корреляционная связь между показателями, характеризующими дефицит факторов системы свертывания, и снижением антипротеиназного потенциала.

Заключение. На основании результатов исследования показателей системы свертывания крови и неспецифических протеиназ при развитии синдрома ишемии-реперфузии установлено, что нарушения в системе гемостаза характеризуются развитием гипокоагуляции на фоне роста активности трипсиноподобных протеиназ и снижения уровня их ингибиторов. Установленные изменения могут быть связаны с развитием дефицита факторов свертывания и ингибиторов протеиназ и иметь общие механизмы развития.

Ключевые слова: свертывающая система крови, синдром ишемии-реперфузии, неспецифические протеиназы.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Работа выполнена при поддержке Программы развития ФГАВО «Крымский федеральный университет имени В.И. Вернадского» на 2015–2024 гг. в рамках реализации академической мобильности по проекту «Сеть академической мобильности «Развитие научных исследований в области экспериментальной медицины – РНИЭМ», а также при финансовой поддержке Российского фонда фундаментальных исследований «Механизмы участия провоспалительных и апоптотических факторов в инициации повреждения органов при экстремальных состояниях», проект № 17-415-92010р_а.

Соответствие принципам этики. Исследование одобрено комитетом по этике ФГАОУ ВО «КФУ имени В.И. Вернадского» (протокол № 2 от 11.09.2015).

Для цитирования: Писарев А.А., Петренко В.И., Кубышкин А.В., Харченко В.З., Фомочкина И.И., Кузичкин Д.С. Изменение показателей свертывающей системы крови и неспецифических плазменных протеиназ при развитии синдрома ишемии-реперфузии. *Бюллетень сибирской медицины.* 2020; 19 (3): 67–75. https://doi.org: 10.20538/1682-0363-2020-3-67-75.

INTRODUCTION

Disorders of the blood coagulation system complicate the course of various critical conditions. Coagulation disorders, along with multiple organ dysfunction syndrome and systemic inflammatory response syndrome, which can cause the development of disseminated intravascular coagulation (DIC) [1], significantly affect the course of any underlying, concomitant, or background diseases, as well as the efficacy of treatment and patient mortality. It has become more common for doctors from a variety of specialties to encounter ischemia-reperfusion injury, which is important for disciplines including angio-surgery, transplantology, traumatology, various fields of internal medicine (myocardial infarction, acute cerebrovascular accident), and emergency medicine [1, 2]. Multiple organ dysfunction syndrome is a significant component in the pathogenesis of ischemia-reperfusion injury. The main manifestations of this syndrome are disorders of the coagulation system and non-specific proteinases [2, 3].

Tissue damage as the result of reperfusion disturbances and the associated development of an inflammatory reaction contribute significantly to the pathogenesis of coagulation and vascular-thrombocytic disorders. They tend to cause endothelial dysfunction, increased platelet activity, activation of plasma coagulation factors, hypoactivity of physiological anticoagulants, and suppression of fibrinolysis. These disorders can vary from subclinical variants (as in local venous thrombosis) to severe disturbances of hemostasis, such as disseminated intravascular coagulation (DIC), which is characterized by massive systemic thrombosis followed by episodes of bleeding due to the depletion of coagulation factors [5].

Pro-inflammatory reactions in patients with extreme conditions are accompanied by complex humoral and cellular interactions, which involve activation of numerous signaling pathways, including the generation or expression of thrombin, complement, cytokines, neutrophils, adhesion molecules, and other inflammatory mediators. Excessive inflammatory cascades coupled with the unfavorable course of the underlying disease lead to multiple organ dysfunction, which can manifest as coagulopathy, myocardial dysfunction, respiratory or renal failure, and neurocognitive defects. Coagulation and inflammation are also closely interconnected through networks of both humoral and cellular components, including coagulation factors, non-specific proteinases, and fibrinolytic cascades [6, 7]. Despite the significant number of recent studies devoted to the research of the blood coagulation system and proteolysis under critical conditions, many topics related to its pathogenesis and treatment policy remain controversial. In this regard, the aim of this work is to determine the general principles responsible for pathogenetic changes in the blood coagulation system and in non-specific proteinases and their inhibitors during the experimental development of ischemia-reperfusion injury, and to substantiate etiopathogenetic approaches to experimental correction.

MATERIALS AND METHODS

The studies were carried out on 48 white male Wistar rats weighing 180–200 g. Animals were housed under identical standard conditions in accordance with the Guide for the Care and Maintenance of Laboratory Animals. Research and euthanasia were carried out in accordance with state and international standards for the humane treatment of animals, and in compliance with the main provisions of regulatory legal acts [8–10].

Experimental studies of pathogenetic changes in the blood coagulation system and non-specific

proteinases and their inhibitors were carried out using a model of ischemia-reperfusion injury achieved by applying rubber-band tourniquets to both hind limbs at the level of the inguinal folds for a period of 6 hours [3]. The width of the tissue clamped by the tourniquet was 2–3 mm. The criteria for correct application of the tourniquet was the absence of edema in the limbs and their pale color. Revascularization was performed simultaneously by cutting the tourniquets 6 hours after they were applied [3]. Animals were placed in groups using simple randomization as follows: Control group: intact animals (n = 15); Group 2: 6 hours reperfusion group (n = 12); Group 3: 12 hours reperfusion group (n = 11); and Group 4: 24 hours reperfusion group (n = 10). Blood for analysis was obtained by cardiopuncture (4 mL) and placed in single-use Vacutainer glass tubes with 0.05 M EDTA for 10–15 s. The blood was centrifuged for 15 minutes at 1,200 g. Euthanasia of the animals was carried out by decapitation after preliminary narcotization with sodium thiopental (40 mg/kg) [11].

To assess the condition of the coagulation system, the following indicators were determined: prothrombin time (PT) (s); activated partial thromboplastin time (APTT) (s); fibrinogen concentration (FBG) (g/L); soluble fibrin-monomer complexes (SFMC) (mg/L); antithrombin III (AT III) (%); plasminogen (PG) (%); α2-antiplasmin (APL) (%); and protein C (PC) (%). Hemostasis values were measured on a CA 1500 automatic coagulometer (Sysmex, Japan) using standard commercial Siemens reagent kits (Germany). The concentrations of FBG, PT, and APTT were measured using clotting methods. The concentrations of AT III, APL, PC, and PG were determined using chromogenic methods. The content of SFMC was evaluated by the manual paracoagulation method using Technology-Standard reagent kits (Russia).

To evaluate the activity variables of non-specific proteinases and their inhibitors, trypsin-like, elastase-like, and antitryptic activities (TLA, ELA, and ATA), as well as the level of acid-stable inhibitors (ASI), were determined. The component activity of the proteinase inhibitor system was studied using enzymatic methods with a Biomat 5 spectrophotometer (UK) [12, 13]. Trypsin-like activity was determined by measuring the speed of N-benzoyl-L-arginine cleavage from the synthetic substrate of ethyl ester N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Sigma, USA). Elastase-like activity was assessed by studying the hydrolysis rate of the Boc-L-alanine-4-nitrophenil ester synthetic substrate (Boc-Ala-ONp) (Sigma, USA). Antitryptic activity was assessed by inhibition of BAEE cleavage by trypsin. Similarly, the activity of acid-stable inhibitors was studied after preliminary preparation of serum by heating it in an acidic environment.

The data obtained during the research were analyzed statistically using the MedStat certified computer data-processing package for Windows. The main statistical variables determined were mean (M), error of mean (m), and standard deviation (s). All indicators are expressed quantitatively and the distribution did not differ from normal according to the Shapiro – Wilk test [14]. Student's t-test was used for comparison of group means in two groups. The results of statistical processing of the hemostatic system indices are represented as relative differences with the control group measured as a percentage. To assess the degree of relationship, a correlation analysis was performed, and the Pearson correlation coefficient was calculated using Microsoft Excel 2016. The differences were considered statistically significant at $p < 0.05$.

RESULTS

The analysis of the blood coagulation system of rats with experimentally induced ischemia-reperfusion injury revealed a regular dynamic change in the hemostasis indices. Pronounced changes were observed by the 6th hour of the development of ischemia-reperfusion injury in both the external and internal blood coagulation pathways; PT increased by 112.0% (p = 0.0142) and APTT elongation by 170.0% (p = 0.0147) compared with the control group (Fig. 1). At 6 hours of ischemia-reperfusion, a marked decrease of 29.6% (p = 0.0002) was observed in the level of antithrombin III (Fig. 2), accompanied by a decrease in the plasminogen level of 29.6% (p = 0.0207) and a decrease in the level of antiplasmin of 11.7% (p = 0.0256) in comparison with the control values (Fig. 3).

After 12 hours of ischemia-reperfusion, we observed a 174.2% extension for PT (p = 0.0389) and a marked 4.9-fold extension of APTT (p = 0.0002) compared to the control indices. The level of AT III after 12-hour ischemia-reperfusion decreased by 10.4% (p = 0.0442). The content of SFMC increased by 121.3% (p = 0.0300) compared with the control level (Fig. 4).

An increase in the duration of the reperfusion period to 24 hours was characterized by an increase of 59.6% in SFMC (p = 0.0114), an increase of 11.5% in the content of AT III (p = 0.0371), a decrease of

71.4% in PC (p = 0.0071) (Fig. 3), and a 39.8% increase in Group 3 (p = 0.0494). We also observed a maximum shift in the following indicators: an PT elongation of 186.6% (p = 0. 0346) and a maximum 4.9-fold increase in APTT (p = 0.0147) compared to control values.

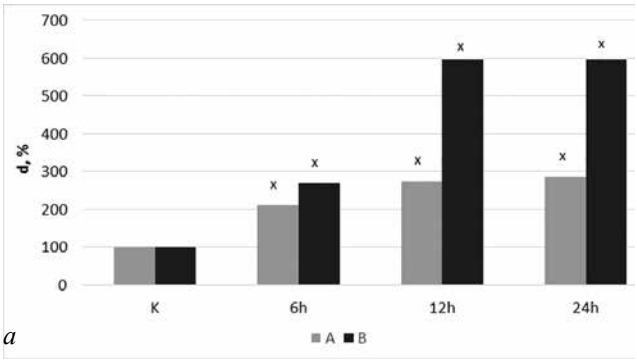

Fig. 1. Prothrombin time (*a*) and activated partial thromboplastin time (*b*) in groups with different durations of ischemia-reperfusion (6 h, 12 h, and 24 h) as a percentage (d, %) related to the control (K) group. X represents the difference from the control group (Student's t-test, $p < 0.05$)

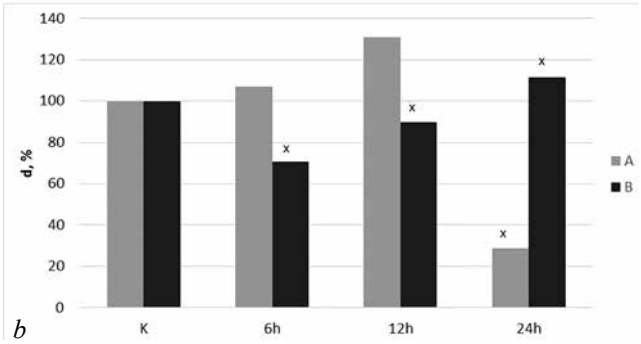

Fig. 2. Protein C (*a*) and antithrombin III (*b*) levels in groups with different durations of ischemia-reperfusion (6 h, 12 h, and 24 h) as a percentage (d, %) related to the control (K) group. X represents the difference from the control group (Student's t-test, $p < 0.05$)

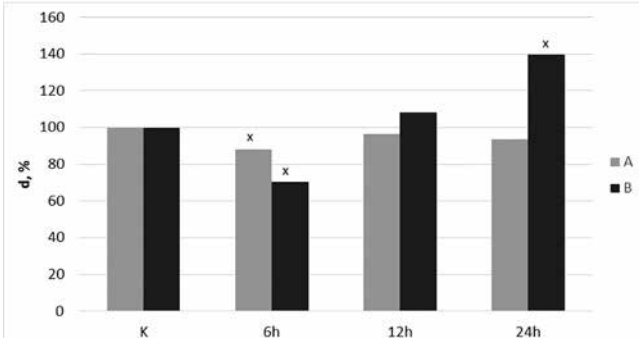

Fig. 3. Plasminogen (*a*) and antiplasmin (*b*) levels in groups with different durations of ischemia-reperfusion (6 h, 12 h, and 24 h) as a percentage (d, %) related to the control (K) group. X represents the difference from the control group (Student's t-test, $p < 0.05$)

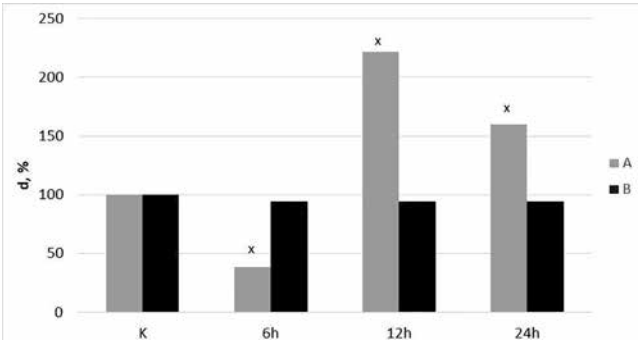

Fig. 4. SFMC (*a*) and fibrinogen (*b*) levels in groups with different durations of ischemia-reperfusion (6 h, 12 h, and 24 h) as a percentage (d, %) related to the control (K) group. X represents the difference from the control group (Student's t-test, $p < 0.05$)

In the first 6 hours, a decrease in PG activity was observed. In the subsequent periods, plasmin inactivation probably occurs in response to fibrinolysis products and/or PG activation does not occur. Free APL is also used for binding to plasmin, and takes part in the inhibition of non-specific proteinases [15]. PG and APL are acute phase proteins, and their levels increased during the 24 hours of the experiment as a result of a relatively reduced due to hypocoagulation consumption. The level of AT III decreased in the first 6 hours in response to the coagulation process activation. However, as thrombin and other AT III cofactors (Xa, XIa, IXa) are consumed, the plasma level of free AT III begins to increase.

In general, it can be assumed that during the 24-hour reperfusion period, systemic tissue damage occurs. It is accompanied by an acute-phase response that is a reaction to stress exposure. Distinct signs of the consumption coagulopathy development appear in these conditions.

During the experiment, somewhat different dynamics were observed in the PC level changes. Unlike AT and APL, cofactor proteins whose levels decrease immediately after binding to targets, protein C is an enzyme. The thrombin-AT complex is destroyed by proteolytic systems in the liver within a few minutes; the half-life of PC in the circulatory system is about 6 hours. Thus, during the course of ischemia-reperfusion, in all likelihood, there is a rapid depletion of functional reserves and hypocoagulation development within 6 hours of tissue damage.

Along with the experimental dynamics of blood coagulation indices, the nature of the shifts in the activity of non-specific proteinases and their inhibitors in

the blood serum of experimental animals was studied in relation to the timing needed for ischemia-reperfusion injury development.

Analysis of the obtained data has shown the following dynamics in non-specific proteinases and their inhibitors' activity changes (Table 1). Six hours after revascularization of the limbs, the elastase-like activity of blood serum was 3.8 times lower than that of the control values (p = 0.0012). After 12 hours, an even greater decrease, up to 19.0% of the control index (p = 0.0008), was observed. The pronounced decrease in ELA likely indicates the activation of natural proteinase inhibitors that neutralize elastase, reducing its serum activity severalfold. Twenty-four hours after the development of ischemia-reperfusion, ELA showed a tendency to increase, although the activity remained 2-fold below the control parameter (p = 0.0011). Apparently, after 24 hours of the ischemia-reperfusion development, the inhibitory control weakened, which led to an increase in the proteolytic activity of blood serum.

Table 1

Changes in the proteolytic activity and inhibitory potential of rat blood using a model of ischemia-reperfusion injury at different observation times, $M \pm m$

Experimental group	ELA, nMol/mL • min	TLA, nMol/mL • min	ATA, IU/mL	ACI, IU/mL
Control group, n = 10	2.19 ± 0.14	0.26 ± 0.02	34.67 ± 1.57	6.83 ± 0.30
Ischemia 6 h, n = 10	2.31 ± 0.09*	0.20 ± 0.01*	38.03 ± 1.33*	3.81 ± 0.36*
Ischemic-reperfusion syndrome 6 h, n = 10	0.57 ± 0.05*	0.50 ± 0.06*	20.61 ± 1.16*	3.18 ± 0.31*
Ischemic-reperfusion syndrome 12 h, n = 10	0.41 ± 0.02*	0.73 ± 0.06*	25.76 ± 1.76*	3.39 ± 0.30*
Ischemic-reperfusion syndrome P 24 h, n = 10	1.10 ± 0.09*	0.46 ± 0.12	16.02 ± 0.79 *	3.08 ± 0.23*

* indicates the reliability of differences in the data (p) in comparison with those of the control group (*$p \leq 0.05$ is statistically significant).

The dynamics of the trypsin-like activity of blood serum during the development of reperfusion injury were characterized by other changes. Six hours after revascularization of the limbs, the TLA index was 48.0% greater than that of the control (p = 0.0006). After 12 hours, the index, having reached the maximum level, exceeded the control indices 2.8-fold (p = 0.0011), which is apparently associated with the influx of a large number of proteinases into the systemic circulation from previously ischemic tissues. Later, a downward trend in the studied index was observed. Thus, 24 hours after reperfusion, TLA, having decreased by 37.0%, remained 43.0% higher than values in the control group (p = 0.0472), which indicates the onset of compensatory mechanisms and a timely increase in inhibitory activity. Interesting data confirming our previous assumptions were obtained in the study of antitryptic activity: 6 hours after reperfusion, a 1.7-fold decrease in ATA was noted (p = 0.0009), which fell to 1.4-fold after 12 hours. The decrease in this parameter progressed, and by 24 hours after reperfusion, the ATA activity had fallen 2.2 times lower than that of the control value (p = 0.0004).

The level of acid-stable inhibitors also largely depended on the duration of the reperfusion period. As a result, 6 hours after revascularization, the level of ASI was 2.2-fold lower than that of the control value (p = 0.0009); after 12 hours, 2-fold lower (p = 0.0012), and after 24 hours, 2. 2-fold lower (p = 0.0007). The described dynamics of the ASI levels is associated with their increased consumption as a result of increased protease activity.

Thus, an increase in the activity of non-specific blood proteinases during the development of reperfusion injury at its early stages should be noted, along with the concomitant increase in an inhibitory activity. With continued reperfusion, the inhibitory capacity tends to decrease and the activity of non-specific blood proteinases in the experimental animals tends to increase.

To clarify the relationship between the coagulogram indices and the state of the non-specific proteinase system and their inhibitors in the experimental ischemia-reperfusion injury, we performed a correlation analysis (Table 2).

A significant positive correlation was found between changes in TLA and PT, APTT, and SFMC in the blood during the process of modeling ischemia-reperfusion injury for 6, 12, and 24 hours (r = 0.78, 0.82 and 0.59, respectively). The more the activity of this proteinase increased, the more the values of blood coagulation indices increased along the external (PT) and internal pathways (APTT). The concentration of SFMC in the blood of experimental animals increased as well. At the same time, for the ATA

and PT indices, a negative correlation with APTT was found (correlation coefficients of –0.82 and –0.83, respectively). Thus, a higher level of antitryptic activity, mainly of α-1-proteinase inhibitor, corresponded to a lower level of the coagulogram indices described above. In addition, it was found that antitryptic activity had a direct proportional correlation with the values of FBG, APL, and PC (correlation coefficients of 0.86, 0.75, and 0.58, respectively). The greater the proteinase system inhibitory potential in the form of increased ATA was, the higher values of these laboratory indices were observed.

Table 2

Results of correlation analysis (r) of coagulogram indices and the activity of non-specific proteinases and their inhibitors

Experimental group	PT	APTT	FBG	SFMC	APL	AT III	PC	PG
ELA	–0.799 $p = 0.002$	–0.869 $p = 0.002$	+0.930 $p = 0.001$	–0.248	+0.670 $p = 0.032$	+0.521 $p = 0.048$	–0.232	+0.153
TLA	+0.781 $p = .003$	+0.820 $p = 0.002$	–0.786 $p = 0.003$	0.594 $p = 0.043$	–0.296	–0.318	+0.358	+0.031
ATA	–0.826 $p = 0.002$	–0.833 $p = 0.002$	+0.867 $p = 0.002$	–0.045	+0.755 $p = 0.006$	–0.003	+0.585 $p = 0.046$	–0.304
ASI	–0.921 $p = 0.001$	–0.959 $p = 0.001$	+0.997 $p = 0.001$	–0.217	+0.752 $p = 0.005$	+0.248	+0.201	–0.127

Note. The *p* value denotes a statistically significant correlation. The – sign indicates an inverse correlation.

A negative correlation between ASI and the PT and APTT indices (correlation coefficients of –0.92 and –0.95, respectively), and a positive correlation between the ASI indices and the FBG and APL levels (correlation coefficients of 0.99 and 0.75, respectively) were noted.

When analyzing the ELA values for ischemia-reperfusion injury and coagulogram indices (PT and APTT), a negative correlation was revealed (correlation coefficients of –0.79 and –0.86, respectively), but when studying the values of FBG, APL, and AT III, a positive correlation was found (correlation coefficients of 0.93, 0.67, and 0.52, respectively) between them.

DISCUSSION

Our analysis of the dynamics of coagulation system indices shows significant differences and stages in the reaction of the hemostatic system during different periods of ischemia-reperfusion injury. Thus, even in short-term, 6-hour ischemia-reperfusion, hypocoagulation predominates, which can be explained by systemic activation of plasmatic systems in response to the acute damage. This is indicated by an increase in SFMC, which is a marker of the consequences of developing thrombinemia in response to acute damage. Increase in the duration of ischemia-reperfusion injury (up to 12 h and, especially, 24 h) leads to further changes, indicative of hypocoagulation disorders aggravation. It seems likely that these changes are associated with depletion of coagulation factors and their inadequate entry into the systemic circulation because of impaired synthetic liver function under conditions of prolonged stimulation and severe functional stress.

Similar changes are observed in the system of non-specific proteinases and their inhibitors. Dynamics analysis of the proteinase and their inhibitors' indices taken in different time periods of reperfusion syndrome indicates a pronounced activation of non-specific proteinases (TLA) in the blood serum of experimental animals with modeled ischemia-reperfusion. It is associated with the direct participation of proteolytic enzymes in the hemocoagulation cascade, and in the body's systemic adaptive response to massive endothelial damage. Thus, during 6-hour ischemia-reperfusion, there is a compensatory increase in proteinase inhibitors with an associated decrease in proteinase activity. With an increase in the duration of ischemia-reperfusion injury (12 and 24 h), there is a depletion of inhibitory potential with an increase in proteinase activity.

The most pronounced changes both in the hemostatic system and in the system of non-specific proteinases and their inhibitors were observed in the 24-hour ischemia-reperfusion group. A disruption of the body's adaptive capabilities represented by depletion of blood coagulation factors was observed in this group. As a result, there was a shift in the hemostasis system indices towards hypocoagulation, and an increase in proteinase activity as the result of an

inhibitory potential deficiency. This may be a predisposing factor for the development of DIC. This assumption corresponds with literature data confirming the development of DIC in response to systemic activation of the coagulation pathway [16]. The intravascular formation of fibrin is enhanced by dysfunction of natural anticoagulant systems, such as antithrombin (antithrombin III) and protein systems (protein C), during the active development of DIC. Subsequently, all components of the coagulation and anticoagulation cascade are depleted, which leads to complete non-coagulation of blood [17].

Statistically significant data from the correlation analysis confirm the assumptions mentioned above and indicate a close relationship between changes in the blood clotting system parameters and proteinase-inhibitor system indices. Apparently, the changes seen in the blood clotting system status during ischemia-reperfusion injury, namely initial hypocoagulation with subsequent further increases in the duration of blood clotting time at 12 and 24 hours (indices of the external and internal pathways), are closely interrelated. This may occur due to the changes in the proteinase-inhibitory system demonstrated by an increase in proteolytic activity and a decrease in inhibitory potential. Possibly, it is the deficiency of inhibitors, together with the lack of blood coagulation factors due to the decreased synthetic liver function at a later stages of ischemia-reperfusion injury, that are the key links in the pathogenesis. For this reason, they are possible therapeutic targets, which can be corrected, not only in response to reperfusion injury, but also during DIC syndrome, acquired thrombophilic conditions, and other blood coagulation disorders.

CONCLUSION

It has been found that, during the development of ischemia-reperfusion injury for 6, 12, and 24 hours, the disturbances in the hemostatic system are characterized by the development of hypocoagulation as the result of an increase in the trypsin-like proteinases activity and a decrease in their inhibitors level. The changes observed are likely to be directly associated with the development of coagulation factors and proteinase inhibitors deficiencies. These changes share common development mechanisms associated with excessive coagulation factors depletion during prolonged activation of the coagulation cascade, and, possibly, with a decrease in the synthetic liver function under a long-term functional load of hepatocytes, as well as their hypoxic and reperfusion injury.

REFERENCES

1. Maegele M., Schöchl H., Cohen M.J. An up-date on the coagulopathy of trauma. *Shock*. 2014; 41 (21): 21–25. DOI: 10.1097/SHK.0000000000000088.
2. Fedosov M.I., Fomochkina I.I., Kubyshkin A.V., Babanin A.A., Pylaeva N.Yu. Pathogenetic significance of proteolytic and cytokine mechanisms in reperfusion syndrome. *Tauride Medical and Biological Bulletin.* 2012; 15 (3): 354–358 (in Russ.).
3. Kharchenko V.Z., Kubyshkin A.V., Anisimova L.V., Fomochkina I.I., Ivantsova N.L., Zhukova A.A. et al. Proteinase inhibitors and antioxidants in the pathogenic therapy of organopathology caused by reperfusion syndrome complicated with blood loss. *Crimean Journal of Experimental and Clinical Medicine.* 2016; 7 (2): 23–25 (in Russ.).
4. Maegele M., Spinella P.C., Schoechl H. The acute coagulopathy of trauma: mechanisms and tools for risk stratification. *Shock.* 2012; 38 (5): 450–458. DOI: 10.1097/SHK.0b013e31826dbd23.
5. Oshiro A., Yanagida Y., Gando S., Henzan N., Takahashi I., Makise H. Hemostasis during the early stage of trauma: comparison with disseminated intravascular coagulation. *Critical Care*. 2014; 18: 61–63. DOI: 10.1186/cc13816.
6. Francis A., Baynosa R. Ischaemia-reperfusion injury and hyperbaric oxygen pathways: a review of cellular mechanisms. *Diving and Hyperbaric Medicine*. 2017; 47 (2): 110–117. DOI: 10.28920/dhm47.2.110–117.
7. Kalogeris T., Baines C.P., Krenz M., Korthuis R.J. Ischemia-Reperfusion. *Comprehensive Physiology.* 2016; 7 (1): 113–170. DOI: 10.1002/cphy.c160006.
8. Karkishchenko N.N., Gracheva S.V. Guide to laboratory animals and alternative models in biomedical research. Moscow: Profil, 2010: 344 (in Russ.).
9. Guidelines for the maintenance and use of laboratory animals; eighth Edition; Trans. I.V. Belozertseva, D.V. Blinova, M.S. Krasilshchikova. Moscow: IRBIS, 2017: 336 (in Russ.).
10. The Council of Europe Convention on the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes, 1986: 37 (in Russ.).
11. Recommendations for euthanasia of experimental animals: Part 1, Part 2. Laboratory Animals. 1996; 30: 293–316. 1997; 31: 1–32 (in Russ.).
12. Ogloblina O.G., Platonova L.V., Paskhina T.S. Measurement of trypsin and elastase-like proteinases of polymorphonuclear leukocytes and the level of their acid-stable inhibitors in human bronchial secretion. Moscow: Izd-vo MGU, 1984: 14 (in Russ.).
13. Kubyshkin A.V., Kharchenko V.Z., Semenets P.F., Aliev L.L., Fomochkina I.I., Anisimova L.V. Methods for determining the activity of non-specific proteinases and their inhibitors in serum and biological fluids. Kiev: Izd-vo KNMU, 2010: 28 (in Russ.).
14. Hanusz Z., Tarasinska J. Simulation Study on Improved Shapiro–Wilk Tests for Normality. *Simulation and Computation*, 2014; 43 (9): 2093–2105. DOI: 10.1080/03610918.2013.844835.
15. Iba T., Kidokoro A. High-dose antithrombin therapy for sepsis: mechanisms of action. *Shock*. 2002; 18 (5): 389–394. DOI: 10.1097/00024382-200211000-00001.
16. Levi M., Toh C.H., Thachil J., Watson H.G. Guidelines for the Diagnosis and Management of Disseminated Intravascu-

lar Coagulation. *British Journal of Haematology*. 2009; 145: 24–33. DOI: 10.1111/j.1365-2141.2009.07600.x.
17. Sawamura A., Gando S., Hayakawa M., Hoshino H., Kubota N., Sugano M. Effects of antithrombin III in patients with disseminated intravascular coagulation diagnosed by newly developed diagnostic criteria for critical illness. *Clin. Appl. Thromb. Hemost*. 2009; 15 (5): 561–566. DOI: 10.1177/1076029608323497.

Authors information

Pisarev Anatoly A., Post-Graduate Student, Department of General and Clinical Pathophysiology, V.I. Vernadsky Crimean Federal University, Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea, Russian Federation.

Petrenko Vitalina I., Post-Graduate Student, Department of General and Clinical Pathophysiology, V.I. Vernadsky Crimean Federal University, Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea, Russian Federation.

Kubyshkin Anatoly V., Dr. Sci. (Med.), Professor, Head of the Department of General and Clinical Pathophysiology, V.I. Vernadsky Crimean Federal University, Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea, Russian Federation.

Kharchenko Vladimir Z., Dr. Sci. (Med.), Professor of the Department of General and Clinical Pathophysiology, V.I. Vernadsky Crimean Federal University, Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea, Russian Federation.

Fomochkina Iryna I., Dr. Sci. (Med.), Professor of the Department of General and Clinical Pathophysiology, V.I. Vernadsky Crimean Federal University, Medical Academy named after S.I. Georgievsky, Simferopol, Republic of Crimea, Russian Federation.

Kuzichkin Dmitry S., Senior Researcher, Laboratory of Medical Biochemistry and Neuroendocrinology, Institute of Medical and Biological Problems, Russian Academy of Sciences, Moscow, Russian Federation.

(✉) **Kubyshkin Anatoly V.,** e-mail: kubyshkin_av@mail.ru.

Received 01.08.2019
Accepted 25.12.2019