UDC 612.015.1/32-02:616.36-06:616.137.87-005.4-007.271]-092.9 https://doi.org/10.26641/2307-0404.2020.4.221227

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COMPARATIVE CHARACTERISTICS OF LIPID PEROXIDE OXIDATION ACTIVITY AND ANTIOXIDANT PROTECTION IN RAT'S LIVER ON THE BACKGROUND OF EXPERIMENTAL ISCHEMIC-REPERFUSION LIMB SYNDROME

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Цитування: Медичні перспективи. 2020. Т. 25, № 4. С. 39-47
Cited: Medicni perspektivi. 2020;25(4):39-47

Key words: *ischemia-reperfusion syndrome, liver, trauma, blood loss, haemostatic tourniquet, lipid peroxidation*
Ключові слова: *ішемічно-реперфузійний синдром, печінка, травма, крововтрата, гемостатичний джгут, пероксидне окиснення ліпідів, антиоксидантна система*
Ключевые слова: *ишемически-реперфузионный синдром, печень, травма, кровопотеря, гемостатический жгут, перекисное окисление липидов, антиоксидантная система*

Abstract. Comparative characteristics of lipid peroxide oxidation activity and antioxidant protection in rat's liver on the background of experimental ischemic-reperfusion limb syndrome. Volotovska N.V. *The organism's reaction to pathogenic impacts such as blood loss and ischemia can appear either on systemic or on ultrastructural level. The tourniquet inflation pressure on the extremity triggers local lipid peroxidation. However, the systemic influence of tourniquet is not completely studied. This experiment was dedicated to study of the changes that occur in the liver on the background of ischemic-reperfusion syndrome of the limb. In our experiment the animals were divided into 5 groups, in which the effect of ischemia-limb reperfusion, blood loss, mechanical injury of the thigh bone and their combination on the concentration of malonic dialdehyde and glutatoin peroxidase activity was studied. Biochemical investigation of the liver has shown that each of these experimental interventions had caused activation of lipid peroxidation in the liver and proper increase of the activity of the antioxidant protection enzyme in the blood. The peculiarities of the reaction were an increase of the first index, which was the highest among all experimental groups – on the background of blood loss combined with the use of a tourniquet; at the same time the critical suppression of antioxidation was noted as well. Namely here 2 periods of exhaustion of the wounded organism were established – on the 3rd and 14th day. At the same time, the content of malonic dialdehyde was higher in the group where the imposition of the tourniquet was combined with mechanical trauma, comparatively to isolated mechanical trauma of the thigh, this indicated the role of the tourniquet as a factor complicating the course of posttraumatic period due to ischemic reperfusion syndrome. Understanding the pathogenesis of traumatic disease will allow to find a new way of valuing the effects of limb decompression, to cope with oxidative stress and more successfully treat multiple organ failure.*

Реферат. Сравнительная характеристика активности перекисного окисления липидов и антиоксидантной защиты в печени крысы на фоне экспериментального ишемически-реперфузионного синдрома конечности. Волотовская Н.В. *Реакция организма на патогенные факторы, которыми может быть кровопотеря и ишемия, проявляется как на системном уровне, так и на ультраструктурном. Применение кровоостанавливающего жгута локально активирует перекисное окисление липидов конечности. Однако системное влияние турникета до сих пор изучено недостаточно. Данное исследование проведено с целью выявления изменений, возникающих в печени на фоне ишемически-реперфузионного синдрома конечности. В нашем эксперименте животные были разделены на 5 групп, в которых изучали влияние ишемии-реперфузии конечности, кровопотери, механической травмы бедренной кости и их сочетания на концентрацию малонового диальдегида и активность глутатионпероксидазы. Биохимическое исследование печени показало, что каждый из этих видов вмешательства вызывал активацию перекисного окисления липидов в исследуемом органе и соответствующее ему увеличение активности фермента антиоксидной защиты в крови.*

Особенностями реакции было такое повышение первого показателя, который был самым высоким среди всех экспериментальных групп – на фоне кровопотери, сопряженной с применением кровоостанавливающего жгута; при этом зафиксировано и максимальное подавление антиоксидации. Именно здесь установлено 2 периода истощения раненого организма – 3 и 14 сутки. При этом содержание малонового диальдегида было выше в группе, где сочеталось наложение жгута с механической травмой, по сравнению с изолированной механической травмой бедра, что указывает на роль турникета как фактора, который усугубляет течение посттравматического периода в связи с развитием ишемического реперфузионного синдрома. Понимание патогенеза травматической болезни позволит по-новому воспринимать последствия декомпрессии конечности, бороться с оксидативным стрессом и успешно проводить лечение полиорганной недостаточности.

From ancient times until now, first aid, which consists in applying a tourniquet, is a factor that can save the life of the wounded [17]. At the same time, injuries and blood losses, which are an inevitable consequence of military conflicts both in the world and in Ukraine, require meticulous study of the mechanisms of ischemic-reperfusion syndrome due to the use of tourniquets. The consequences of intense release of toxins into the systemic bloodstream after decompression of the limb is oxidative stress, which not only promotes peroxidation in the place squeezed by the tourniquet, but also disturbs metabolism and is a trigger of related protective reactions of the organism in general [11].

The essence of ischemic-reperfusion syndrome is that after removal of the hemostatic tourniquet harmful biologically active substances enter the circulatory system, while the functional load on the liver increases [9], which in conditions of hypoxia caused by blood loss prevents its full self-repair. Although in general the available sources of information contain results that highlight the pathogenic effects of ischemic-reperfusion syndrome (IRS) on the liver and other internal organs, the information about the activity of peroxidation processes in its tissue against this background compared with changes in antioxidant response, as well, as the study of the patterns of IRS against the background of modifications of the injury is absent.

The increase in lipid peroxidation is accompanied by an increase in the activity of the antioxidant system [12] – glutathione peroxidase. According to the literature, the malfunction of the antioxidant system contributes to the progression of lipid peroxidation (LPO) and oxidative modification of proteins, which leads to necrotic-apoptotic changes in tissues suffering from ischemia-reperfusion [6].

The peculiarities of the reaction of the glutathione system, in particular in the liver and kidneys have been reported by scientists but there is not enough data on the effect of IRS on the systemic content of these enzymes.

The purpose of the study is a comparative characterization of changes that occur in the main detoxification gland of the body - the liver - against the background of ischemic-reperfusion syndrome and against the background of modifications of the injury.

MATERIALS AND METHODS OF RESEARCH

The experiment was performed on 260 white nonlinear male rats (5-5.5 months) kept in traditional vivarium conditions. To achieve this goal, six experimental groups (EG) were formed: animals of the control group (CG) were removed from the experiment without intervention; EG1 – imposition of a tourniquet on the thigh for 2 hours. (isolated ischemia-reperfusion); EG2 – modeling of blood loss; EG3 – a combination of ischemia-reperfusion with blood loss; EG4 – application of mechanical trauma on the femur in order to simulate a fracture using the device ShchP-1; EG-5 – a combination of ischemia-reperfusion with mechanical trauma.

The experiment was performed under conditions of thiopental sodium anesthesia (40 mg/kg) in compliance with the general rules and regulations of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986), Resolution of the First National Congress of Bioethics, 2011) and the law of the Ministry of Health of Ukraine № 690 of September 23, 2009 [5]. Thus, the ischemic-reperfusion syndrome was modeled by applying a 1 cm wide strip of rubber tourniquet on the upper 1/3 of the femur, calculating the force of pressure under the control of marking applied on the tourniquet. It was left for 2 hours, after which the animal was released. To simulate blood loss, 40% of the circulating blood was drawn from the femoral vein. Animals were removed from the experiment 1 hour after decompression or other interventions, on day 1, 3, 7 and 14 after injury by total bleeding from the heart.

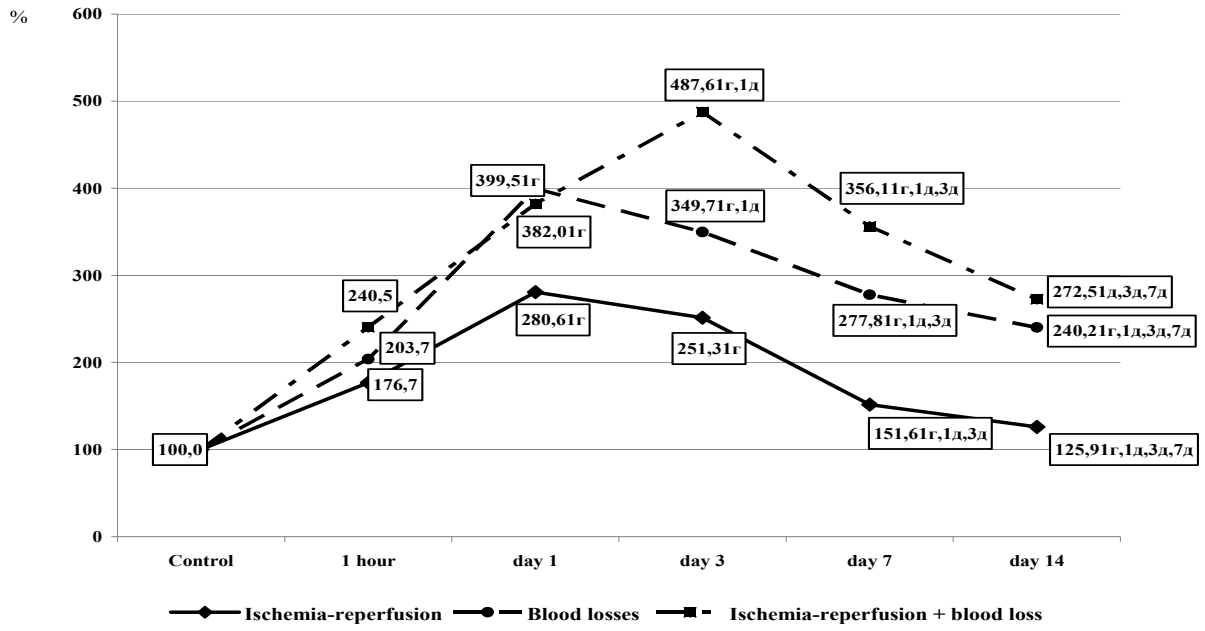
To detect TBA-reactive substances (metabolic products that react with thiobarbituric acid), 10% homogenate was prepared from liver tissue. It is known that the secondary products of lipid peroxidation (LPO), namely malonic dialdehyde (MDA), in interaction with thiobarbituric acid (TBA) under conditions of high temperature and acidotic pH have the ability to form a colored complex with optical density, possible for registration, at a wavelength of 532 nm. The amount of

MDA was calculated based on the molar extinction coefficient of the stained complex, which is equal to 1.56x105cm-1m-1, and expressed in μmol/l of serum or μmol/kg of tissue [7]. Glutathione peroxidase activity was determined spectrophotometrically by increasing the content of oxidized glutathione - a routine method described by G.O. Kruglikova and U.M. Stuttman [8]. Statistical processing of the obtained digital material was performed using computer programs Statistica 6.1 (StatSoftInc., Serial number AGAR909E415822FA) and "Microsoft Excel" (Open License 67528927). The probability of the difference was determined using Student's t-test [1]. Differences were considered significant with a probability of null hypothesis of not more than 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

According to modern scientific studies, a hemostatic tourniquet not only causes temporary ischemia but can cause complications such as impaired blood supply and nerve damage in the tourniquet pressure area, edema of the limb, pale skin, muscle weakness without paralysis, pain [19]. In our experiment we paid attention to the processes of peroxidation in the liver. Although the tourniquet is an effective means of stopping critical blood loss, its use for 2-3 hours can trigger irreversible processes in the tissues precisely because of the lack of blood supply and in the most difficult cases - lead to amputation of the limb [18].

Thus, the dynamics of LPO in the liver was as follows. As can be seen from Figure 1, in the conditions of ischemia modeling, the content of TBA-reactive products increases significantly. Thus, against the background of isolated ischemia-reperfusion (EG1) 1 hour after, on day 1, 3 and 7 this figure was higher than the same indicator of the control group (CG), respectively, by 76.7%, 2.8 times, 2.5 times and 51.6% ($p<0.05$). On day 14, the indicator did not return to the initial level, exceeding the control by 25.9% ($p>0.05$). Against the background of isolated blood loss (EG2), the content of TBA-reactive products of LPS after 1 hour was higher than this indicator in CG by 2 times, 1 day after the intervention – 4 times, 3 days – 3.5 times, 7 days – 2.8 times, 14 days – 2.4 times ($p<0.05$). In the conditions of ischemia-reperfusion combined with blood loss (EG3), the increase in LPO activity was the most pronounced, compared with the previous groups: after 1 hour. the indicator exceeded the control by 2.4 times, on day 1, 3, 7 and 14 was higher by 3.8 times, 4.9 times, 3.6 times and 2.7 times ($p<0.05$) respectively.



Note: here and in other figures 1h, 1d, 3d, 7d - differences in relation to 1 hour, day 1, day 3 and day 7 of observation are statistically significant ($p<0.05$).

Fig. 1. Content of TBA-reactive products of LPO in a 10% liver homogenate (percent relative to the control group) against the background of isolated IRS of a limb, blood loss and their combination

Similar dynamics which confirmed the pathogenic effect of IRS of the limb was found in the following comparison groups. Thus, against the background of isolated mechanical trauma (MT) of the thigh (EG4) 1 hour after the intervention, the activity of the LPO increased by 32.8% ($p>0.05$). Significant increase in LPO was recorded on day 1, 3, 7 after injury when compared with the control, the rate was higher by 65.6%, by 86.2%, by 54.5% ($p<0.05$). On day 14 the indicator did not differ significantly from the control. The activity of LPO against the background of MT combined with the imposition of the tourniquet (EG5) was higher. So, in 1 hour the indicator was higher than the same in CG by 66.1%, then, on day 1 and 3 remained at the same level – was higher than the CG indicator by 2.8 times and 2.9 times, and on day 7 and 14 – exceeded it by 86.2% and 31.8%, respectively ($p<0.05$).

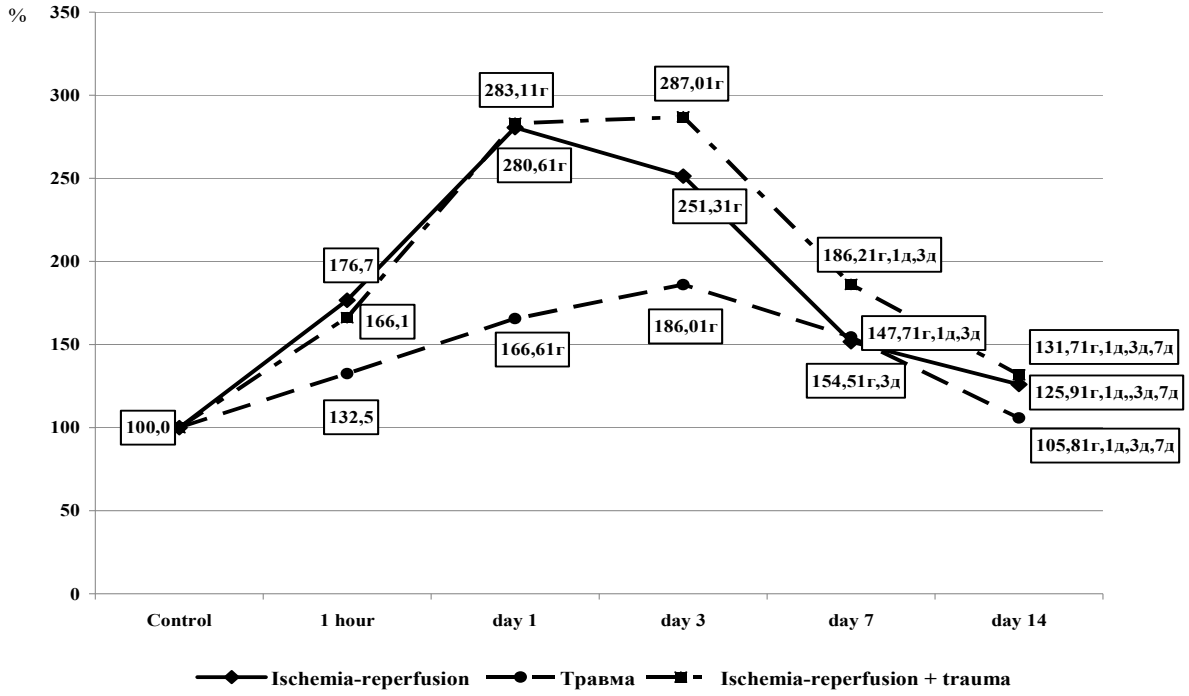


Fig. 2. Content of TBA-reactive products of LPO in a 10% liver homogenate percent relative to the control group) on the background of isolated IRS of a limb, mechanic trauma and their combination

When comparing the concentration of the studied indicator between groups with different degrees of severity of the intervention, it was found that in 1 hour the indicators were distributed as follows. The rate in EG3 was higher than that in EG1 and EG2 by 36.2% and 18.9%, respectively ($p<0.05$). In addition, the indicator in EG5 was statistically significantly higher by 6% in EG1 and by 25.1% in EG4, respectively ($p<0.05$). On day 1 the indicator in EG3 exceeded the data in EG1 by 38.3% ($p<0.05$) and was lower than the data in EG2 by 4.4% ($p>0.05$), and the indicator in EG5 was higher than in EG1 by 2.5% ($p>0.05$) and 70.9% higher compared to EG4 ($p<0.05$). On day 3 a further increase in LPO activity in EG3 and EG5 groups and a decrease in EG1, EG2 and EG4 was recorded. EG3 was higher than similar in EG1 and EG2 by 94.1% and 39.5% ($p<0.05$), respectively. Also, the indicator in EG5 was higher than in of EG1 and EG4 by 14.3% and 52.3%, respectively ($p<0.05$). On day 7 the dynamics was a sharp decrease in all study groups, while the activity of LPO in EG3 remained higher than that in EG1 and EG2 by 2.3 times and 28.2%, respectively ($p<0.05$). As for the EG5 data, the indicator was higher than the EG1 and EG4 data by 24.4% and 20.5%, respectively ($p<0.05$). On day 14 the indicator in EG3 exceeded the indicators in EG1 and EG2 groups by 2.2 times ($p<0.05$) and 13.5% ($p>0.05$), respectively. As for EG5, its indicator was higher than in EG1 by 4.6% ($p>0.05$) and in EG4 by 24.5% ($p<0.05$).

In our study the results obtained which show a sharp increase in LPO in liver tissue indirectly indicate the depletion of anti-oxidant enzymes in the detoxification gland - the liver. A group of scientists who studied the effect of other pathogenic factors on the thiol link found that the concentration of the glutathione peroxidase enzyme usually decreases [10].

Table 1

Content of TBA-reactive products (in μmol/kg) of LPO in a 10% liver homogenate after ischemia-reperfusion of the limb and blood loss (Me (LQ; UQ)) - median (lower and upper quartiles)

Experimental group	The term of the reperfusion period				
	1 year	day 1	day 3	day 7	day 14
Control = 1.89 (1.69; 2.05) (n=10)					
EG1 Isolated IRS	3.34 (3.27; 3.32)* (n=10)	5.22 (4.91; 5.41)* (n=10)	4.75 (4.72; 4.78)* (n=10)	2.87 (2.84; 2.92)* (n=10)	2.38 (2.29; 2.52) (n=10)
EG2 Isolated blood loss	3.85 (3.45; 4.04)* (n=7)	7.55 (7.37; 7.68)* (n=7)	6.61 (6.34; 6.80)* (n=6)	5.25 (5.06; 5.78)* (n=7)	4.54 (4.22; 4.91)* (n=7)
EG3 IRS combined with blood loss	4.55 (4.21; 50.6)* (n=6)	7.22 (7.05; 7.31)* (n=6)	9.22 (8.74; 9.59)* (n=6)	6.73 (6.46; 6.99)* (n=6)	5.15 (5.10; 5.21)* (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 Isolated trauma	2.51 (2.26; 2.63) (n=10)	3.13 (2.87; 3.36)* (n=10)	3.52 (3.17; 3.73)* (n=10)	2.92 (2.63; 3.02)* (n=10)	2.00 (1.86; 2.20) (n=10)
Group 5 IRS combined with trauma	3.14 (2.94; 3.30)* (n=9)	5.35 (5.08; 5.45)* (n=9)	5.43 (5.17; 5.62)* (n=8)	3.52 (3.48; 3.68)* (n=9)	2.49 (2.40; 2.60)* (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 and 3; 2. p_{2-3} – the probability of differences in relation to experimental groups 2 and 3; 3. p_{1-5} – the probability of differences in relation to experimental groups 1 and 5; 4. p_{4-5} – the probability of differences in relation to experimental groups 4 and 5.

As can be seen from Table 2, the activity of glutathione peroxidase was most depleted on day 3 against the background of IRS combined with blood loss. When comparing the activity of GP between the experimental groups, the following patterns were found: 1 hour after the intervention, the concentration of GP in EG3 was by 3 times lower as compared with EG1 and EG2 and by 34.2% ($p<0.05$); also in EG5 it was lower as compared with EG1 and EG4 by 23.3% and by 42.4% ($p<0.05$). On day 1, EG3 was by 4.5 and 3.8 times lower than in EG1 and EG2, respectively ($p<0.05$); also the indicator in EG5 was lower than the indicator in EG1 and EG4 by 34.5% and by 25.9% ($p<0.05$). As for day 3, when the highest enzyme suppression was observed in all study groups, except for EG3, EG3 indicator was lower than EG1 and EG2 data by 2.6 and by 23.9% ($p<0.05$), and in EG5 it was lower as compared with EG1 and EG4 by 48.4% and by 30.5%, respectively ($p<0.05$). On day 7 when the activity of LPO decreased, the resource of GP began to recover and had the following features: in EG3 was lower than in EG1 and EG 2 by 46.8% and by 22.1%, respectively, and was lower in EG5 as compared with EG1 and EG4 by 42.1% and by 31.1%, respectively ($p<0.05$). On day 14 day after the intervention, the rate of GP, although increased compared to previous experimental periods but still

did not reach the level of the control group and in EG3 was lower than in EG1 and EG2 by 36.5% ($p<0.05$) and by 9 .8% ($p>0.05$); also the indicator in EG was lower than the data of EG1 and EG4 by 21.2% and by 12.3% ($p<0.05$).

Table 2

Content of glutathione peroxidase (mmol/(min * kg^{-1})) after ischemia-reperfusion of the limb, blood loss and mechanical trauma (Me (LQ; UQ)) - median (lower and upper quartiles)

Experimental group	The term of the reperfusion period				
	1 hour	day 1	day 3	day 7	day 14
Control = 0.290 (0.271; 0.302) (n=10)					
Group 1 Ischemia-reperfusion	0.287 (0.283; 0.308) (n=10)	0.233* (0.217; 0.250) (n=10)	0.225* (0.214; 0.236) (n=10)	0.233* (0.214; 0.242) (n=10)	0.274 (0.260; 0.299) (n=10)
Group 2 Blood loss	0.146* (0.140; 0.160) (n=7)	0.196* (0.180; 0.204) (n=7)	0.113* (0.099; 0.122) (n=6)	0.159* (0.149; 0.183) (n=7)	0.193* (0.181; 0.217) (n=7)
Group 3 Ischemia-reperfusion+ blood loss	0.096* (0.088; 0.098) (n=6)	0.052* (0.048; 0.058) (n=6)	0.086* (0.073; 0.093) (n=6)	0.124* (0.120; 0.128) (n=6)	0.174* (0.165; 0.193) (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
Group 4 Trauma	0.382* (0.245; 0.409) (n=10)	0.205* (0.189; 0.219) (n=10)	0.167* (0.155; 0.192) (n=10)	0.196* (0.192; 0.197) (n=10)	0.246* (0.243; 0.254) (n=10)
Group 5 Ischemia-reperfusion+trauma	0.220* (0.207; 0.234) (n=9)	0.152* (0.149; 0.155) (n=9)	0.116* (0.103; 0.126) (n=8)	0.135* (0.124; 0.146) (n=9)	0.216* (0.187; 0.225) (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 and 3; 3. p_{2-3} – the probability of differences in relation to experimental groups 2 and 3; 4. p_{1-5} – the probability of differences in relation to experimental groups 1 and 5; 5. p_{4-5} – the probability of differences in relation to experimental groups 4 and 5.

Analysis of the modern literature has shown that the development of ischemic-reperfusion syndrome caused by the use of a tourniquet, and, apparently, as a consequence, liver failure may play a significant role in influencing the general condition of the body. The fact that the liver is involved in the pathological process is a predictable reaction, which is also part of the syndrome of multiple organ failure. The definition of IRS as the cause of such pathogenic effects on the body has been proven by many scientists [16]. In addition, we previously studied the effect of blood loss and mechanical trauma on the femur – and all this also led to increased lipoperoxidation which manifested itself in statistically proven impairment of liver function, namely its bile-forming function [2].

Thus, the results obtained by us indicate the activation of LPO against the background of various factors that caused ischemia: tourniquet and blood loss. The combination of both of these factors within 2 hours, generally recognized as safe (the time of compressiing the limb with a tourniquet), was nevertheless further manifested by increased degeneration of cell membranes. In our experiment, it was clearly seen when the studied indicator in EG3 exceeds the data of all other groups at appropriate time intervals.

A proven increase of glycolytic metabolites and high-energy phosphate content in skeletal muscles, which occurred against the background of ischemia caused by the use of a tourniquet during surgery on the bleeding area is among the results of other scientists. Against the background of moderate growth of phosphocreatinin in the ischemic limb a pronounced (by 4-5 times) growth of lactate was revealed. Apparently, the reason for the moderate increase in glucose and glucose-6-phosphate was the activation of glycogenolysis in cases where the ischemia lasted for 30-90 minutes. However, even when it lasted only 5 minutes, similar changes were again revealed [15].

Summarizing the above, one of the main reasons that activate the pathogenetic chain of ischemic-reperfusion injury is rhabdomyolysis and hypoxia. The first is manifested in excessive sudden entry of toxins into the systemic bloodstream after decompression of the limb. The cause of rhabdomyolysis, in turn, is muscle edema with the subsequent development of hypovolemia, hemoconcentration and massive release of myoglobin from the compressed tissues. Potassium and biologically active substances, in turn, are also able to trigger multi-organ damage [13]. Hyperconcentration of myoglobin in the renal tubules in the conditions of pH shift to acidosis provokes intra-tubular obstruction and renal dysfunction [4]. Another factor – hypoxia is known as a cause that stimulates LPO and in our experiment it was provoked by two factors – blood loss and tourniquet, which caused local and systemic ischemia, which in turn causes not only lack of oxygen but also local pressure with subsequent rhabdomyolysis. Thus a closed pathological circle is formed.

The dynamics of LPO in the liver is considered by us to be a protective reaction, because it stimulates AOD. The most severe period of LPO with a peak on day 1 was recorded in EG1, EG2 and EG4 and against the background of isolated IRS, blood loss or mechanical trauma, as well as on day 3 against the background of IRS combined with blood loss in EG3 and IRS, combined with mechanic trauma in EG5. Confirmation of this is in the submitted statistical materials. This also indicates the factor that triggered LPO of hepatocyte membranes - it should be toxins and hypoxia.

Also, in our previously published studies, the reaction from the liver AOD system was indeed present [3]. Our results are consistent with the achievements of a group of other scientists. Orlova E. and others found that the highest SOD activity is in the liver, the lesion of which is the result of compression of the limb with tourniquet, signaling the likelihood of damage to other organs due to the development of IRS of the limb [14].

The acquired knowledge on this topic is important, because in today's conditions the number of situations that are combined with bleeding from the main vessels has increased significantly. As a result, massive blood loss becomes one of the leading causes of death in such conditions. And the use of a tourniquet still remains one of the simplest and most important ways to control blood loss. Therefore, its importance cannot be denied and the side effects proven by us, their pathogenic effects at the systemic level require more careful attention of the scientific community.

CONCLUSIONS

Thus, the results showed an increase in LPO activity in the liver against the background of factors that cause ischemia, such as tourniquet and blood loss. Combining them during the recognized safe period still more enhanced the lipid peroxidation of cell membranes. Among the reasons that activated the pathogenesis of ischemic-reperfusion syndrome, we can highlight the effect of toxins as a result of rhabdomyolysis, which entered the systemic bloodstream after the release of the limb from the tourniquet. In turn, the stress of liver function is a protective reaction to a pathogenic factor. The critical period of growth of LPO activity was day 1 - in groups with isolated IRS, or isolated blood loss, or trauma (1, 2 and 4 EG) and day 3 - in groups of combined impact - IRS with blood loss (EG3) and IRS with mechanical trauma (EG5).

Conflict of interest. The author declares the absence of a conflict of interest.

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The article was received
2020.07.09