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ARTICLE

Remote ischemic postconditioning as well as blood plasma from double-conditioned donor ameliorate reperfusion syndrome in skeletal muscle

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ABSTRACT

The aim of this study was to verify the possibility of preparation and effectiveness of the use of blood plasma containing an effector of ischemic tolerance activated by applying two sublethal stresses to a donor. As sublethal stresses, two periods of 20-minute hindlimb ischemia were used with a two-day interval between them. Active plasma was isolated six hours after the second hindlimb ischemia. The effectiveness of active plasma as well as remote postconditioning was tested after three hours of tourniquet-induced ischemia on the gastrocnemius muscle. The wet/dry ratio of gastrocnemius muscle (degree of tissue oedema), nitroblue tetrazolium reduction (tissue necrosis), and CatWalk test (hind limb functionality) were evaluated 24 h after the end of ischemia. Three hours of ischemia increased muscle oedema and necrosis in comparison to control by 26.72% ($p < 0.001$) and 41.58% ($p < 0.001$) respectively. Remote ischemic postconditioning as well as injection of conditioned blood plasma significantly prevented these changes, even when they were applied one or three hours after the end of ischemia. Equally effective double-conditioned plasma appears to have better prospects in life-threatening situations such as stroke and myocardial infarction.

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Ischemia; reperfusion; skeletal muscle; remote ischemic postconditioning

Introduction

Onset of ischemia in trauma cases is usually unpredictable, so there is almost no way of protecting tissues from ischemia/reperfusion (IR) injury. Free tissue transplantations are lengthy procedures which result in prolonged tissue ischemia. In the last two decades increased attention has been devoted to the study of the ischemic tolerance phenomenon. Stress impulse can induce changes in the organism ranging from activation of the endogenous protective mechanism known as ischemic tolerance via apoptosis of particularly vulnerable cells (e.g. neurons), to necrosis or even pan-necrosis (i.e. infarction) of all cellular elements exposed to the stress.

Conditioning procedures are the most powerful cardioprotective interventions in animal experiments. However, ischemic preconditioning cannot be used to reduce infarct size in patients with acute myocardial infarction because its occurrence is not predictable [1]. The decisive factor for activation of ischemic tolerance is combination of two stresses [2,3]. This phenomenon is a two-stage process; the first tier is obligatory in any case; however, for full tolerance activation the application of the second stress is absolutely necessary as well. A great advantage is that the two stresses need not be of the same nature (cross tolerance). This cross tolerance not only enables the first stress to have the form of planned stress (preconditioning), but it can be a pathological event as well, and then planned stress can be used as the second stress, i.e. postconditioning (hundreds of publications confirm this fact).

Remote tolerance means that to achieve tolerance in the whole organism, it is necessary to stress only a part of it, e.g. part of a limb or an organ (for a review, see Riksen et al. [4]). Tolerance then spreads with the blood throughout the body [5,6]. Shimizu et al. [7] even demonstrated cross-species transfer of tolerance by blood. It is also apparent that substances present in the blood are able to protect various tissues against damage, or even to prevent death.

Remote ischemic postconditioning is a phenomenon in which the therapeutic effect of induced sublethal ischemia in a specific organ leads to attenuation of IR injury in the remote target organ [8]. Tourniquet postconditioning causes a significant reduction in systemic inflammatory response (TNF-alpha, oxygen-derived free radicals). Laboratory and histological samples showed a significant decrease in remote organ (lung and renal) dysfunction after postconditioning [9]. Carrying out postconditioning on a healthy limb is easy. We hypothesized that in the blood of a double-conditioned donor, full-strength tolerance would appear, which would be effective if delivered to an ischemic patient.

Materials and methods

Forty-seven adult albino Wistar rats of both sexes weighing 250–350 g which were free of any clinically evident disease were group-housed and maintained on a 12-h light/dark cycle, with *ad libitum* access to water and rodent chow. The animals were bred in a registered animal colony (SK PC 20011) at the Institute of Neurobiology, Košice, Slovakia. Experiments were performed in accordance with European Community legislation. The Ethics Committee at the Institute of Neurobiology as well as the State Veterinary and Alimentary Administration of the Slovak Republic approved the experiments. The animals were randomized into groups as shown in Figure 1.

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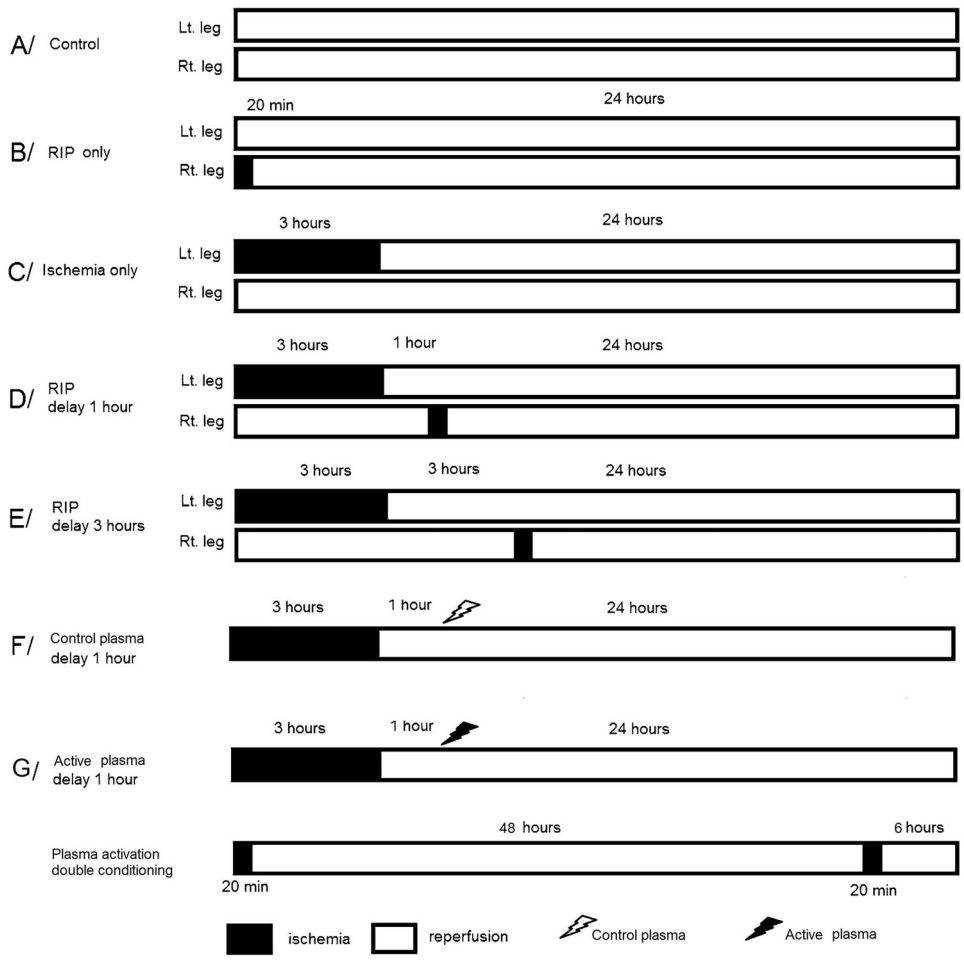

Figure 1. Experimental protocols. The rats were randomized into following groups: A – intact control ($n = 10$), B – 20 min right hind limb ischemia ($n = 8$), C – ischemic control = 3 hours of left hind limb ischemia ($n = 5$), groups D + E – 3 hours of left hind limb ischemia followed by 20 min right hind limb ischemia 1 or 3 hours later ($n = 5$each), groups F and G were treated one hour after 3 hours of ischemia with blood plasma (1 ml i.a.) isolated from control or conditioned rats ($n = 5$ each). The last four animals underwent two 20 min episodes of ischemia with a two-day interval between them. Active plasma was obtained from blood taken 6 hours after the second ischemia.

Hind limb ischemia

The ischemic procedure on the left (3 h of ischemia) or right (20 min of postconditioning) posterior limb was carried out with an external elastic band application (tourniquet), placed as proximally as possible [10]. The effectiveness of arterial occlusion was monitored through a laser-Doppler flowmeter. Chloral hydrate (300 mg/kg i.p., Sigma-Aldrich, St. Louis, MO, USA, 10% solution in saline) was used throughout the ischemic time to sedate the animals and allow hassle-free measurement of blood flow and pressure. We did not use barbiturates or volatile anaesthetics due to their known ability to interfere with ischemia/reperfusion induced injury [11].

All animals underwent a functionality test 24 h after the end of ischemia and were then given an overdose of chloral hydrate (400 mg/kg, i.p., 10% solution in saline). Muscle samples from the belly of the gastrocnemius muscle were immediately removed and processed.

Preparation of conditioned blood plasma

The rats were conditioned with two periods of 20 min hind limb ischemia with 48 h interval between them. Blood was obtained six hours after the second ischemic event by puncture of the left ventricle and collected in heparinized tubes (50 IU/ml, Heparin, Leciva, sol. Inj. 50 K, Zentiva k.s., Prague, Czech Republic). Blood plasma was isolated by means of 20 min centrifugation at 4000 rpm and 4 °C (SW5 rotor, Velocity 18 R, Dynamica Pty Ltd., Victoria, Australia).

Monitoring of muscle ischemia/reperfusion efficiency and blood flow

Monitoring of muscle ischemia was performed with the laser probes of a Doppler flow-meter (Periflux System 5000, Perimed AB, Järfälla, Sweden). After a skin incision, a laser probe was attached with the help of a circular plate directly on the belly of the gastrocnemius muscle surface and fixed with circular stitching. Circulation was monitored at 3-s intervals and evaluated using PSW 2.5.5 software (PeriSoft for Windows, Perimed AB Parr Medical Technology (Peking) Co., Ltd.) [12].

Monitoring of blood pressure

Non-invasive blood pressure measurement was performed using a VetSpecs VSM8 monitor (VetSpecs Inc., Ball Ground, GA, USA) with a cuff and sensor band placed around the tail.

Muscle cell necrosis

Necrosis in gastrocnemius muscle slices was evaluated after reacting with nitroblue tetrazolium (NBT, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Muscle samples were immediately cut transversely into 2 mm thin slices and incubated in triplicates in 0.05% NBT for 20 min in the dark [13]. Viable muscle cells were stained dark blue and dead cells continued to be unstained. Formazan-free controls (considered to be 100% necrotic) were obtained after incubation of intact muscles without NBT. The ratios of necrotic to viable muscle cells were calculated after photographing under a LEICA EZ4HD microscope with LAS EZ v 2.1.0 software (Leica Microsystems, Heerbrugg, Switzerland). Densitograms were scanned and calculated using Image J 1.48i software (National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij). The color density of a formazan-free blank was taken as 100% of necrosis, and the density of an intact control was considered as 0% of necrosis.

Muscle oedema assessment

The extent of muscle oedema was measured as the ratio of wet to dry weight of the gastrocnemius muscle. Muscle samples (100–200 mg wet weight) from the belly part of the gastrocnemius muscle were immediately weighed and then oven-dried at a temperature of 55 °C until constant weight was reached (at 48–72 h). Tissue oedema was detected as a relative increase in the ratio of wet to dry weight [14].

Monitoring of limb functionality after ischemia

Functionality changes were monitored using a CatWalk XT 10.0 (Noldus Information Technology b.v., Wageningen, the Netherlands). The CatWalk is a laser lighting-based device which scans and digitally records more than 20 parameters, including the intensity of the load, time of load, individual parts of the foot area, speed and regularity of movement [15]. All rats passed along the test lane until three compliant results from each animal were obtained. Values were calculated using CatWalk XT v. 10.0 software (Noldus Information Technology b.v., Wageningen, the Netherlands).

Statistical analysis

Data are presented as the mean ± the standard error of the mean (SEM). Groups were analysed with one-way ANOVA followed by Tukey–Kramer's test using GraphPad InStat 3.1 software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at $p < 0.05$.

Results

Changes in blood circulation and pressure

Tightening of the rubber tourniquet led to an immediate cessation of blood circulation in the hind limb. After just 20 min of ischemia, tourniquet release resulted in reactive hyperemia with 30% higher blood flow than the original values, lasting for approximately 20 min (Figure 2(A)). Then the blood flow returned to its pre-ischemic value. During tourniquet ischemia on one leg, an increase of blood circulation in the opposite leg occurred (Figure 2(B)). Ischemia lasting three hours caused slower reperfusion, which was reduced approximately by 30% and remained thus for a number of hours (Figure 2(C)). Postconditioning applied either one or three hours after the same interval of ischemia showed a transient increase in blood flow as in RIP alone but resulted in higher values of perfusion persisting up to 24 h (Figure 2(D)).

Changes in blood pressure caused by RIP are not shown. Blood pressure rose during the first ten minutes of tourniquet ischemia, reaching values 27.44% higher than control ($p < 0.05$), but after 20 min of reperfusion it decreased to its original values. In addition to the increase in pressure a simultaneous increase in blood flow was observed.

Edema of the gastrocnemius muscle

Three hours of ischemia caused the water content in the muscle (*wet/dry ratio)* after 24 h of reperfusion to be significantly ($p < 0.001$) increased by 26.72% (Figure 3). Delayed remote ischemic postconditioning lasting 20 min proved capable of preventing massive increase in the wet/dry ratio. The group with three hours of ischemia and RIP after one hour of reperfusion had water content increased by 4.75%, and the same group but with RIP after the third hour of reperfusion had water content increased only by 4.13%. Both of these results were significantly lower compared to ischemia without RIP ($p < 0.001$). With application of blood plasma obtained from intact rats, oedema triggered by three hours of ischemia was practically not affected (increase by 26.69%). However, injection of the conditioned plasma significantly prevented occurrence of oedema (increase by 1.28%, $p < 0.001$).

Muscle necrosis

Presence of muscle necrosis after three hours of ischemia without and with subsequent postconditioning is presented in Figure 4. Viable muscles stained with nitroblue tetrazolium assume the dark blue colour of created formazan. Three hours of ischemia with subsequent 24 h of reperfusion led to massive 41.58%, ($p < 0.001$) necrosis of the gastrocnemius muscle, caused by loss of enzymatic (oxidoreductase) activity, which can be detected as the inability to produce dark blue formazan. The group with three hours of ischemia and RIP after one hour of reperfusion had 4.31% occurrence of necrosis, and the group with RIP after the third hour of reperfusion had 4.93% occurrence of necrosis (both $p < 0.001$ compared to ischemia without RIP). Equally effective was the application of conditioned blood plasma delayed one hour after the end of ischemia (4.6 ± 0.24% of necrosis, $p < 0.001$ compared to ischemia followed by injection of control plasma).

Limb functionality

Function of muscles after ischemia with or without RIP was evaluated using CatWalk apparatus. It was evident that the control (sham) group of rats used all extremities equally, and with equal intensity. Normally rats place their left hind and right front feet in the same moment. After three hours of ischemia, the rats no longer used their left hind limb, and there was no contact of that limb with the floor, but no changes could be seen in the right hind limb which was used for postconditioning (20 min ischemia). Rats from the groups with RIP and activated plasma used all four limbs (not shown).

Discussion

Sublethal ischemia is known to be able to induce the robust endogenous defence mechanism called ischemic tolerance.

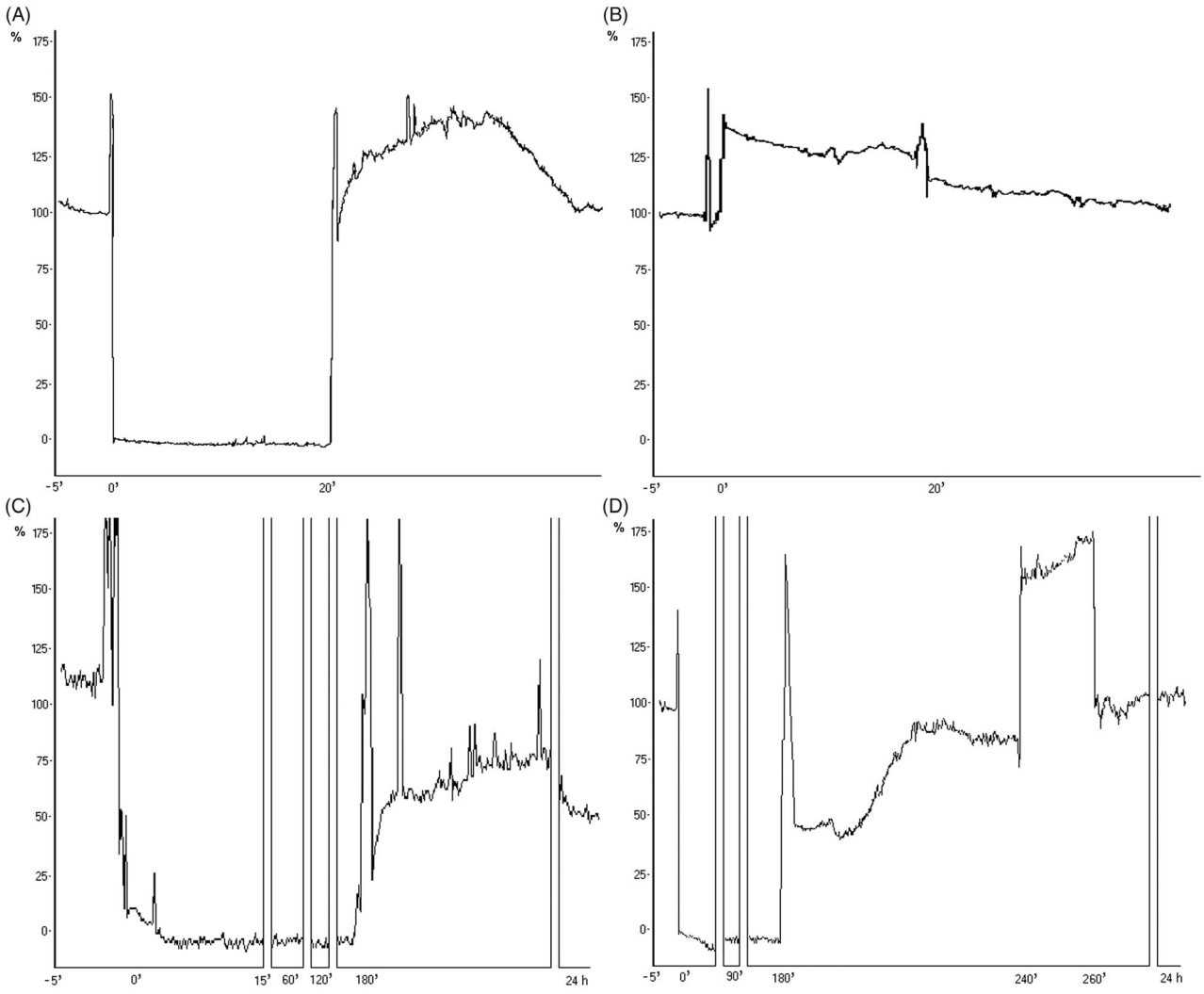

Figure 2. Blood flow during ischemia and reperfusion measured on gastrocnemius muscle. (A) 20-minute ischemia with reperfusion; (B) Blood flow rate measured during ischemia applied to the opposite leg; (C) 180-minute ischemia with reperfusion; (D) 180-minute ischemia followed by RIP applied after 60 minutes of reperfusion.

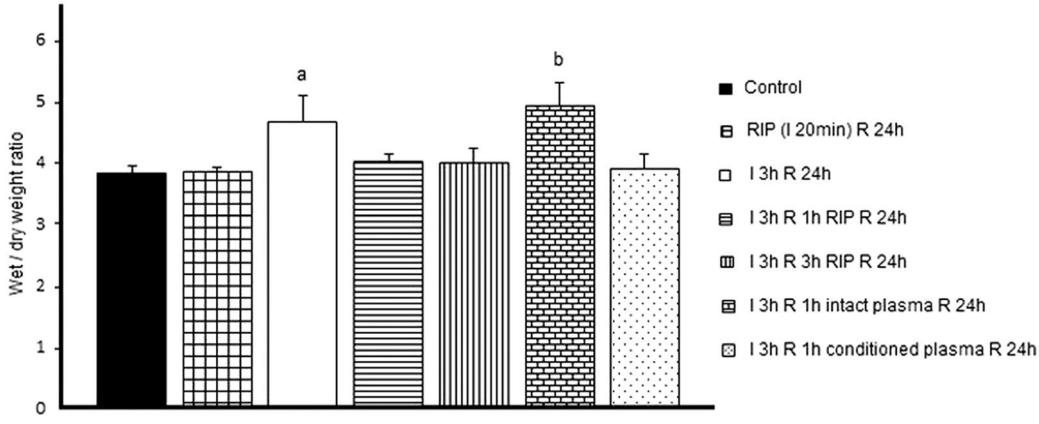

Figure 3. Changes in water content in m. gastrocnemius after 3 h of ischemia and 24 h of reperfusion with or without postconditioning. Data are expressed as average ± S.E.M. Isch.: ischemia; Rep.: reperfusion. a = significantly different in comparison to control, RIP and postconditioning ($p < 0.001$). b = significantly different in comparison to group with c plasma ($p < 0.001$).

Remote postconditioning allows that tolerance to be shared with the whole body. If short ischemia is used as postconditioning in a sufficiently large volume of muscle, it induces two independent synergic beneficial effects: induction of tolerance and elevation of blood pressure, and thus also increased blood flow. The fact that ischemic tolerance operates independently is confirmed by the results when remote ischemia is used before or during lethal ischemia [14,16]. On the other hand, transient increase in perfusion caused by RIP can be of huge benefit at the end of ischemia after free-flap transfer or replantation of amputated parts of the body. In

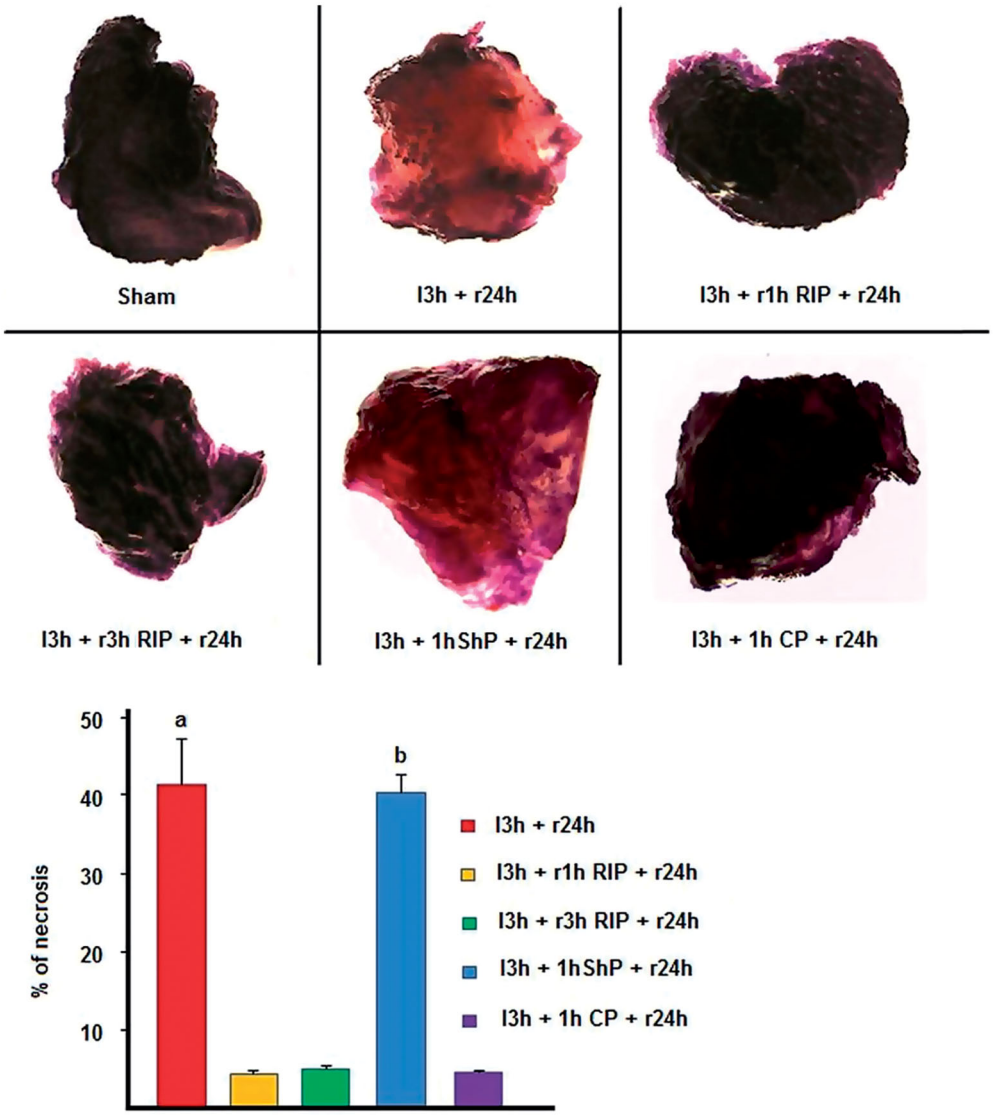

Figure 4. Formazan creation in Gastrocnemius muscle slices after reaction with nitroblue tetrazolium. Viable muscle is dark blue stained by creating formazan, and non-viable muscle unable to react with NBT is unstained. Data are expressed as average ± S.E.M. Sham: sham control; I: ischemia; r: reperfusion; RIP: remote ischemic postconditioning; ShP: plasma from sham control; CP: conditioned plasma. a = significantly different in comparison to control and groups with RIP ($p < 0.001$). b = significantly different in comparison to group with conditioned plasma ($p < 0.001$)

addition to postischemic reactive hyperemia RIP prevents edema, no reflow phenomenon and improves microcirculation [17].

With regard to the potential use of ischemic tolerance in clinical medicine, the traditional application of tolerance is based on classical preconditioning when sublethal stress triggers a temporary tolerance to stresses of various origins, enabling tissues to survive subsequent lethal doses. Classical preconditioning is useful only before elective procedures when ischemic conditions are expected to occur, e.g. free-flap transfer or coronary artery bypass surgery [18]. The reverse use of tolerance procedure, i.e. the application of sublethal stress after the lethal one, called postconditioning, is known in several forms. First there is so-called rapid or immediate postconditioning based on several cycles of repeated short periods of ischemia/reperfusion, used for instance after myocardial [19], liver [20], brain [21] and skeletal muscle ischemia [22]. In terms of clinical use this procedure has a few limitations. It is unusable in cases of cardiac arrest, or with partial ischemia, where it is often impossible to establish the exact beginning of the reperfusion; its application during transport to a medical facility is hardly imaginable; and finally, also based on our personal experience with the use of experimental animals, it is inappropriate for clinical practice due to the appearance of accompanying spasms and convulsions during the alternation of ischemia and reperfusion [23].

Another method of delayed postconditioning which uses the ability of a living organism to activate its defence mechanisms and to stop the process of degeneration from the beginning is based on utilizing appropriate stressors some hours after a lethal impulse. Simply expressed, delayed postconditioning must be used before the start of the irreversible stage of apoptosis/necrosis. The width of the therapeutic window depends on the intensity of the previous attack. However, if we consider the real clinical situation of a patient in critical condition due to long-lasting muscle ischemia or devastating limb injuries, most of the seemingly wide range of stressors is not applicable. It is not possible to perform repetitive ischemic insult, or to use hypoxia, and the utilisation of hyper- or hypothermia is also debatable. The use of lipopolysaccharide induces fever, and 3-nitropropinic acid is mitochondrial poison. Application of bradykinin may result in an increase in internal calcium and subsequent excitotoxic glutamate release. However, the use of remote ischemic postconditioning

(local stress remote from the damaged tissue) provides protection for the whole body.

Twenty years ago Przyklenk et al. first described how 'brief episodes of ischemia in one vascular bed protected the remote virgin myocardium from subsequent sustained coronary artery occlusion' [24]. Later, remote local ischemia was used as postconditioning through repetitive occlusion and release of selected arteries, but this was changed subsequently into the more palatable non-invasive tourniquet ischemia of skeletal muscles or an entire limb [14]. Many attempts have been made to determine the optimal protocol for ischemic postconditioning. The development has moved from several periods of ischemia/reperfusion lasting a few seconds through to longer-lasting repetitive I/R intervals towards single periods of ischemia lasting 10–20 min. We chose single 20-min duration of RIP in line with Pignataro et al. [25], due to the possibility of tourniquet-induced neuromuscular injury pressure. Twenty minutes of postconditioning ischemia did not produce any necrosis, oedema or functional changes in the muscle distal to the tourniquet, but it eliminated massive necrosis, oedema or functional changes induced by three hours of ischemia in the contralateral hind limb, even when it was applied with a delay of one or three hours.

Our method when we used remote ischemic postconditioning consisted of 20 min ischemia of skeletal muscle for either one or three hours after normothermic ischemia of the hind limb muscles. Our results clearly demonstrate that this stressor is able to effectively prevent onset of oedema, consecutive secondary hypoperfusion, and muscle cell death. Similar results have been published by Tsubota et al. [14], the only difference being that they used remote conditioning in mice immediately before reperfusion at the end of lethal ischemia.

In our experiment 20 min of ischemia had no measurable adverse effect on the origin of muscle cell necrosis, or of tissue oedema. These facts were also confirmed by functional testing on the CatWalk apparatus. Three hours of ischemia alone caused long-lasting inability in the rats to use the injured extremity, but 20 min of ischemia used as postconditioning restrained the generation of damage caused by three hours of ischemia.

The three-hour wide therapeutic window (in the case of normothermia or more in hypothermic muscles) can be utilized for a wide range of classical drugs. Efficacy of remote tourniquet ischemic postconditioning in the case of delayed neuronal death (apoptosis) induced by transient global cerebral ischemia or kainate intoxication suggests that conditioning products are able to overcome the blood/brain barrier [26].

Sublethal stress will induce the first stage of tolerance in the body of the donor. The second stress applied after two days of maturation finalizes the complete tolerance. Six hours after the second stress is enough to synthesize the protective substances appearing in the blood plasma. Application of cycloheximide simultaneously with the second stress completely blocked the end effector synthesis, but when cycloheximide was used at five hours after postconditioning the majority of ischemized CA1 neurons survived [2].

Due to the multifactorial changes induced by ischemia, the effector must cause events threatening the life of cells to return to their original values. This concerns the functioning of mitochondria and ion channels, restoration of protein synthesis, and glutamate concentration. The effector circulating in the blood after postconditioning is effective in all the organs studied and is even transmissible between species [7]. In the RPost signalling mechanism, kinases are involved: protein kinase C, PI3, Akt, JAK. The hypothetical end effector in RPost is aldehyde dehydrogenase-2, the transcription factors STAT, Nrf2, and also the BKCa channel [27]. There have been a large number of studies suggesting that the ATP-sensitive potassium channels (KATP channel) play a significant role as mediators or end effectors in RIPC [28].

In assessing the possibility of using ischemic tolerance in the clinic, it is necessary to differentiate between acute, life-threatening conditions such as stroke and heart attack, occurring especially in the elderly, suffering from additive diseases and taking various drugs. Positive results have been achieved through the application of remote ischemic conditioning on the microcirculation of human pedicled and free surgical flaps [17,29]. However, the prevailing result of translating cardioprotective strategies to clinical practice is disappointment [30]. The translation of findings from healthy, young animals with acute coronary occlusion/reperfusion to patients of older age, with a variety of co-morbidities and co-medications, suffering from different scenarios of myocardial ischemia/reperfusion remains a challenge [31]. In addition, the use of any method from a wide variety of conditioning methods causes considerable discomfort to the affected patient. On the other hand, the method we are proposing allows us to prepare in the body of a healthy, young and unmedicated donor an instantly usable medicament really containing 'the switch' between cell death and survival.

Conclusions

Based on our results, it can be stated that atraumatic remote postconditioning is able to effectively prevent the formation of oedema, necrosis and loss of muscle functionality, even if it is used one or three hours after the end of ischemia. 20-min tourniquet ischemia (RIP alone) causes no damage to muscle in the sense of formation of oedema, necrosis or damage to limb functionality.

The temporary increase in blood pressure and related blood flow during tourniquet postconditioning can work as an additional effect to the phenomenon of ischemic tolerance, enhancing reperfusion at the end of time-consuming reconstruction in plastic and orthopedic surgery. It is important to note that tourniquet application increases blood pressure to about 30% higher values than before its application, which can be dangerous in the event of possible bleeding.

Administration of substances of complete tolerance by injection enables their immediate therapeutic effect. This can fundamentally change the possibility of exploiting this robust defence phenomenon in clinical medicine, and it could markedly decrease the risk of reperfusion syndrome in revascularisation and replantation surgery.

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Disclosure statement

No potential conflict of interest is reported by the authors.

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