Structural changes in skeletal muscles of the hind limbs of rats in acute ischemia-reperfusion and its correction by carbacetam, detected by polarization microscopy

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Introduction
The incidence of acute ischemia of the extremities is 140 cases per 1 million populations per year, which is 0.1 % of all patients with surgical profile, and is one of the most common in urgent peripheral vascular surgery, with

Arterial tourniquets are used in clinical practice for angioplasty and arthroplasty, and in case of limb injuries, their use often occurs according to vital signs. After removing the tourniquet and blood supply restoration to the limb arises a multifactorial lesion of tissues both ischemic and distant from the site of ischemia. A number of publications have been devoted to the study of morphological disorders in muscle tissue in acute ischemia-reperfusion in the medical literature. However, the researches for effective means for drug correction of these disorders still continues. The aim of the study was to explore peculiarities of skeletal muscle remodeling of the hind limbs of rats, detected by polarization microscopy, in acute ischemia-reperfusion, caused by the application of an arterial tourniquet, and in the correction of reperfusion disorders by carbacetam. Microscopic examination of histological sections of skeletal muscles of the hind limbs of 60 rats below the site of application of the tourniquet under conditions of experimental acute ischemia-reperfusion was performed. Acute ischemia for all animals was caused by application of SWAT rubber bands on the hind limbs of animals, 5-6 mm in width, at the inguinal fold level within 2 hours under thiopental anesthesia. A reperfusion was modeled by removing the tourniquet. Half of the experimental animals in the reperfusion period for the purpose of correction intraperitoneally was administered the nootropic drug 1-oxo-3,3,6-trimethyl-1,2,3,4-tetrahydroindolo[2,3-c]quinoline (carbacetam) at a dose of 5 mg per kilogram of body weight once a day during the entire reperfusion period. The histological specimens of the skeletal muscles were stained with hematoxylin and eosin, and were examined with a light microscope with polarization nozzle. Studies with using the polarization microscopy have shown that in the early reperfusion period morphological criteria for skeletal muscle remodeling expressed by deformation and anisotropy of muscle fibers, disappearance of their transverse striation, cracks and ruptures of fibers, and in the most severe cases there were signs of necrosis of the fibers with their fragmentation into separate lumps. Subject to the correction of reperfusion disorders by carbacetam, there is a decrease in the degree of damage and consistent acceleration of restoration of the skeletal muscles structure, which was the most pronounced in groups of animals with reperfusion terms after 1 and 14 days. Complex of features indicates, that at the tissue level the administration of carbacetam reduces the ischemic-reperfusion lesion of the muscular fibers, as also accelerates the mechanisms of reparative rhabdomyohistogenesis. Thus, structural changes in the skeletal muscles of the limb after two-hour ischemia and subsequent reperfusion increased in the early reperfusion period and reached its peak after 1 day of reperfusion, and in the late period of reperfusion their reverse development took place. With the correction of disorders by carbacetam, the degree of damage was reduced and the recovery of the skeletal muscle structure of the limb was accelerated.

Keywords: acute ischemia, reperfusion, skeletal muscles, polarization microscopy, arterial tourniquet, carbacetam.
significant morbidity and mortality [1, 3, 22].

Acute ischemia of the extremities is one of the main reasons for the deterioration of the quality of life of patients and their early disability, especially in the context of an increasing number of car accidents, natural disasters and hostilities [7]. From 5.5 % to 14.6 % of cases of acute limb ischemia lead to a decrease in the quality of life of the patient or limb amputation [7]. The number of deaths ranges from 9.7 % to 35 %, and in elderly patients reaches 42 % [3, 22]. Among survivors, 20 % are at risk of high amputation [3]. Acute ischemia on the background of trauma, in particular damage to the main vessels is from 6-8 % to 10 % of all gunshot wounds in the structure of combat trauma [3]. In 67 % of cases, a tourniquet is applied in case of vascular damage at the prehospital stage [4, 9, 13]. After removal of the tourniquet and restoration of blood supply to the limb, a multifactorial lesion of both primary ischemic and distant from the site of ischemia tissue develops [6, 16], which is called ischemic-reperfusion syndrome [10, 24].

A number of studies [8, 14, 23] have studied the structural changes in the soft tissues of the extremities in the development of this syndrome, but polarization microscopy is rarely used to describe structural disorders in skeletal muscle, which is a very informative method of morphological examination of muscle tissue. Also requires a comprehensive study of the effectiveness of nootropic drugs for the correction of morphological disorders in ischemia-reperfusion [11, 25]. An attempt was made to determine the effectiveness of carbacetam in ischemic-reperfusion injury of rat skeletal muscle by analyzing the results of polarization microscopy of rat skeletal muscle.

The aim of the study was to investigate the features of skeletal muscle remodeling of the hind limbs of rats detected by polarization microscopy in acute ischemia-reperfusion caused by the imposition of an arterial tourniquet, and in the correction of reperfusion disorders by carbacetam.

Materials and methods

Histological examination of the skeletal muscles of the hind limbs of 60 rats under experimental acute ischemia-reperfusion was performed. There were 8 intact animals in the control group.

The experiment was conducted in the Central Research Laboratory of I. Horbachevsky Ternopil National Medical University of the Ministry of Health of Ukraine and consisted in modeling ischemia by applying a SWAT rubber tourniquet on the hind right limb of the animal, at the level of the inguinal fold under thiopental-sodium anesthesia (40 mg/kg of body weight). The width of tissue compression was 5-6 mm. Reperfusion was simulated by restoring blood circulation in the previously ischemic limb after removal of the tourniquet 2 hours after its application.

The animals were euthanized by decapitation under thiopental-sodium anesthesia (500 mg/kg body weight intraperitoneally), after which muscle tissue was collected. The experiment was conducted in compliance with the provisions of the Law of Ukraine № 3447 - IV of 21.02.06 "On protection of animals from cruel treatment" (2006), "Rules for work with experimental animals", approved by the Ministry of Health of Ukraine № 755 of 12.08.1997, "European conventions on the protection of vertebrate animals used for research and other scientific purposes" (Strasbourg, 1986), Council of Europe Directive 2010/63 EU on animal experiments.

During the experiment, the experimental animals were divided into three groups: the first experimental group - rats with simulation of ischemic-reperfusion damage by applying a tourniquet on the hind limb for 2 hours (30 rats); the second experimental group - rats with simulation of ischemic-reperfusion damage by applying a tourniquet on the hind limb for 2 hours, which in the reperfusion period intraperitoneally administered 1-oxo-3.3.6-trimethyl-1.2.3.4-tetrahydroindolo [2.3-c] quinolines (carbacetam) at a dose of 5 mg per kilogram of body weight once a day throughout the perfusion period (30 rats); control group - intact animals (8 rats).

Animals of the first and second experimental groups were divided into 5 subgroups of the study, 6 animals in each. The model of the early post-ischemic period in both groups was represented by three subgroups of animals with reperfusion changes at 1 and 2 hours and 1 day, and the model of the late reperfusion period by two subgroups of animals with post-ischemic changes at 7 and 14 days after tourniquet removal.

Histological examination was performed at the Department of Pathological Anatomy with a sectional course and forensic medicine of I. Horbachevsky Ternopil National Medical University of the Ministry of Health of Ukraine. Skeletal muscle sections below the 4-5 μm thickness of the tourniquet, made on the MS-2 microtome, were stained with hematoxylin and eosin according to conventional methods [19].

Histological specimens were examined using a MICROmedSEO SCAN laboratory microscope with a polarizing nozzle and photographed using a Digital Camera for Microscope Science Lab DCM 820. Evaluation of the type and degree of ischemic damage to skeletal muscles of the extremities in a polarization study was performed according to the gradation described by Cellarius Yu. G. with co-authors and Nepomnyashchikh L. M. [2, 17, 18].

Results

Analysis of histological preparations of skeletal muscle of experimental animals of the control group revealed that the muscles in the longitudinal sections are represented by fibers that vary slightly in thickness. Sarcomplasm is homogeneous, transverse striation on the longitudinal sections is revealed in the form of straight and evenly spaced strips (A-band), the nuclei of the myosymplast are oriented with their axes along the muscle fibers (Fig. 1).

Examination of the skeletal muscles of the hind limbs
of animals by polarization microscopy 1 hour after removal of the tourniquet revealed damage to the muscle fibers of the femoral region, mainly I degree, which was manifested by increased anisotropy of A-bands of myofibrils in some areas (Fig. 2). Damage to myofibrils of second-degree muscle fibers, which were expressed by the convergence of A-band, was observed only in some places.

Reperfusion lesion two hours after restoration of blood supply in the limb was characterized mainly by the second degree of myofibril damage, which was visualized (in addition to the characteristic for the first degree of increased anisotropy of A-band) and their balanced convergence relative to each other (Fig. 3).

At the end of day 1 of the experiment, polarization microscopy revealed areas of fusion of A-bands, which is characteristic of III degree of muscle fiber damage, as well as the phenomena of fragmentation and disaggregation of myofibril, which corresponds to the most severe IV degree of contractile type of myofibril damage. Numerous breaks and cracks of fibers were noted. In some cases, myocytolysis was also observed, in which the destruction of myofibrils with the decay of necrotized muscle fibers on the edge of the tubercle was detected (Fig. 4).

In the model of animals of late reperfusion lesion after 7 days there were signs of restoration of the structure of myofibrils of muscle fibers, and in animals with reperfusion after 14 days there were signs of regeneration of fibers with small sarcosomes around the myofibrils. At the same time, areas of both enhanced anisotropies of A-bands and fibers with convergence of these disks, but without their fusion into a continuous conglomerate, were detected. Such changes corresponded to the I-II stages of contractual remodeling of myofibrils (Fig. 5).

In the group of animals with correction, polarization microscopy in the early reperfusion period, namely 1 and 2 hours after the restoration of blood supply to the ischemic limb, revealed changes that are characteristic of the I-II degree of muscle fiber damage. Similar changes were found in a group of animals without corrective therapy. However, type II contractile damage, characterized by convergence of A-band and enhanced anisotropy of myofibrils, in rats with correction was somewhat less common than in the group of animals without correction (Fig. 6).
A polarization study of skeletal muscle performed 1 day after restoration of blood supply in most animals with correction showed fusion of anisotropic discs, as well as damage to the integrity of myofibrils, which corresponds to type III contractural remodeling, but without signs of intracellular myocytolysis. We did not find any characteristic signs of cat IV stage of myofibril damage in the femoral segment of rats after 1 day of reperfusion (Fig. 7).

In addition, in animals with correction of the sign of remodeling after 7 days of reperfusion approximately corresponded to those in animals without the introduction of carbacetam with reperfusion after 14 days. And in rats with a period of reperfusion after 14 days, the phenomena of regeneration and reduction of contractile disorders of the femoral muscle fibers to the structure of myofibrils of intact animals were detected. And only in some cases type I myofibril damage with enhanced anisotropy without noticeable convergence of A-band and with preserved transverse striation was detected (Fig. 8).

Discussion
To date, there are several mechanisms that can neutralize or mitigate the effects of reperfusion injury [15]. A special place among the groups of drugs used in the treatment and prevention of ischemic disorders are nootropics, their feature is that, in addition to nootropic, mnemonic, cognitive, metabolic, neuroprotective, antiplatelet, antioxidant properties have antioxidant properties, as they smooth the phenomena of hypo- and hyperperfusion in the post-ischemic period (during reperfusion), improve microcirculation in tissues and do not have pronounced side effects characteristic of psychotropic and sedative drugs [5]. However, the study of this group of drugs in order to find new biologically active substances with a wide range of properties and to expand the indications for their use continues.

The effectiveness of carbacetam has been repeatedly described in the restoration of cognitive impairment, including the restoration of research reactions and memory
processes in experimental TBI, reduction of endogenous intoxication and oxidative stress in polytrauma, reduction of NO2 content and NOS synthesis and activity, and increased catalase activity in the hippocampus of rats in Alzheimer's disease [20, 21]. Studying its effect on endogenous intoxication in the conditions of polytrauma, a decrease in the level of endogenous intoxication, manifested by normalization of serum MSM in 14-28 days, was revealed, and on the basis of previous experiments it was found that, in addition to nootropic properties, protection of liver, heart and lung tissues and caused a pronounced antioxidant effect in the tissues of internal organs [12].

Polarization microscopy revealed morphological changes in the skeletal muscles of the hind limbs of rats below the site of arterial tourniquet in both carbacetam-free and ischemic-corrected animals, but the severity of such changes was different in the compared groups.

Two-hour imposition of an arterial tourniquet on the hind limb of rats developed ischemic-reperfusion remodeling of skeletal muscles below the site of plate imposition, the most pronounced disorders of which appeared 1 day after restoration of blood supply to the limb and reached stage IV with changes in contractile damage with a gradual reduction of changes to stage I-II in animals after 14 days of reperfusion.

Experimental ischemia and reperfusion are characterized by progressive remodeling of muscle fibers, mainly by contraceptive type, but in the group of animals without correction, there were also isolated cases of myocytolysis.

In the stages of postischemic perfusion, with the correction of carbacetam, there was a consistent decrease in the degree of damage and acceleration of the recovery of the skeletal muscle structure of the limb. If, after 1 and 2 hours of perfusion, the structural differences of skeletal muscle in animals without and with carbacetam correction were not significant, then there was a positive increase in the dynamics of rats administered carbacetam.

The difference between the groups of animals with and without carbacetam correction was most pronounced in the groups with a perfusion period after 1 day. In particular, in animals with correction, no damage to stage IV myofibrils was observed, which was common in this period in animals without correction. Also, animals with correction did not show myocytolysis of muscle fibers after 1 day of reperfusion.

Structural changes in muscle fibers in the group of animals with correction after 7 days of reperfusion were approximately similar to those in the group without correction with reperfusion after 14 days. In animals of the late reperfusion period (reperfusion after 14 days) with correction revealed reduction of contractural disturbances to structure of myofibril of control group or, in some cases, to the I stage of contractural remodeling of skeletal muscles whereas in group of animals without correction in this period were observed, characteristic of the I and II stages of contractural damage to skeletal muscles.

Conclusions

It was found that structural changes in the skeletal muscles of the limb during two-hour ischemia and subsequent reperfusion increased in the early reperfusion period and reached its peak after 1 day of reperfusion, and in the late reperfusion period their reverse development occurred. If the disorders are corrected with carbacetam, the degree of damage is reduced and the skeletal muscle structure of the ischemic limb is restored.

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