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Research Article

The End Effector of Ischemic Tolerance Present in Blood Plasma from Double Conditioned Donors Ameliorates Trimethyltin Provoked Damage in Brain

Rastislav Burda [1], Viera Danielisová [2], Jozef Burda [2,*]

1. University Hospital of L. Pasteur, Rastislavova 43, Košice, Slovakia; E-Mail: burda@netkosice.sk
2. Slovak Academy of Sciences, Institute of Neurobiology of Biomedical Research Center, Šoltésovej 4, Košice, Slovakia; E-Mails: danielis@saske.sk; burda@saske.sk

* **Correspondence:** Jozef Burda; E-Mail: burda@saske.sk

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Abstract

***Background*:** Many experiments have been done to demonstrate robust ischemic tolerance efficiency using mostly young and healthy animals. However, the translation of these results to usually elderly and sick patients moreover taking many various medicines has to date been disappointing. 3-Methyltin (TMT) poisoning and short-term transient cerebral ischemia cause similar damage, especially, to selectively vulnerable brain regions such as hippocampal CA1 and CA3.

Methods: Using dual conditioning, we activated the full ischemic tolerance the products of which are located to blood plasma. As sublethal stresses, two periods of 20-minute hind-limb ischemia was used with a two-day interval in rats. Active plasma was isolated 6 h after the second hind limb ischemia. Brain damage was detected by visualizing dead neurons by Fluoro-Jade B staining and neuronal survival by NeuN immunoreaction. The functional capabilities of the surviving neurons were monitored by the Morris water maze.

Results: The use of activated plasma dramatically reduced the hippocampal degenerative CA1 neurons from 56.59% to 7.51%. NeuN positivity was increased from 23.82% to 78.67%. Time spent searching for a hidden island in the Morris water maze was shortened from 48.46 to 21.63 s.

Conclusions: By using double conditioning it is possible to provoke the synthesis of substances present in the blood plasma, providing the organism with significant protection against acute degenerative processes.

Keywords

Trimethyltin intoxication; hippocampus; neurodegeneration; remote ischemic postconditioning

1. Introduction

Ischemic tolerance is surprisingly strong defence mechanism of the organism gives cells that survived the lethal stress or met with sublethal stress to become tolerant to in other circumstances fatal stress. Specifically, the most sensitive neurons in the brain able to survive without blood supply for approximately 5 min are after the previous conditioning able to survive and to retain the functionality for two times longer time without oxygen and glucose supply. The existence of this phenomenon of tolerance has been demonstrated in all kinds of tissues and different species studied so far including human.

To achieve complete tolerance, a combination of two stresses is necessary [1]. The first fundamental characteristic of this phenomenon is the cross-tolerance, where resistance activated by one type of stress allows tissue to overcome the stress of a different origin. Cross-tolerance allows mild stress to be used as an activator used before the lethal stress, but also in an arrangement where, following a pathological event, mild stress is used as a therapeutic intervention. The second important characteristic is the phenomenon of remote tolerance, which means that in order to obtain the whole body tolerance just exposure of the part of the organism to stress is required [2]. Tolerance is then spread via blood throughout the body [3, 4]. Shimizu and co-workers [5] even demonstrated cross-species transfer of tolerance by the blood plasma.

A major milestone in the study of ischemic tolerance was that in addition to using the first, mild stress (preconditioning), comparable and surprisingly strong efficacy can be achieved even if moderate stress (postconditioning) was used after of lethal stress in hearth [6] and brain [1, 7]. It should be pointed out that the phenomenon of tolerance cannot save irreversibly damaged cells; however, further damage can be prevented, thus it is essential to use postconditioning in the range of the therapeutic window. The therapeutic window differs for different kinds of tissues or cells and depends on the type of stress, its intensity, and the length of exposure as well as the temperature of the affected tissue. The delayed neuronal death occurring after global cerebral ischemia as well as some apoptosis-inducing intoxications offer two-day wide therapeutic window and enables to effectively use delayed postconditioning even 48 h after pathology [1, 7, 8]. These studies indicated that for the acquisition of full tolerance, a combination of two stresses is necessary.

Another problem is the use of appropriate stressors. Among a whole range of possible biological, chemical, and physical stressors (substances or treatments), it is very difficult to choose the most suitable. In case of a patient with a very serious condition, such as being in coma after a heart attack or stroke, the suitable method to induce local stress is to stop blood flow in a part of the limb using tourniquet. This procedure also has its drawbacks, however, by increasing the blood pressure and the flow rate, which rules out its use in cases or risk of bleeding into the brain. The method of local stress is already in use in clinical medicine since 2007 [9]. Published clinical tests using remote postconditioning in the case of traumatic brain damage are clearly positive [10]. However, the translation from numerous successful animal experiments on cardioprotection beyond that by reperfusion to clinical practice has to date been disappointing [11]. The problem is various types of conditioning, times of application, but mainly health, higher age and co-medication of patients. Optimal time for the application of preconditioning for the development of a fully functional tolerance is 2 days [10]. However, postconditioning can be used repeatedly. The drugs that clearly interfere with conditioning are naloxone [12] and antioxidants that block the effect of pre- and post-conditioning [5, 8, 13, 14]. Since cancer treatment inevitably acts as a stressor, e.g. tolerance-promoting conditioner, the application of antioxidants simultaneously with anticancer therapy can eliminate the tolerance activation. Therefore, the philosophy of cancer treatment can be changed to the daily application of smaller doses of the drug to substantially increase its efficacy [8]. Trimethyltin (TMT) is a potent neurotoxin that selectively induces neuronal death in the limbic system of humans and other animals, in particular in the hippocampal formation. Animals exposed to TMT develop dose dependent behavioural alterations (hyperactivity and aggression), cognitive impairment (memory loss and learning impairment), and spontaneous seizures. In rats, TMT administration is characterized by a subacute pattern of actions, which replicate some peculiar features of human neurodegenerative diseases [15], resulting in progressive neuronal death of CA1 and CA3/hilus pyramidal neurons.

TMT is used in experimental models simulating brain ischemia. TMT-induced neurodegeneration is a complex event resulting from different pathogenic mechanisms that include calcium overload, excitotoxicity, neuroinflammation, oxidative stress, and mitochondrial dysfunction [16, 17]. Since the localization of damage and the typically delayed death of neurons are very similar to certain types of acute neurodegeneration, TMT can be used in experimental models to simulate global ischemia of the brain. The results obtained in the study of ischemic tolerance inspired us to compare options and use this model in the study of mechanisms of ischemic/reperfusion damage and therapy in the CNS.

Our results using the remote tolerance [18] led us to the idea to apply combination of two sublethal stresses, “preconditioning and postconditioning”, was applied on donor animals. This led to the development of full strength tolerance in the blood of the donors. In this work we are testing efficacy of active plasma isolated from the blood of donor applied to the bloodstream of the recipient submitted to intoxication of brain.

2. Material and Methods

Forty-two adult albino Wistar rats of both sexes weighing 250–350 g free of 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 the registered animal colony (SK PC 20011) of

the Institute of Neurobiology, Kosice, Slovakia. The experiments were performed in accordance with European Community legislation and were approved by the Ethics Committee at the Institute of Neurobiology as well as the State Veterinary and Alimentary Administration of the Slovak Republic approved the experiments.

2.1 Experimental Groups

The rats were randomized into 6 groups. Control n=8, control plasma n=5, double conditioned plasma n=5, trimethyltin (TMT, 8 mg/kg i.p.) n=8, TMT + control plasma n=8, TMT + conditioned plasma n=8.

2.2 Control Plasma

The control plasma from five rats was obtained by puncture of the left ventricle and collected in heparinized tubes (50 IU/mL, Heparin Leciva sol inj 50K, Zentiva k.s., Praha, Czech Republic). The blood plasma was isolated by centrifugation at 4000 rpm for 20 min at 4°C (SW5 rotor, Velocity 18R, Dynamica Pty Ltd., Victoria, Australia). The yield of plasma from a single animal was 3 mL.

2.3 Conditioned Active Plasma Preparation

At 48 h of the interval, two episodes of sublethal ischemia for 20 min were induced. The duration of ischemia was chosen following Pignataro [19]. The procedure for ischemia on the right posterior limb was carried out by applying an external elastic band (tourniquet) placed as proximally as possible [20]. 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. The effectiveness of arterial occlusion was monitored through a laser-Doppler flow-meter. Activated plasma was obtained 6 h after 2nd 20 min ischemia by puncture of the left ventricle and collected in heparinized tubes. Blood plasma was isolated similarly as described for the control animals.

2.4 Monitoring of Muscle Ischemia/Reperfusion Efficiency and Blood Flow

Monitoring of muscle ischemia was performed with laser probes using 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. Blood circulation was monitored at 3-second intervals and evaluated using PSW 2.5.5 software [21].

2.5 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.

2.6 Trimethyltin (TMT) Intoxication

Next three groups were used for experiment with TMT intoxication. The TMT dose was chosen to cause neurodegeneration of 50% of the hippocampal CA1 neurons 7 days after administration.

The first TMT intoxication group was subjected to a single injection of TMT (8 mg/kg i.p.) followed by saline injection after 1 h (1 mL i.a., n=8). The rats in the second group after 1 h of TMT were treated by control plasma (1 mL i.a., n=8). In the third group, the rats after 1 h of TMT injection were treated by active plasma (1 mL i.a., n=8). The animals were killed 7 days after TMT intoxication by transcardiac perfusion/fixation.

2.7 Brain Fixation

Transcardiac perfusion via the left ventricle was performed under chloral hydrate anesthesia (300 mg/kg, i.p.). Perfusion started with a washout of blood vessels with 200 mL of 0.9% NaCl. Brains were perfusion fixed with 4 % paraformaldehyde solution in phosphate buffered saline, removed, and postfixed overnight in the same fixative prior to vibratome sectioning. Four 33 µm thick coronal sections of the brain from each animal were prepared at the level of bregma - 3.3 ± 0.2 mm for the hippocampus and randomly selected for Fluoro Jade B staining, which was used to label all degenerating neurons present in the CA1 region 7 days after the TMT intoxication with or without plasma application, regardless of the mechanism of cell death.

2.8 Fluoro Jade B Staining

The sections were mounted on 2% gelatine-coated slides and then dried on a slide warmer at 50°C for 30 min. The slides were then immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 10 min, and subsequently rinsed in distilled water for 2 min. After 20 min in the staining solution containing 0.0004% Fluoro Jade B dye (Histo-Chem Inc., Jefferson, AR, USA), the slides were rinsed three times with distilled water for 1 min each [22]. The excess water was removed by briefly draining the slides (about 15 s) vertically on a paper towel. The slides were then placed on a slide warmer, set at approximately 50°C, and until they were fully dry (5-10 min). The dried slides were cleared by immersion in xylene for at least 1 min before putting a coverslip with DPX (Sigma-Aldrich, St. Louis, MO, USA). The slides were examined under a Leica DM2500 fluorescence microscope with a Leica camera and LAS V3.6 software (Leica Microsystems, Wetzlar, Germany).

2.9 Immunocytochemistry

Immunocytochemistry was performed on the prepared coronal free-floating 33 µm thick vibratome sections. The sections containing the hippocampus were immunostained for NeuN, a neuronal marker. Briefly, the sections were incubated overnight at 4°C with monoclonal mouse NeuN antibody (Chemicon Int., Temecula, CA, USA; 1:500) in 0.1 mol/L PBS (pH 7.4) with 0.2% Triton X-100. After washing with 0.1 mol/L PBS (pH 7.4) containing 0.2% Triton, secondary anti-mouse IgG antibody raised in a horse (Vector Lab-oratories, Burlingame, CA, USA) was applied for 90 min at room temperature. After further washing, avidin/biotin complex formulation kit (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA, USA) was used for 90 min, then the slides were rinsed with PBS followed by Tris Buffer (pH 7.6), and reacted with DAB (0.1 mol/L Tris, 0.04% DAB, 0.033% H_2O_2). The reaction was stopped with phosphate buffer. The slides were dehydrated, cleared, and coverslipped for analysis.

2.10 Morris Water Maze Test

Cognitive and memory functions of the rats underwent experimental procedures were tested with the Morris water maze [23] on the sixth and seventh days of reperfusion. Approximately 500 mL of milk was added to the water, making it opaque. A submerged escape platform (20 cm tall and 15 cm diameter) was located in the southeast quadrant of the maze. A variety of extra-maze visual cues were visible within the maze. The experimenter was unfamiliar with the treatment received by the subject and remained at a fixed location approximately 0.5 m away from the outside edge of the tank during each trial. The water maze training procedure lasted for two days. On the sixth day after TMT intoxication, each rat underwent two trials. During a trial, a rat was placed in the water facing the same place at the edge of the pool. The rat was allowed 60 s to locate the platform. If after 60 s it did not find the escape platform, it was guided by the experimenter and allowed to remain on the platform for 10 s. The inter-trial interval for each subject was 5 min, during which the rat was dried and returned to the home cage. On the second day, the decisive probe trial was performed: all rats started from the same starting position opposite from the quadrant where the submerged escape platform had been positioned during the tests. The escape latency (the time a subject required to locate the hidden platform after being released) of each subject was measured. The maximum value for each trial considered for statistical analysis was 60 s.

2.11 Statistical Analysis

Data are presented as the mean ± standard error of means (SEM). Groups were analyzed 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 $p<0.05$.

3. Results

3.1 Changes in Blood Circulation and Pressure

The tightening of a rubber tourniquet led to an immediate cessation of blood circulation in the hind limb. After 20 min of ischemia, the tourniquet was released. It resulted in reactive hyperemia with 30% higher blood flow than the original values, lasting for approximately 20 min (Figure 1A). Then the blood flow returned to its pre-ischemic value. During tourniquet ischemia on one leg, an increase in the blood circulation was noted in the opposite leg (Figure 1B). The changes in blood pressure caused by RIP are not presented. Blood pressure was increased during the first 10 min of tourniquet ischemia, reaching a value 27.44% higher than the control ($p<0.05$), and 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.

3.2 Hippocampal CA1 Neurons Degeneration/Survival

TMT was chosen in the dose able to destroy approximately a half of these cells (Figure 2). Seven days after the application of TMT (8 mg/kg, i.p.) with control plasma injection 1 h, 168.65 ± 8.01 Fluoro Jade B positive neurons (degeneration 56.59 ± 8.1%) were seen. The treatment with active plasma dramatically decreased the number of degenerated CA1 neurons to 25.37 ± 5.07

cells (7.51 ± 1.5%, p<0.01). Survival (NeuN positivity) was increased from 80.47 ± 9.45 (23.82 ± 2.80%) CA1 neurons to 265.77 ± 29.19 cells (78.67 ± 8.64%).

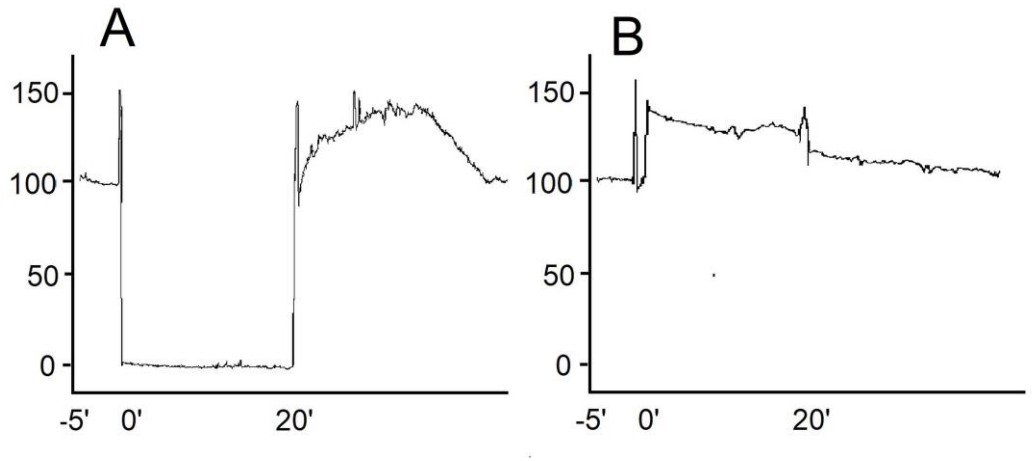

Figure 1 Blood flow during ischemia and reperfusion measured on gastrocnemius muscle. A: 20 min ischemia with reperfusion**.** B: The blood flow rate measured during the ischemia applied on the opposite leg.

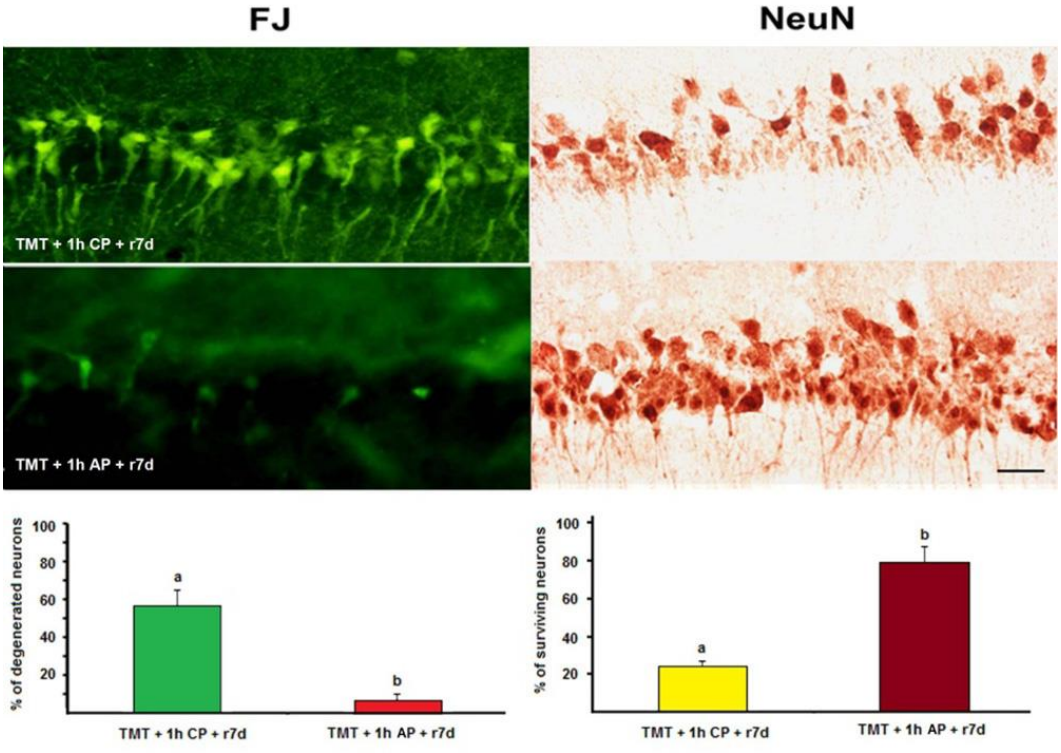

Figure 2 Occurrence of neurodegeneration and survival of neurons in hippocampal CA1 region after trimethyltin intoxication with doubleconditioned plasma injection 1 h after TMT. The animals were killed 7 days after the TMT administration. Data are expressed as average ± S.E.M. FJ = Fluoro-Jade B staining for neurodegeneration; NeuN = immunoreaction with the neuronal nuclear marker; a = significantly different in comparison to the control plasma (p<0.001); b = significantly different in comparison to the group with conditioned plasma (p <001).

3.3 Morris Water Maze Test

The function of CA1 neurons in terms of their ability to participate in learning and memory after TMT intoxication, with control or conditioned plasma (Figure 3), was evaluated using the Morris water maze test. Despite the fact that the chart contains all the data from the test, the probe on the second day of the test are the only important results. Significant changes in the time needed to find the hidden platform were observed. Finding the hidden platform took significantly ($p<0.001$) longer, from 14.05 ± 2.41 s in sham control rats to 47.12 ± 5.85 s in the TMT group with control plasma application. The animals treated with double conditioned plasma 1 h after TMT intoxication were able to locate the platform in less than half of the time (19.32 ± 2.05 s) needed by the TMT with control plasma group. These data confirm not only that conditioned plasma is able to prevent neuronal death but also that surviving neurons retain a substantial part of their function with the ability to learn and remember.

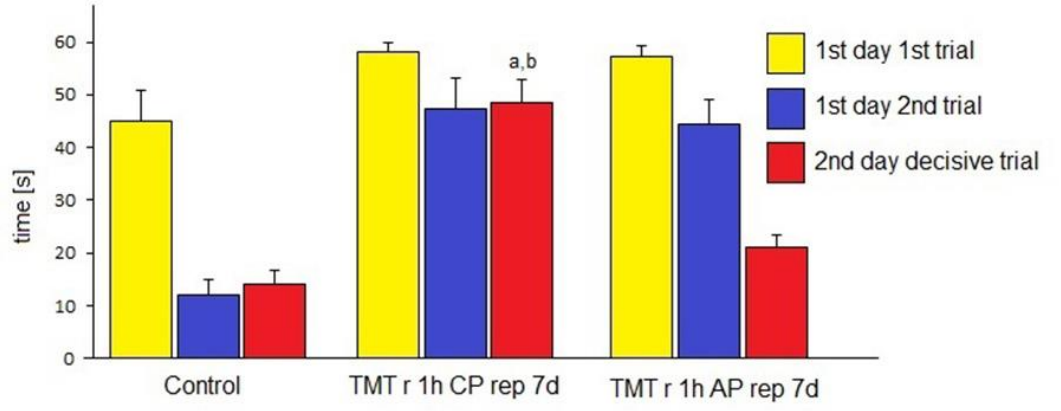

Figure 3 The results of Morris water maze test performed on day 6 and 7 after ischemia with or without activated plasma injection given 1 h after TMT. a = significantly different in comparison to control plasma ($p<0.001$); b = significantly different in comparison to group with conditioned plasma ($p<0.05$).

4. Discussion

Ischemic tolerance seems to be more effective than the currently used treatment. The mechanism of its activation includes two steps. The activation initiates the first stress, which may be: 1) projected sublethal stress (preconditioning); 2) pathological situation (ischemia/hypoxia/intoxication), which can be lethal for a group of cells; however, for the rest of the cells it would be a sublethal stress; 3) as a first stress is necessary for the treatment aimed at eliminating an undesirable cell populations what is confirmed by the finding that stressor activating tolerance could also be gamma radiation [24]. Several physical procedures and biological, chemical, or pharmacological compounds can serve as a stressors activating first or, after previous pathological stress, finalizing full tolerance. The second stress switches the tolerance to the second stage - full tolerance - which is capable to stop the processes leading to the death or complete cellular damage. The products of the full tolerance are similar to that of the first stage distributed by blood throughout the body. An important point is that the full tolerance must be achieved even within the therapeutic window. This therapeutic window takes certain

time depending on the type and duration of the pathological stress and the process of cell death. To synthesis of protein (s) providing a tolerance after the second stress period of 5 or more hours is required [8].

One concern is what kind of pre-, per-, or post-conditioning can be used. Individual laboratories, using otherwise similar protocols, often vary in intensity, length, and dose of the stress. In the clinic, it is even more complicated because some procedures cannot be used and some diagnoses do not allow other conditioners to be used. The easiest way is to deploy tourniquet on the arm or leg. Here, however, it should be kept in mind that this causes an increase in blood pressure and flow that could be dangerous in cases of traumatic injury or may lead to bleeding in the brain.

Our solution eliminates annoying, dangerous, poorly effective or late use of conditioning. The use of two mild atraumatic stresses separated from each other for 48 h, is very easy protocol to "produce" the complete tolerance in organism of donor. This process of activating blood plasma proteins in the blood of patients has numerous advantages: - it can be used immediately after the arrival of the doctor to the patient literally before accurate diagnosis estimation, - therapeutic effect is immediate, thus outpacing the efficiency of application stressor or trigger for several hours. The effectiveness of active plasma has been established in apoptosis inducing intoxication of the brain as well as cerebral and skeletal muscle ischemia (not shown). Brain protection after application into the bloodstream indicates that active substances are able to overcome the blood-brain barrier. The effect of plasma in the brain and skeletal muscle suggests that the active substances occurring in the active plasma will effectively operate in ischemic, apoptosis-inducing and partly also the traumatic conditions of other organs, particularly the heart.

5. Conclusions

Double tourniquet ischemic conditioning carried out for a period of 48 h allowed obtaining active blood plasma with efficacy comparable to other ischemic tolerance practices. The advantage of activated plasma is that it is immediately effective after administration, regardless of age, the health of patient, and possibly the co-medication used.

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Author Contributions

Rastislav Burda - data analysis and interpretation, drafting the article; Viera Danielisová - data collection, data analysis; Jozef Burda - the conception and design of the work, critical revision of the article.

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Competing Interests

The authors have declared that no competing interests exist.

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