Original article

Experimental Liver Peroxidation Against the Background of Limb Ischemia - Reperfusion Injury – Is There a Pathogenic Difference Between Its Modifications?

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Abstract

Introduction: Use of the haemostatic tourniquet is an important step in providing first aid in open bleeding injuries. Tourniquet pressure on the extremity triggers local lipid peroxidation. However, the systemic influence of tourniquet thereof has not been fully studied.

Aim: This study aimed to evaluate the changes that occur against the background of ischemia-reperfusion syndrome (IRS) and modifications of trauma in the main gland for detoxification – the liver.

Material and Methods: In order to estimate the liver condition under the effects of a haemostatic tourniquet, animals were divided into five experimental groups, where modifications of hypoxia were performed as a result of bleeding, IRS and trauma due to mechanical fracture of the thigh.

Results: Biochemical study of the liver has shown that each type of such interventions caused the activation of lipid peroxidation in this organ. The highest increase of the malonic dialdehyde rate was observed in response to haemostatic tourniquet combined with blood loss. Additionally, its content was higher in the group combining mechanical trauma and the tourniquet compared to isolated trauma.

Conclusion: All types of interventions caused hypoxia – as a result of isolated bleeding and cessation of blood flow due to the tourniquet. However, the release of overconcentration of toxic derivatives of rhabdomyolysis, which entered the blood stream after limb release, activated the pathological mechanisms of IRS, which included intensified lipid peroxidation in the liver.

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Introduction

Nowadays, as in the past, the use of the haemostatic tourniquet is an important step in providing first aid [1, 2]. However, a careful study of the mechanisms of ischemia-reperfusion syndrome due to the use of haemostatic bandage is required [3]. The latter was proved to be an important cause of oxidative stress [4, 5], which promotes lipid peroxidation in the limb region under tourniquet pressure, and affects metabolism and protective reactions in the entire organism [6, 7].

The essence of ischemia-reperfusion syndrome (IRS) is that after the release of the tourniquet, a large amount of toxins enters systemic blood circulation [8, 9], which, in turn, causes an increase in functional liver activity [10-13]. However, against the background of hypemic hypoxia, full liver repair is impossible. At the moment, there is not enough data that could comprehensively explain the correlation between the IRS and the liver, especially lipid peroxidation (LPO) in the liver in the condition of IRS

Material and Methods

The experiments were performed on 260 white non-linear male rats 5-5.5 months old. The animals were removed from the experiment at the 1st hour after intervention, and on the 1st, 3rd, 7th and 14th day after trauma on the basis of thiopental-sodium anaesthesia (40 mg/kg of body weight intraperitoneal) by total bleeding from the heart. Such number was based on the need to obtain statistically significant data in each group, as well as at each time point – in order to see the stages of development of posttraumatic disease, in particular lipid peroxidation activity, caused by haemostatic tourniquet. The high number of experimental animas used is also due to higher mortality of rats in EG3 and EG5 groups (explained bellow) compared to other experimental groups Animals were divided into 5 groups (N=10 animals per group): control group (CG), where rats were administered only thiopental-sodium anaesthesia (40 mg/kg of body weight intraperitoneally); EG1 (rubber tourniquet was applied to the upper third of the thigh for 2 hours, reperfusion lasted 1 hour (isolated IRS); EG2 (simulated blood loss in the amount of 40% of the volume of circulating blood from the femoral vein); EG3 (tourniquet on thigh was combined with 40% blood loss from the femoral vein on the other lower limb), EG4 (mechanical trauma that caused fracture of femur), EG5 (tourniquet on thigh was combined with fracture of femur of the other lower limb). Given the onset of severe pain in EG4 and EG5, an injection of a 2%-solution of lidocaine was administered for 7 days in the posttraumatic period. Animals in other groups were administered analgesics twice – on the day of intervention and on the following day.

The experiments were performed in the vivarium of I. Horbachevsky TNMU in the morning. The special room had a stable temperature (18-22 °C), relative humidity (40-60%) and illumination of 250 lux.

All experimental stages of work were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the resolution of the First National Congress on Bioethics (Kyiv, 2001) and the Order of the Ministry of Health of the Ukraine No. 690 of 23 September 2009.

The activity of active thiobarbituric acid derivatives in 10% of liver homogenate samples was determined using a method based on the ability of secondary products of lipid peroxidation (LPO), especially malonic dialdehyde, during a reaction with thiobarbituric acid at high temperatures and in acidic pH, to form a coloured complex with optic density that can be registered by spectrophotometry on waves of 532 nm [14].

Statistical analysis

Statistical analysis of the obtained data was performed using Excel (Microsoft, USA). The

statistical significance of the differences between independent indices was determined using Student's t-test at a normal distribution and by non-parametric methods in other cases. The correlation coefficient was significant at $p < 0.05$.

Results

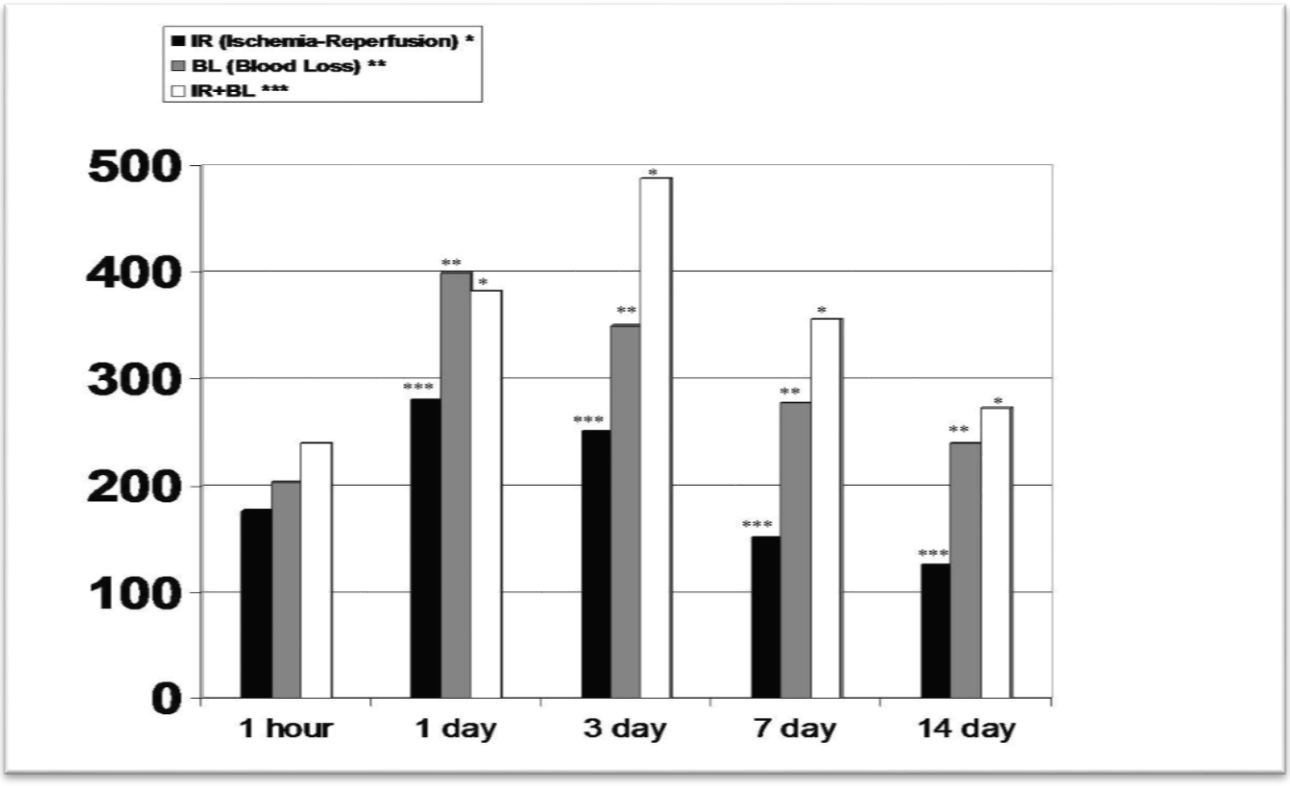
As can be seen in the data in Figures 1 and 2 against the background of ischemia modelling, the content of TBA-active products increases significantly.

Thus, in isolated ischemia-reperfusion injury (EG1) after the 1st hour, on the 1st, 3rd and 7th day, there was an increase of the index by 76.7% ($p < 0.05$), 2.8 times ($p < 0.05$), 2.5 times ($p < 0.05$) and 51.6% ($p < 0.05$), respectively, compared to the control. On the 14th day, the indicator did not return to baseline, exceeding the control by 25.9% ($p > 0.05$).

Against the background of isolated blood loss (EG3), the content of TBA-active derivatives of lipid peroxidation after the 1st hour was 2 times higher than said index in CG ($p < 0.05$), on the 1st day after intervention 4 times ($p < 0.05$), on the 3rd day 3.5 times ($p < 0.05$), on the 7th day 2.8 times ($p < 0.05$) and on the 14th day 2.4 times higher ($p < 0.05$).

As for EG3 – tourniquet on thigh was combined with 40% blood loss from the femoral vein on the other lower limb – that group included the most obvious increase in peroxidative activity compared to other groups: after the 1st hour, the index exceeded the control 2.4 times ($p < 0.05$), on the 1st, 3rd, 7th and 14th day, it was 3.8 times ($p < 0.05$), 4.9 times ($p < 0.05$), 3.6 times ($p < 0.05$) and 2.7 times higher ($p < 0.05$), respectively.

Figure 1 – Dynamics of TBA-active derivatives of lipid peroxidation in the liver (in % compared to the control level) after ischemia-reperfusion of limb and blood loss



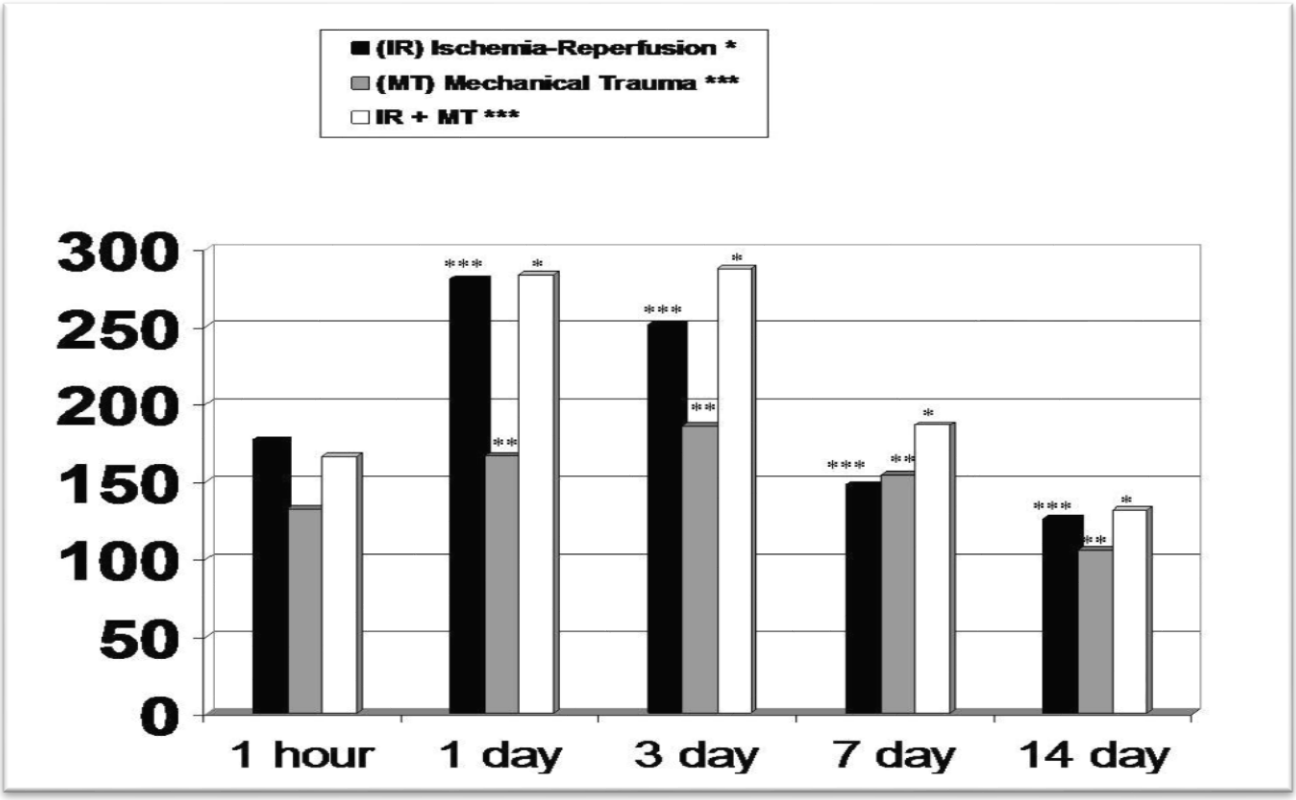
Notes: statistical differences between the 1st day, 3rd day, 7th day and 14th day in comparison with the 1st hour, 1st day, 3rd day and 7th day, respectively are significant, $p < 0.05$)
In the 1st group – * statistical significance compared to previous day
In the 2nd group – ** statistical significance compared to previous day
In the 3rd group – *** statistical significance compared to previous day

Comparison of the following groups with the effect of isolated ischemia-reperfusion of the limb on the LPO in liver tissue confirmed its pathogenic effect. Thus, against the background of isolated mechanical trauma (MT) of the thigh (EG4) in the 1st hour after the intervention, the activity of the LPO increased by 32.8% ($p > 0.05$). Significant increase was observed on the 1st, 3rd and 7th day after trauma, when, compared to the control, the index was higher by 65.6% ($p > 0.05$), 86.2% ($p < 0.05$) and 54.5% ($p < 0.05$), respectively.

On the 14th day, the index did not differ significantly from the control.

At the same time, its activity against the background of MT combined with the tourniquet (EG5) was higher. Thus, after the 1st hour, the index was higher than in CG by 66.1% ($p < 0.05$). On the 1st and 3rd day, it remained at the same level – it was higher compared with the control by 2.8 times ($p < 0.05$) and 2.9 times ($p < 0.05$); on the 7th and 14th day, it exceeded it by 86.2% and 31.8%, respectively.

Figure 2 – Dynamics of TBA-active derivatives of lipid peroxidation in the liver after IR of limb and mechanical trauma



Notes: Data are presented in % compared to the control level. statistical differences between the 1st day, 3rd day, 7th day and 14th day in comparison with the 1st hour, 1st day, 3rd day and 7th day, respectively, are significant, $p < 0.05$)
In the 3rd group – *statistical significance compared to previous day
In the 4th group – **statistical significance compared to previous day
In the 5th group – ***statistical significance compared to previous day

The dynamics of changes in the studied index had specific patterns caused by both ischemia and additional effects of mechanical trauma. With varying intensity, depending on the combination of pathogenic effects, the activity of LPO increased by the 3rd day, but decreased by the 14th day, although it did not reach the normal level of CG.

Thus, there was a significant increase of the index in EG1 – on the 1st day after the intervention, it remained increased by 58.8% ($p < 0.05$), compared with the activation of the POL in the 1st hour, after which a decrease was noted. At the same time, on the 3rd day it exceeded the activity in the 1st hour by 42.2% ($p < 0.05$). On the 7th and 14th day, the index was lower than the most acute period of the 1st day by 46% ($p < 0.05$) and by 1% ($p < 0.05$), respectively, and significantly lower than the 1st hour index.

In EG2, the dynamics of activity of LPO derivatives was similar, with the highest score on the 1st day, when the index exceeded the data of the 1st hour by 96.1% ($p < 0.05$), after which it decreased markedly. Thus, activity on the 7th day was lower by 30.5% ($p < 0.05$) and by 20.6% ($p < 0.05$) than indices of the 1st and 3rd day, respectively.

With regard to the value of the index in EG3 on the 1st day, compared to the 1st hour, the index increased significantly by 58.8% ($p < 0.05$), on the 3rd day it increased by 27.6% ($p < 0.05$) compared to the 1st day, and 2 times compared to the 1st hour. The 7th day index was 27% lower than the 3rd day index ($p < 0.05$), and the 14th day index was 23% lower ($p < 0.05$) compared to the 7th day period.

A significant increase in LPO activity was recorded on the 1st day in EG4, when the index exceeded the data obtained in the 1st hour after the intervention by 25.7% ($p < 0.05$). The highest activity score was on the 3rd day, when the index exceeded the data of the 1st hour by 40.4% ($p < 0.05$), after which it gradually decreased, becoming lower than the analogous index after the 1st hour, the 1st, 3rd and 7th day by 20.2% ($p < 0.05$), 36.5% ($p < 0.05$), 43.1% ($p < 0.05$) and 36.5% ($p < 0.05$), respectively.

LPO activity in EG5, in contrast to EG1 and EG4, in which the 1st day was a critical period, continued rising on the 3rd day, when it exceeded the level of the 1st hour and 1st day by 70.4% ($p < 0.05$) and 72.8% ($p < 0.05$), respectively. After that, LPO activity decreased sharply – on the 7th day it decreased by 35.1% ($p < 0.05$) compared to the 3rd day, and on the 14th day it decreased by 29.3% ($p < 0.05$), compared to the 7th day.

As can be seen in Table 1, by comparing the values of the studied index in groups with different severity of ischemia, it was found that in the 1st hour, in EG3 the index was higher than in EG2 and EG1 by 26.5% ($p < 0.05$) and by 15.3% ($p < 0.05$), respectively. In addition, the index in EG1 was significantly higher than in EG4 – by 25% ($p < 0.05$). The results in EG5 exceeded EG4 by 20.2% ($p < 0.05$). Thus, dynamics of lipid peroxidation were as follows: on the 1st day, the estimated index was lower than in EG2 and EG3 by 29.8% ($p < 0.05$) and by 26.5% ($p < 0.05$), respectively. Also, in EG1, which was almost identical to EG5, it was higher than in EG4 (isolated trauma) by 40.6% ($p < 0.05$). On the 3rd day, there was a further increase in the activity of LPO in EG3 and EG5 and a decrease in the groups EG1, EG2 and EG4. Index of EG3 was higher by 48.5% ($p < 0.05$) and by 28.3% ($p < 0.05$) than in EG1 and EG2, respectively. A comparison between the indices in EG1, EG4 and EG5 found that the index of EG1 was slightly lower than in EG5 – by 12.4% ($p < 0.05$) and higher than in EG4 by 26% ($p < 0,05$). On the 7th day, a sharp decrease of index dynamics was observed in all studied groups. The activity of LPO in EG3 remained higher than in EG2 and EG1 by 22% ($p < 0.05$) and by 57.4% ($p < 0, 05$), respectively, while the score in EG5 remained higher than in EG1 by 17% ($p < 0.05$). On the 14th day, the index in EG3 exceeded the indices in EG2 and EG1 by 11.9% ($p < 0.05$) and 2.2 times ($p < 0.05$), respectively. At the same time, the index of EG5 on the 14th day remained statistically significantly higher than in group 4, by 19.7% ($p < 0.05$), which confirms the

attachment of tangential pathogenic effects caused by ischemia-reperfusion syndrome.

Table 1. Content of TBA-active derivatives of lipid peroxidation in 10% of rat liver homogenates based on variants of ischemia-reperfusion syndrome and isolated blood loss and trauma (micromolkg-1)

Group	Reperfusion period				
	1st hour	1st day	3rd day	7th day	14th day
			Control = 1.89 (n = 10)		
Group 1 **isolated ischemia-reperfusion**	3.34 (n = 10)	5.22* (n = 10)	4.75* (n = 10)	2.87* (n = 10)	2.38 (n = 10)
Group 2 **blood loss**	3.85* (n = 7)	7.55* (n = 7)	6.61* (n = 6)	5.25* (n = 7)	4.54* (n = 7)
Group 3 **ischemia-reperfusion + blood loss**	4.55* (n = 6)	7.22* (n = 6)	9.22* (n = 6)	6.73* (n = 6)	5.15* (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**	2.51 (n = 10)	3.13* (n = 10)	3.52* (n = 10)	2.92* (n = 10)	2.00 (n = 10)
Group 5 **ischemia-reperfusion + trauma**	3.14* (n = 9)	5.35* (n = 9)	5.43* (n = 8)	3.52* (n = 9)	2.49* (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 comparison with the control group are statistically significant (p ⍰ 0.05);*
2. p1-3 – the probability of differences in relation to experimental groups 1 and 3;
3. p2-3 – the probability of differences in relation to experimental groups 2 and 3;
4. p1-5 – the probability of differences in relation to experimental groups 1 and 5;
5. p4-5 – the probability of differences in relation to experimental groups 4 and 5

Discussion

This experimental study was dedicated to identifying the effects of ischemia-reperfusion syndrome of the limb on indices of lipid peroxidation in the liver tissue. It was estimated that even with isolated use of a tourniquet, as well as with isolated blood loss or injury, peroxidation processes in the liver were intensified. However, the addition of IRI statistically significantly complicated the course of injuries, indicating a side effect of the use of the tourniquet. Knowledge of the periodization of traumatic disease against the background of the studied pathology is important for the development of sanogenic effects in order to minimize this pathogenic factor.

According to the latest research, haemostatic tourniquet causes not only temporary ischemia, but is accompanied by complications such as: ischemia and nerve damage in the pressure zone of the tourniquet, limb oedema, skin pallor, muscle weakness without paralysis and pain [9, 15]. In our experiment, we paid attention to the processes of peroxidation in the liver. Although the tourniquet is an effective tool for rapid cessation of critical bleeding, its use for 2-3 hours can trigger irreversible processes of tissue death due to absence of blood supply and, in severe cases, can lead to limb amputation [16-18].

Correlation analysis showed that in the development of ischemia-reperfusion syndrome, caused by the application of a haemostatic tourniquet, obviously insufficient liver function could play a great role [19]. Formation of the active hepatic response is a predictable reaction, which is part of the syndrome of multiple organ failure. Contribution of the IRS as the reason for such pathological effects to the organism was already

demonstrated [20 - 25]. In addition to that, increased lipid peroxidation is manifested in certain liver dysfunction, especially bile formation [26].

Present results confirmed the increase of liver lipid peroxidation in the presence of different conditions that cause ischemia: tourniquet and bleeding, combination of blood loss with haemostatic plait during the period, which is considered to be safe, but unfortunately further increases the degeneration of cell membranes. Thus, the results of different scientists examined the concentration of glycolytic metabolites and the content of high energy phosphates in skeletal muscles, which were analysed at various times of tourniquet ischemia during operation in bloodless field. Against the background of moderate growth of phosphocreatine in the ischemic limb, a pronounced increase of lactate (4-5 times) was observed. It is obvious that the reason for a moderate increase of glucose and glucose-6-phosphate was the activation of glycogenolysis. Since ischemia lasted from 30 to 90 minutes in these cases, the changes in indices were detected after 5 minutes [27]. Present study did not directly show a sharp increase of LPO in the tissue possibly due to depletion of the antioxidant system in the liver.

Similar results, extraordinary increase (14 times) of lactate, were obtained by another group [28]. They likewise determined a decrease of skin temperature from 35.9 to 33.5 degrees, which quickly dropped to that level in the first 15 minutes, after which it remained stable at this level [28].

Summing up, the main causes for activation of pathogenesis branches of ischemia-reperfusion injury became known as the following: rhabdomyolysis and hypoxia. The former resulted in hyperejection of toxins into the bloodstream after release of limb from tourniquet. Its basic pathogenesis – muscle oedema with subsequent development of hypovolemia, haemoconcentration and massive release of myoglobin, potassium and biologically active substances that could trigger multiorgan failure from compressed tissue [29]. Increased concentration of myoglobin in renal tubules in the conditions of acidic pH promotes intratubular obstruction and kidney dysfunction [30-32]. Another factor is hypoxia, which is known to be the cause that stimulates lipid peroxidation [33] – and in our experiment it was activated by two causes – bleeding and ischemia. The latter provided not only the lack of oxygen, but also local compression with subsequent rhabdomyolysis. Thus, a pathological circle was formed.

In turn, liver function was recognized in this study as a protective reaction. The most severe period of LPO activity decrease was the 1st day – in the groups with isolated IRI or isolated blood loos or isolated trauma (EG1, EG2, EF4) – and the 3rd day – in the groups with combined effects – ISI with blood loss (EG3) and IRI with mechanical trauma (EG5). Present study suggests that, something caused peroxidation of lipid membranes of hepatocytes, e.g. toxins and hypoxia.

In previously published results, the active response of the liver antioxidant system was represented by increased and subsequently depleted activity of antioxidant enzymes [34]. This studies coincide with other authors' results. Orlova E. et al. estimated that SOD activity is highest in the liver and advised a correction of its insufficiency with "Vin-Vita" [35]. Liver tissue damage as a result of application of tourniquet signalled the possibility for development of multiple organ failure due to the ischemia-reperfusion limb syndrome [36, 37].

To sum up the above, the highest increase of malonic dialdehyde rate was observed against the background of a combination of haemostatic tourniquet on the thigh with blood loss. Additionally, malonic dialdehyde content was higher in the group with a combination of trauma and the tourniquet in comparison with isolated mechanical trauma. This fact confirms the role of the tourniquet as a factor that complicates the course of traumatic disease due to the development of the ischemia-reperfusion syndrome. All of our interventions caused hypoxia – as a result of isolated bleeding and cessation of blood flow due to haemostatic

tourniquet. Among the causes that activated pathological mechanisms of ischemia-reperfusion injury, attention should be paid to the influences of toxic derivatives of rhabdomyolysis, which entered the bloodstream in excessive concentrations after limb release from tourniquet. Intensification of the liver function was the result of a protective response to pathogenic effects.

The knowledge gained is very important, as in recent years the number of situations accompanied by bleeding from large blood vessels increased significantly. In addition, the use of haemostatic tourniquets is still one of the most efficient and easy way to control blood loss. Thus, their importance cannot be denied, but side effects and their pathogenic effects on the systemic level require more detailed research.

Conclusion

Intensification and decrease of liver function was the result of a protective response to pathogenic effects. Periods of lipid peroxidation activity allowed us to find 2 periods of exhaustion of wounded organisms against the background of blood loss, combined with the use of haemostatic tourniquets and consequently affected by ischemia-reperfusion syndrome – they are on the 3rd and 14th day against the background of bleeding combined with IRS. Knowledge of this will help to better understand the pathogenesis of traumatic disease and with a new form of oxidative stress and development of the best treatment.

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Competing interests. None to declare.

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[i] **Author contribution**: single author article