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Amount of generators of reactive oxygen species as a marker of organism damage on the background of experimental limb ischemia-reperfusion

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Abstract

Massive blood loss resulting in hemorrhagic shock requires the use of a hemostatic tourniquet. According to the literary sourced using of tourniquet for 2 hours is safe, but the newest investigations have shown ambiguous consequences for the body, coused by both local changes in the tissues under the tourniquet's pressure, and the outflow of metabolic products into the general bloodstream from primarly ischemic limb.

The aim was to identify the interralation between the level of production of reactive oxygen species by neutrophils of peripheral blood due to ischemia-reperfusion of the limb, as well as its combination with volumetric blood loss or mechanictal trauma.

Materials and methods. The experiment was perfomed on 260 white male rats (200-250 g), which were divided into 5 groups: control, EG-1 – simulation of isolated limb ischemia-reperfusion (IR), EG-2 – simulation of isolated volumetric blood loss, EG -3 – combination of limb IR with blood loss, EG-4 – modeling of isolated thigh mechanical injury,

EG-5 – combination of limb IR and mechanical injury. The percentage of neutrophils-producers of reactive oxygen species (ROS) was determined in the blood by flow cytofluometry.

Results. It was found that ischemia-reperfusion of the limb, acute massive blood loss and their combination led to an increase in the number of ROS producers in the peripheral blood in all study periods. In the EG-2 on 1st and 3rd days it was higher than in the KG in 2,41 times and in 2,39 times accordingly, and also on 7th day exceeded level of the KG in 2,39 times and remained raised in 2 times on 14th day . In the EG-3 on the 1st day after injury exceeded the CG data in 2,5 times, then slightly decreased and exceeded the CG level in 2,3 times on the 3rd day. However, on the 7th and 14th day the index exceeded the CG level in 2,6 times and 3,3 times. The peculiarities which indicate the effect of mutual burden was that on the 14th day the level of ROS producers in the EG-3 was statistically significantly higher compared to the EG-1 in 3 times, and compared to the EG-2 – by 63,7%. Also, on the 14th day, the level of ROS in the EG-5 was in 2,4 times higher than the data of the EG-1, and by 58,3% higher than the data of the EG-4. In this case, after modeling only the ischemia-reperfusion of the limb, the figure was increased till the 14th day, on which it beame close to the initial rate.

Key words: Ischemia reperfusion injury; oxidative stress; reactive oxygen species; acute blood loss; sceletal trauma; mechanical inhury, hemostatic tourniquet.

Introduction

The use of a tourniquet for injuries of the main vessels of the extremities still remains an effective and simple mean of the first aid [1]. Mortality from volumetric blood loss due to the development of mixed hypoxia in the Europe and USA is 7-10%, and peculiarity is that in the structure of potentially non-fatal lesions, in 90% of that cases, death occurs due to bleeding [2]. Thus, temporary aid is as extremely important, as the timing of application. However, there are now more and more literature data that complications caused by pressure on the underlying tissues, they are both local and distant [3, 4]. Due to the bleeding of this area, hypoxia develops first, resulting in increasing of generation of reactive oxygen species [5, 6].

As we have previously established [7] against the background of even isolated ischemic-reperfusion injury (IRI) there there was an increase of lipid peroxidation in the internal organs, which was particularly expressed and malignant against the background of IRI, combined with massive blood loss. That factors caused "oxidative stress" as a

manifestation of an imbalance between antioxidant protective systems and the activity of oxidants [8].

A significant contribution to the development of intra- and extracellular changes that occur against the background of ischemia-reperfusion, belongs to the reactive oxygen species (ROS) [9]. By itself, reperfusion injury is paradoxical, because after the release of the limb or organ from the tourniquet/ligature, blood enters the body, which brings enough oxygen. This, instead of improving the condition of tissues that have been in hypoxia, on the contrary, triggers a chain of dangerous reactions [10]. In this case, ROS generated in one source can cause long-term effects in the outlying internal organs, such as the gastrointestinal tract, heart or brain, liver or kidneys.

The mediator between the local response due to the increase in the concentration of ROS is the inflammatory response – reaction activated by endothelial cells. That is the base for the following, systemic damage [11]. The literature extensively describes the damaging mechanisms that are triggered by such a vital restoration of blood supply and reoxygenation of the ischemic organ – a change in pH and imbalance in the concentration of electrolytes $Na+$ Ca^{2+} [12], the generation of high amount of free radicals, which can lead aither to mitochondria damage with subsequent cell death or to the restoration of mitochondria [13]. Whereas in the modern literature cases of military medicine are analyzed, so the main cases are about the isolated lesions of the numerous injured [14, 15]. However, there are many victims who have a combined injury, so those undeniable changes that occur in ischemic muscle that do not lead to death from isolated blood loss can be fatal due to a combination of several damaging factors. And the tourniquet can play an important role.

The aim of the study is to establish – to establish a connection between ischemia-reperfusion injury (IRI) of the limb, combined with massive blood loss or skeletal trauma and the activity of the production of reactive oxygen species in the blood of experimental animals.

Materials and methods of research. The assumption that the use of a tourniquet? combined with massive blood loss (hypovolemic shock) and subsequent reinfusion of ischemic tissues lead to significant changes with disruption of internal organs became the working hypothesis of our experimental study. To achieve this goal 260 nonlinear white male rats (200-250 g) were used in the experimental study. Some of them died in the postoperative period, so Table 1 shows the number of survived animals. All animals were divided into 5 groups: EG-1 included animals that were performed with simulated ischemia-reperfusion of the limb. Under thiopental-sodium anesthesia (40 mg • kg^{-1} body weight intraperitoneal), SWAT-T (US) tourniquet with width10 mm was applied to the thigh of an animal and

adequately corresponds pressure of the tourniquet when applied to the thigh of an adult human. According to the literature, such a tourniquet is characterized by minimal negative traumatic effects on the underlying tissues due to its width and long-term pain threshold [16]. The tourniquet was tightened according to the applied effective pressure marking, which is able to stop the blood flow.

In EG2 under conditions of anesthesia, acute volumetric blood loss (up to 40% of volume of circulating blood) was modeled by puncture of the femoral vein with farther hemostasis. In EG3, these two injuries were combined. In EG4 animals were done with mechanical trauma of the femur with aim of closed fracture simulation. We applied on the thigh a single dosed blow with a specially designed device, which caused a closed fracture (impact surface area was 0.5 cm2, impact force - more than 120 kg / cm2)

The combination of ischemia-reperfusion of the thigh with mechanical trauma was modeled in EG5. Animals were eliminated from experiment at the 1st hour after intervention, and on the 1st, 3rd, 7-th and 14-th days after trauma on the base of thiopental-sodium anaesthesia by total bleeding from the heart. In the case of KG animals, they were anesthetized with an equivalent dose of sodium thiopental and material were collected for the study, as from the experimental groups.

The dyer with blocked fluorescence – Dichlorofluorescein diacetate (DHF-DA by Sigma Aldrich, USA) – was used for measurement the level of reactive oxygen species in blood neutrophils. After passive penetration into the cell and cleavage of the acetate group under the action of esterases, DXF-DA turns into a polar compound, which is not capable of diffusion from the cell. As a result of interaction with hydrogen peroxide and other free radicals, DXF-DA becomes a fluorescent compound. In a clean polystyrene tube 90 mcl of a suspension of mononuclear leukocytes and 10 mcl of a working solution of DCF-DA were placed. Cells were resuspended and incubated for 20 min at 37 ° C. Then were centrifuged at 1000 rotations per min during 10 min, supernatant was drained the and 400 mcl of phosphate-buffered saline were added. Samples were placed on ice, the level of ROS production was analyzed by the intensity of the glow of the dye (FL-1 channel) on a flow cytofluorimeter Epics XL ("Beckman Coulter", USA). The values of the studied parameter were expressed as a percentage.

The study design was considered with the rules of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (European Convention, 1984) and Decree Ministry of Health of Ukrain [17], considered by the commission at a meeting of the commission on bioethics of I. Ya. Gorbachevsky Ternopil

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A statistical analysis on obtained data was performed by Excel (Microsoft, USA). In addition to the absolute values, which are presented in the tables in the form of median (Me), lower and upper quartiles (LQ; UQ), the deviation of each indicator as a percentage to the control level (100,0%) was calculated. To rate the probability of differences we defined the pequliarities of the variational grouping of indicators in the compared groups. Due to the lack of a normal grouping, the nonparametric Mann-Whitney test was used. The differences were considered as a true when the probability of the null hypothesis was not more than 5 % ($p<0.05$).

Results and discussion

Basically, the results of this study in the first здфсу are intended to show that even a 2-hour isolated use of hemostatic tourniquet in a healthy animal caused an increase in the concentration of reactive oxygen species within 1 hour after the start of reperfusion. Our results coincide with the investigation of early features of reperfusion reactions, which were reflected in the largest oxygen capacity in the lungs, and, at the morphological level, at this time did not affect the liver, but stimulates mitochondrial respiration in the kidneys [18].

As could be seen from the data of Table 1, comparing changes in the concentration of ROS generators with the CG data, the following features were found: in the EG-1 on the background of isolated ischemia-reperfusion of the limb after the 1st hour the level of ROS generators increased by 40,5 %. On the 1st, 3rd and 7th days the level of ROS was significantly increased and exceeded the CG data by 2,2, 2,9 and 2 times, concordantly. On the 14th day, the percentage of ROS-producing leukocytes decreased by 11,4 % (at $p> 0,05$). On the background of isolated blood loss in the EG-2 after the 1st hour, the amount of ROS exceeded the level of the CG by 60,5 %. Also on the 1st, 3rd and 7th days it was higher from the CG in 2,41 times, in 2,39 times concordantly. Also remained elevated in 2 times and even on the 14th day. As for the level of ROS on the background of IR, combined with massive blood loss in the EG-3 it was found that after the 1st hour following the intervention the level exceeded the CG data by 61,1 %. On the 1st day after the injury, it exceeded the CG data in 2,5 times, after which it slightly decreased and exceeded the CG level in 2,3 times on the 3rd day. However, on the 7th and 14th day the rate increased, exceeding the level of the CG in 2,6 times and 3,3 times.

On the background of isolated trauma in the EG-4 after the 1st hour following experimental intervention, the ROS index exceeded the CG data by 16,75 %. On the 1st and 3rd day it grew, exceeding the level of the CG by 55 % and 98,7 %, concordantly. The highest growth, compared with the CG, was set on the 7th day, when the figure exceeded the data in

2,1 times. On the 14th day, it remained elevated by 67,6 %. As for the skeletal injury combined with IR, if after 1st hour the index was higher than in the CG data by 49,1 %, then in the 1st day it exceeded the CG level in 2,6 times. On the 3rd day a significant decrease of the ROS generators concentration was established, although the level was higher than in the CG by 67,4 %. However, on the 7th and 14th day the level exceeded the CG in 2,.3 times and 2,7 times, concordantly.

Table 1 - The content of leukocytes in the blood that generate ROS (%), after ischemia-reperfusion of the limb, blood loss and skeletal injury (Me (LQ; UQ)) – median (lower and upper quartiles)

Experimental group	Reperfusion period				
	1st hour	1st day	3rd day	7th day	14th day
KG Control group = 0,114 (0,105; 0,120) (n=10)					
EG1 isolated ischemia reperfusion	16,02* (15,34; 17,03) (n=10)	25,30* (25,20; 27,79) (n=10)	20,85* (19,49; 22,20) (n=10)	22,88* (21,24; 24,24) (n=10)	12,70 (12,10; 13,89) (n=10)
EG2 Blood loss	18,30* (17,08; 19,49) (n=7)	27,58* (25,87; 29,35) (n=7)	27,21* (25,86; 28,28) (n=6)	27,30* (26,34; 28,51) (n=7)	23,24* 21,19; 23,82) (n=7)
EG3 ischemia-reperfusion + Blood loss	18,36* (16,62; 19,15) (n=6)	28,08* (27,37; 29,29) (n=6)	26,35* (25,66; 26,43) (n=6)	29,62* (27,30; 31,53) (n=6)	38,05* (35,70; 39,40) (n=5)
p_{1-3}	>0,05	>0,05	<0,05	<0,05	<0,05
p_{2-3}	>0,05	>0,05	>0,05	>0,05	<0,05
EG4 Sceletal trauma	13,31* (12,67; 14,19) (n=10)	17,67* (17,00; 19,99) (n=10)	22,65* (21,08; 23,48) (n=10)	23,60* (22,80; 24,89) (n=10)	19,11* (17,51; 20,91) (n=10)
EG5 ischemia-reperfusion + scaletal trauma	17,00* (16,04; 18,90) (n=9)	29,15* (27,50; 31,16) (n=9)	19,08* (17,45; 20,50) (n=8)	26,32* (25,18; 28,75) (n=9)	30,26* (29,32; 34,24) (n=9)
p_{1-5}	>0,05	>0,05	>0,05	<0,05	<0,05
p_{4-5}	<0,05	<0,05	<0,05	<0,05	<0,05

Notes: 1.* – differences in relation to the control group are statistically significant (p<0,05);

2. p_{1-3} – the probability of differences in relation to experimental groups 1 i 3;

3. p_{2-3} – the probability of differences in relation to experimental groups 2 i 3.

4. p_{1-5} – the probability of differences in relation to experimental groups 1 and 5 (p<0,05);

5. p_{4-5} – the probability of differences with respect to experimental groups 4 and 5.

Depending on the degree of body damage due to experimental interventions, the generation of ROS had its own characteristics.

As can be seen from Table 1 and Figure 1, in the EG-1, like in the others, had a fairly high rate of ROS production with a peak the 1st day, when the level of this period exceeded the data estimated after the 1st hour following release from the tourniquet, by 57,9 %. Then, on the 3rd day, although the level of activity decreased compared to the 1st day by 17,6 %, it was higher than the data of the 1st hour by 30,1 %. On the 7th day, the index increased slightly and exceeded the level of the 1st hour by 42,8 %; while it remained below the level of the 1st day by 9,6 %. And on the 14th day the estimated level decreased sharply, compared with the 7th day – by 44,5 %. At the same time, it was lower than the level of the 1st hour by 20,7 %, compared to the level of the 1st day – by 49,8 % and compared to the 3rd day – was lower by 39,1 %.

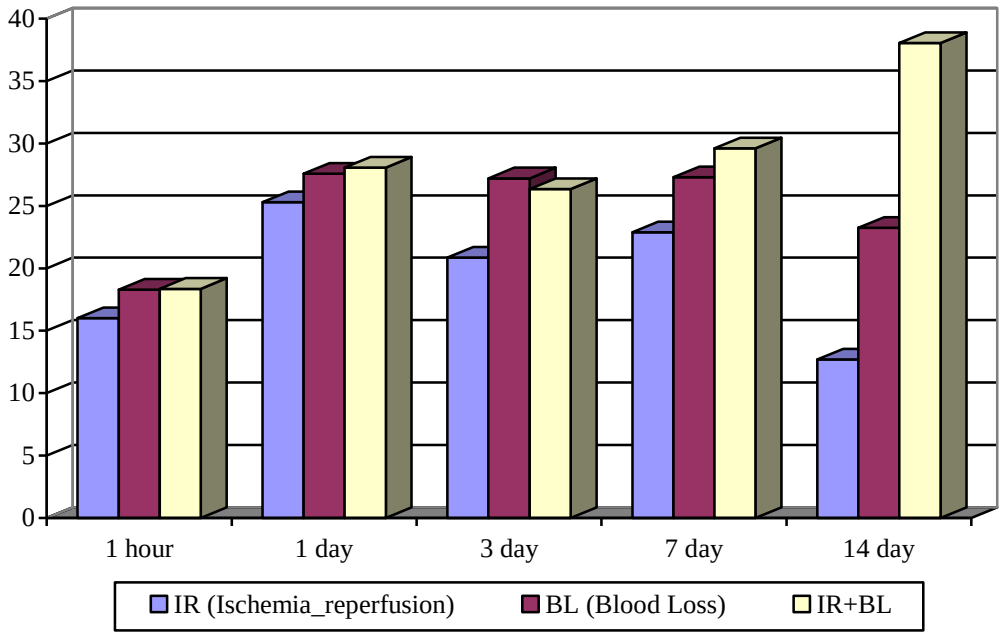

Figure 1 – Dynamics of the content of leukocytes-producers of ROS in a blood (as a percentage of the control level) after ischemia-reperfusion of the limb and blood loss

In the EG-2, mixed hypoxia on the background of isolated volumetric blood loss led to even more expressed changes in the first hours after intervention. Thus, on the 1st day the index exceeded the level fixed after the 1st hour by 50,7 %, and on the 3rd day exceeded the level of the 1st hour by 48,7 %. On the 7th day the concentration of neutrophils-generators of ROS increased, compared with the 1st hour by 49,2 %. At the same time, on the 14th day, being higher than the data of the 1st hour by 27 %, the index was still lower than the data of

the 1st, 3rd and 7th days by 15,7 %, 14,6 % and 14,8 %, concordantly. In the EG-3 the dynamics of pathogenesis was similar to the EG-2, but significantly expressed. Thus, for the 1st day the amount of ROS generators, compared with the 1st hour, increased in 1,5 times. On the 3rd day the index remained higher than the level of the 1st hour by 43,5 %, although it was decreased relatively to the level of the 1st day by 6,2 % ($p > 0.05$). On the 7th day the index exceeded the level of the 3rd day by 12,4 % and was higher than the level of the 1st hour by 61,3 %. It requires particular attention that concentration of ROS generators on the 14th day continued to grow, when the index exceeded the level of the 7th day by 28,5 %. It was also higher than the level of 3rd day by 44,4 %, higher than the level of the 1st day by 35,5 %, and higher than the level of the 1st hour in 2,1 times.

As could be seen from the data of Table 1 and the Figure 2 on the 1st day after the simulation of skeletal injury in the EG-4 there was a moderate increase of the amount of ROS generators by 32,8 %, compared with the 1st hour, and on the 3rd day the level exceeded the level of the 1st hour by 70,2 %, also exceeded level of the 1st day by 28,2 %.

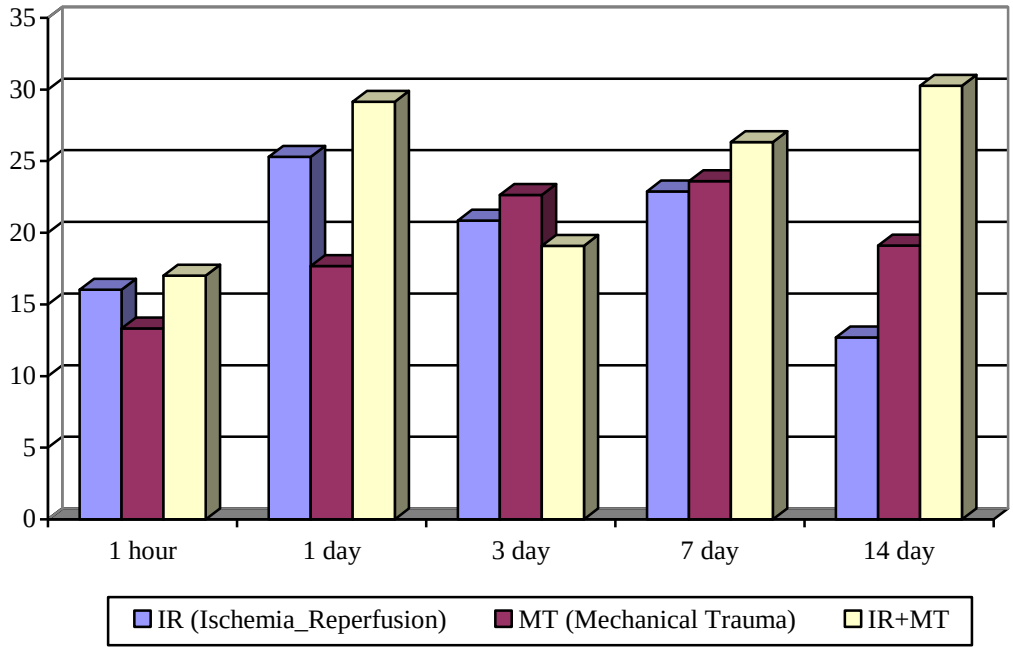

Figure 2 - Dynamics of the content of leukocytes-producers of AFO in the blood (as a percentage of the control level) after ischemia-reperfusion of the limb and mechanical trauma

On the 7th day the generation of ROS grew moderately, exceeding the level of the 1st hour by 77,3 %, and the level of the 1st day by 33,6 %. Also on the 14th day, despite the decrease in pathogenetic activity, compared to the 7th day by 81 %, compared to the 3rd day – by 15,6 %, it still remained higher than the level of the 1st hour by 43,6 %.

On the background of IRI, combined with mechanical trauma in EG-5 dynamics was slightly more expressed, compared to EG-4. Thus, on the 1st day the index exceeded the data of the 1st hour by 71,5 %. On the 3rd day a sharp decrease in ROS generation was investigated, compared to the 1st day by 34,5 %. On the 7th day a sharp rise in the concentration of ROS was found again, which exceeded the level of the 3rd day by 37,9 %, as well as the level of the 1st hour by 54,8 %. And on the 14th day the index continued to grow and was higher than the level of the 7th day by 15 %. It was also higher than the level of the 1st hour by 78 %, and compared to the level of 3rd day – by 58,6 %.

Also, when comparing the degree of ROS growth between groups with modifications of the experimental intervention, it was found that the presence of IRI worsened the course of both isolated blood loss and isolated mechanical trauma.

Thus, after the 1 hour following the intervention and on the 1st day, a statistically significant difference between the level of ROS generators in the EG-1 and in the EG-3, as well as between the EG-2 and the EG-3 was not established, although the level of ROS generators was increased in all groups. Knowing that blood loss in itself is a great stress for the organism, especially volumetric hemmoradge, such a relatively low level of difference could be explained by the mechanisms of compensatory hemodilution, when fluids from the interstitial space were involved. That process, according to the literature, should occur up to 2-3 days [19]. At the same time, on the 3rd day the level of ROS in the EG-3 was higher than the level of ROS in the EG-1 by 26,4 % ($p < 0,05$) and relative to the level of ROS in the EG-2 – was higher by 3,2 % ($p > 0,05$). On the 7th day the differences were similar when the level of ROS in the EG-3 exceeded the data of the EG-1 and the EG-2 by 29,5 % ($p < 0,05$) and by 8,5 % ($p > 0,05$), concordantly. And only on the 14th day the level of ROS generators in the EG-3 was statistically significantly higher compared to both groups: for the EG-1 in 3 times, for the EG-2 – by 63,7 %.

As for the effect of IRI on the consequences of mechanical trauma, which in itself was accompanied by a moderate increase of the concentration of ROS generators such features were revealed: after the 1st hour following the intervention it was found that in the EG-5 the ROS level was statistically significantly higher than in the EG-4 – by 27,4 %.

After the 1st day the level of ROS in the EG-5 exceeded the level of ROS in the EG-1 by 15,2 % ($p > 0,05$) and in the EG-4 by 65 % ($p < 0,05$). On the 3rd day after the intervention the level of ROS in the EG-5 was higher than the data of the EG-1 by 8,5 % ($p > 0.05$) and by 15,8 % in the EG-4 ($p < 0,05$). However, already on the 7th day a statistically significant difference was found in comparison with both groups. Thus, the EG-5, compared with the

EG-1, had a higher level of ROS generators by 16,3 % and, compared with the EG-4, by 12,8 %. Also, on the 14th day the level of ROS generators in the EG-5 was in 2,4 times higher than the data of the EG-1 and by 58,3 % higher than the data of the EG-4.

As it could be seen from the obtained data, the increase in the systemic concentration of reactive oxygen species is apparently a consequence of local limb ischemia, as well as (in the EG-3 and in the EG-2) it is the result of hemic hypoxia, but they also led to secondary damage in internal organs, which is confirmed by our previous studies [20] and other authors [18, 21].

Also, our data confirm that in the experimental modeling of ischemic-reperfusion injury of the limb, such an index as the concentration of reactive oxygen species is a sensitive proof of the compensatory functions of the internal organs. In our previous studies, we found a certain periodization in the growth of TBA-active products of peroxidation in the homogenates of internal organs, which coincides in particular with the growth of active forms of oxygen, described in this article. The peculiarity is that not very active growth of ROS on the background of isolated blood loss or blood loss combined with the limb IR, on the 1st 1nd 3rd days after the intervention is obviously a consequence of hemodilution.

Further study of individual links in the pathogenesis of ischemia-reperfusion of the limb is unambiguously necessary, because the reason that the victim does not have a local compartment syndrome may be that his muscle mass is less developed [22]. At the same time, prevention of possible risks and development of a scheme of therapeutic influence on the limb before the blood circulation in the injured limb is restored is especially important. On the battlefield, where there is a large number of wounded and limited time to determine who needs antioxidants and who does not, there is no time to determine more developed muscular system – it is time to stop bleading properly and not to demage.

Conclusions

1. It was found that ischemia-reperfusion of the limb, acute massive blood loss and their combination led to an increase in the number of ROS producers in the peripheral blood in all study periods.

2. In the EG-2 on 1st and 3rd days it was higher than in the KG in 2,41 times and in 2,39 times accordingly, and also on 7th day exceeded level of the KG in 2,39 times and remained raised in 2 times on 14th day . In the EG-3 on the 1st day after injury exceeded the CG data in 2,5 times, then slightly decreased and exceeded the CG level in 2,3 times on the 3rd day. However, on the 7th and 14th day the index exceeded the CG level in 2,6 times and 3,3 times. The peculiarities which indicate the effect of mutual burden was that on the 14th

day the level of ROS producers in the EG-3 was statistically significantly higher compared to the EG-1 in 3 times, and compared to the EG-2 – by 63,7%. Also, on the 14th day, the level of ROS in the EG-5 was in 2,4 times higher than the data of the EG-1, and by 58,3% higher than the data of the EG-4. In this case, after modeling only the ischemia-reperfusion of the limb, the figure was increased till the 14th day, on which it beame close to the initial rate.

The prospects for futher research are to gether all next investigation results for substantitation that hemostatic tourniquet could have ambiguous systemic influence for the affected with blood loss or mechanical trauma.

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