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The Influence of Remote Ischemic Conditioning on Focal Brain Ischemia in Rats

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Abstract

Despite obvious progress in the treatment of acute forms of ischemic stroke, the risk of this condition remains unacceptably high. Brain infarction in the middle cerebral artery basin occurs in patients with atherosclerosis. The onset of the brain infarction is facilitated by the cessation of circulation (embolism) in conditions of insufficient collateral circulation. The extent of the infarct zone is determined by neuronal death and impaired microcirculation. The development of new methods for effective targeted restorative stroke therapy is crucial for restorative treatment and reducing the risk of mortality after stroke. Remote ischemic conditioning (RIC) is an approach to limiting reperfusion injury in the ischemic region of the brain after focal ischemia. One of the most commonly used *in vivo* models in stroke studies is the filament model of Middle Cerebral Artery Occlusion (MCAO) in rats. In our experiment, it was performed for 30 min (J. Koizumi) with subsequent 48-hour reperfusion. Within the first 24 hours after the start of reperfusion several short episodes of ischemia in low limbs were induced. After 48 hours of reperfusion the brains were harvested and stained with TTC. Then we evaluated the effect of RIC within 24 hours *ex vivo* in rats' brains, as well as syndecan-1 plasma concentration. Infarct area was assessed by means of Image-Pro program with statistical analysis. Infarct volumes in the model group ($31.97\% \pm 2.5\%$) were significantly higher compared to the values in the RIC group 48 hours after ischemia-reperfusion ($13.6\% \pm 1.3\%$) (*$P < 0.05$). A significant reduction in the area of infarction after RIC is likely due to the effect on the regulation of collateral blood flow in the ischemia area. On the second day after ischemia-reperfusion, tissue swelling was reduced in the RIC group compared to the model group. Analysis of the average concentration of Syndecan-1 revealed the difference between model and RIC groups. Syndecan-1, endothelial glycocalyx protein, might be the regulator which performs vascular control of the interaction with inflammatory cell and is responsible for mediate effect of remote ischemic conditioning on the restriction of ischemic-

reperfusion injury.

1. Introduction

Despite obvious progress in the treatment of acute forms of ischemic stroke, the risk of this condition remains unacceptably high. In some cases, the chosen treatment does not achieve the necessary level of effectiveness. The development of new methods for effective targeted restorative stroke therapy is crucial for restorative treatment after stroke and reducing the risk of mortality after ischemic stroke. Brain infarction in the middle cerebral artery basin occurs in patients with atherosclerosis. The onset of brain infarction is facilitated by the cessation of circulation (embolism) in conditions of insufficient collateral circulation. The extent of the infarct zone is determined by neuronal death and impaired microcirculation. Hemodynamic factor takes part in regulation of cerebral circulation. The hemodynamic factor depends on the reactivity of the vessel wall, the properties of which are contractibility and elasticity associated with exchange factors. Although the mechanisms of regulation of collateral cerebral blood flow during ischemia have not been sufficiently studied [1], immediate remote ischemic conditioning (RIC) provides neuroprotection, in part, by enhancing collateral circulation, as has been shown in a mouse model of ischemic stroke [2].

One of the most commonly used *in vivo* models in stroke studies is the Endovascular Suture or Filament Model in rats with Middle Cerebral Artery Occlusion (MCAO), which causes reproducible infarcts in the MCAO area [3]. The MCAO model is implicated in most pathophysiological or neuroprotector studies [4]. Reperfusion accompanied by rapid recovery of blood flow does not directly correspond with the pathophysiology of spontaneous human stroke. However, reperfusion in the experiment more accurately mimics the therapeutic operation of mechanical thrombectomy which is expected to be applied to stroke patients [3]. For many reasons, however, preclinical studies of stroke have low translational success rates [4].

Conditioned animals with ischemia showed a smaller stroke area compared to the ischemic group. These results were achieved by temporarily restricting blood supply to the limb immediately after the stroke [5]. RIC is one of the approaches that are considered effective in increasing ischemic damage prevention by implementing brief cycles of transient ischemia and reperfusion. Preclinical animal studies have shown that RIC has neuroprotective effect which opens up broad prospects for using RIC in the treatment of cerebrovascular diseases. Studies of rodent hypoxia-ischemia models with immediate or delayed reperfusion (up to

24 hours) showed protective effects of RIC on the brains of adult and newborn animals [6]. RIC is regarded as a novel approach to limiting reperfusion injury in the ischemic region of the brain after focal ischemia [7] [8] [9] [10] [11]. Recently, RIC has been adopted in a wide range of ischemia-reperfusion experiments on various organs including kidneys, pancreas, liver, lungs, skin flaps, intestines and stomach [12].

Remote ischemic conditioning of limbs is a promising approach, as it can be used during or after initial ischemic stroke [13]. However, there are several issues which have not been fully clarified: optimal RIC protocol and mechanisms through which RIC protects the brain have not yet been discovered; there are no sensitive and specific RIC biomarkers; it is unclear whether RIC increases blood flow in the brain through collaterals after ischemia-reperfusion and whether it attenuates endothelial dysfunction through vascular regulation. All these pending issues prevent the introduction of RIC into clinical practice [14].

The novelty of this study is that syndecan-1 is suggested to be a marker of RIC and associated with improved outcome in acute stroke.

2. Materials and Methods

Forty male Wistar rats (180 - 235 g) were purchased from Laboratory Animal Nursery "Rappolovo". Animals were housed not more than 5 in a cage, with a 12-hour light/dark cycle and ad libitum access to food (standard rat chow K-120 provided by OOO Laboratorkorm, Russia) and water. The temperature was maintained at 22˚C - 25˚C, humidity—50% - 70%. Air ventilation and UV sterilization with a quartz lamp were carried out every day (the quartz lamp was switched on every 2 hours for 15 min).

Experimental animals were randomly divided into three groups:

1) Sham group (n = 10)

2) Model group (n = 15)

3) RIC group (n = 15)

Lethality was measured as a ratio of non-surviving animals to the total number of animals in a group 48 hours after the start of the experiment.

Left Middle Cerebral Artery Occlusion (LMCAO) was induced in the area of left middle cerebral artery with subsequent transient focal brain ischemia. Rats were anesthetized with chloral hydrate (450 mg/kg, i.p.). Surgical intervention was carried out on thermostatic operating table (TCAT-2LV, Physitemp Instruments Inc., Clifton, NJ, USA), and a rectal transducer was used to maintain core body temperature at 37.0˚C ± 1.0˚C. The animals underwent surgery in the neck area and received single intraperitoneal injection of 1 ml/kg normal saline. Intraluminal filament method was used for middle cerebral artery occlusion [5]. 4-cm Poly-L-Lysin-Coated nylon thread (size 0.34) was inserted into internal carotid artery. MCAO was maintained for 30 min., and then the thread was taken out from the vessel to allow reperfusion to the injured area. We measured regional cerebral flow (r CBF) in the area of the left hemisphere by high-frequency

ultrasonic Doppler. It was carried out using the Minimax Doppler-K (MM-D-K) NET series device (Minimax, Russia).

The sham group underwent surgery in the neck area and received single intraperitoneal injection of 1 ml/kg normal saline without being exposed to MCAO. During sham operation the same manipulations were performed, without artery clipping.

Remote ischemic conditioning protocol was conducted on the left low limb by inducing 5 min. of ischemia by means of a tourniquet, followed by 15 min. of reperfusion. This cycle was repeated 3 times within the first hour of reperfusion, then the cycle was repeated 2 more times with a six-hour interval (Figure 1). Brain ischemia was performed by means of MCAO for 30 min with subsequent low limb ischemia for 5 min and reperfusion for 15 min.

Neurological assessment was performed using Garcia behavioral test. Baseline assessments were conducted before the injury followed by Middle Cerebral Artery Occlusion 3 hours after the stroke and 24 hours after the stroke. The following results were observed: 1) spontaneous activity; 2) symmetry in the movement of four limbs; 3) forepaw outstretching; 4) climbing; 5) responses to body touch; 6) responses to touch of vibrissae [15].

2,3,5-triphenyltetrazolium chloride (TTC) was used to stain brain tissue to check the size of the ischemic injury area. Anesthetized rats were sacrificed 48 hours after reperfusion. The brains were harvested, cleaned, and solidified by immersing in pre-cooled normal saline (4˚C) and keeping in the refrigerator (−20˚C) for 30 min. Then six brain sections (2 mm thick) were prepared. The sections were incubated in 2% TTC (Sigma Aldrich, USA) phosphate buffer at 37˚C until the normal area turned red, while the ischemic injury area remained white; then the sections were fixed in 10% paraformaldehyde.

Brain infarct area was measured using a computerized image analysis system (Karl Storz, Austria) provided by Image-Pro program (MediaCybernetics, USA). The total infarct area of the brain was estimated by adding up infarct areas in all
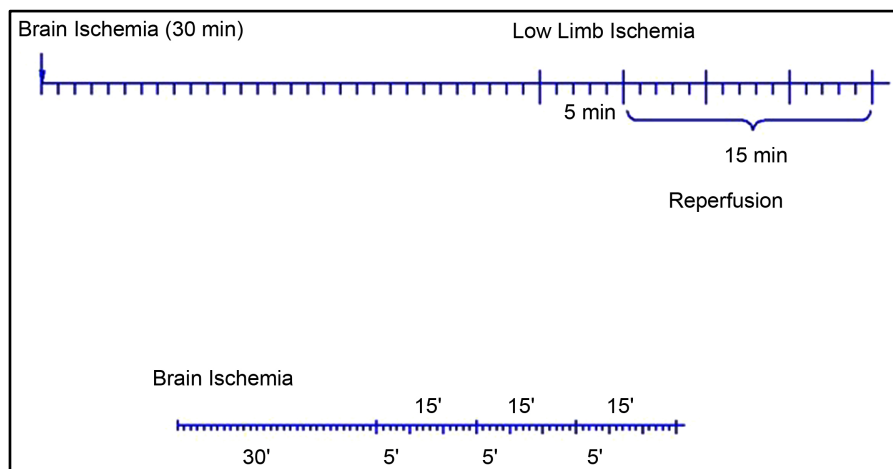

Figure 1. Brain ischemia was performed by means of MCAO for 30 min with subsequent low limb ischemia for 5 min and reperfusion for 15 min.

sections. The infarct volume percentage was calculated according to the formula:

$$\text{Infarct volume} (\%) = \left[\text{right brain infarct area} / (2 \times \text{left brain area}) \right] \times 100.$$

Forty-eight hours after reperfusion the rats were sacrificed, and their brains were removed. Olfactory bulb and brain stem were removed, and the total volume of hemispheres was determined based on the infarct volume calculation method [16]. Tissue swelling percentage was calculated using the following formula:

$$\text{Tissue swelling} (\%) = \left[V_{\text{Right Hemisphere}} - V_{\text{Left Hemisphere}} / V_{\text{Left Hemisphere}} \right] \times 100$$

All data are presented as mean ± SD. Statistical analysis of the data in different groups was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls method. Q value is the test statistics. P value < 0.05 was considered statistically significant [17].

Model success ratio = (total number of rats − rats that died after MCAO/R − rats with failure after MCAO/R)/total number of rats.

An enzyme immunoassay (ELISA, RayBiotech, USA) was used for quantification of plasma proteins ANXA5 and SDC-1; tablet spectrophotometer (Clariostar Plus, Germany) with 450 nm wave length was used for plasma proteins assay. Statistical validation of ELISA results was based on the binomial model. The data was verified for compliance with Gaussian distribution. Statistical significance of group alterations was evaluated using *t*-test (**P < 0.01).

3. Results

We induced middle cerebral artery occlusion for 30 min. 33 animals had complete data across stroke scale. Four animals had missing data of neurological assessment three hours after MCAO, and three animal died 24 hours after MCAO. Model success ratio = 0.825. Garcia scale showed statistically significant changes between the baseline and the 3-hour neurological analysis results in the Model group and RIC group (**P < 0.01. Table 1).

3.1. Evaluation of Brain Infarction

Infarct volumes were measured by staining brain sections with 2,3,5-triphenyltetrazolium chloride. TTC staining results showed no infarction areas in the sham group (Figure 2(a)). By contrast, cerebral infarct lesions were observed in the Model group with left MCAO and in the RIC group. Infarct volumes in the Model group (31.97% ± 2.5%) were significantly higher compared to the values

Table 1. Garcia mean stroke values ± SE by assessment time.

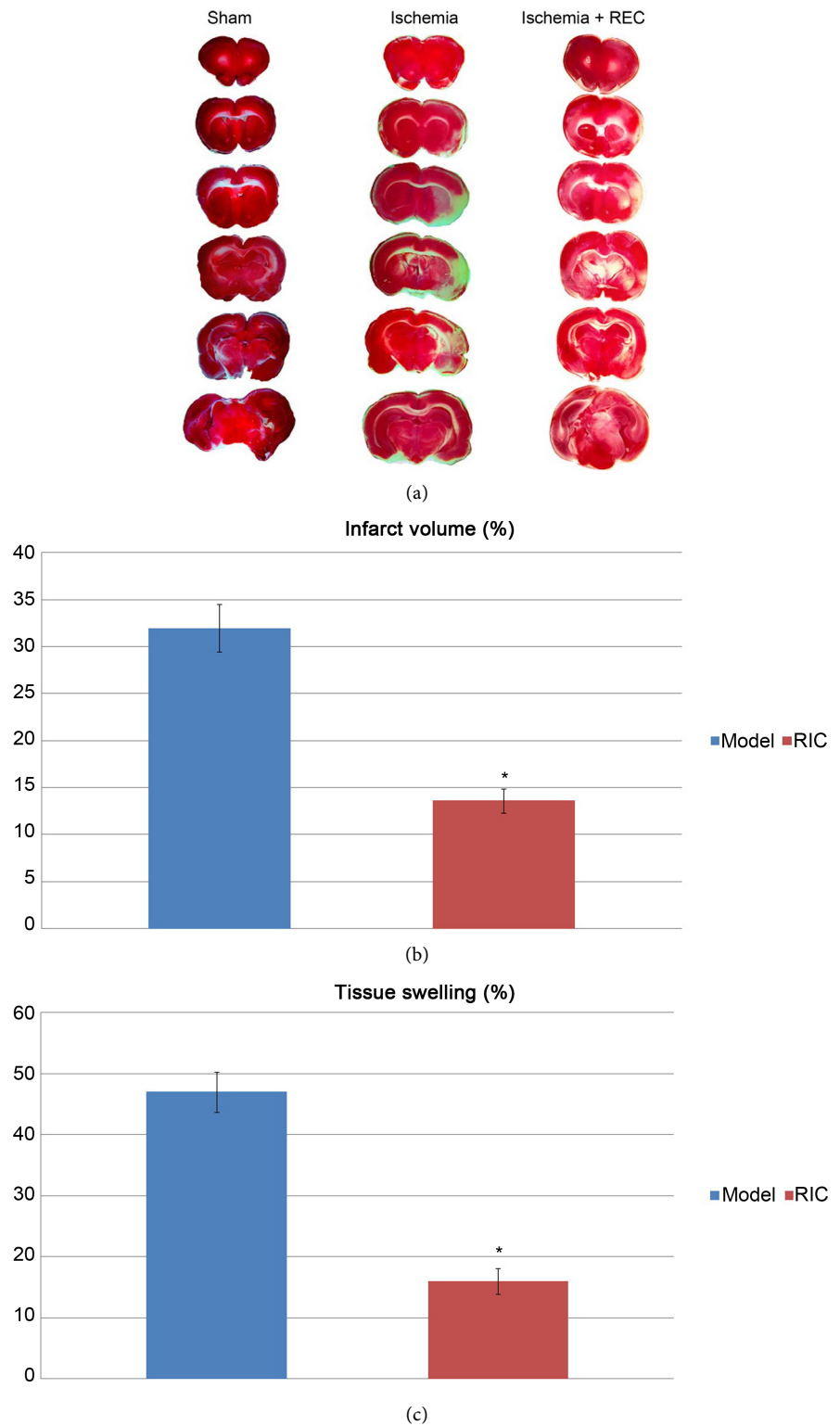| Group	Baseline	3 h post-MCAO	24 h post-MCAO
Sham group	15.00 ± 0.00	15.00 ± 0.00	15.00 ± 0.00
Model group	15.00 ± 0.00	10.7 ± 0.23**	13. 00 ± 0.25
RIC group	15.00 ± 0.00	11.5 ± 0.35**	12.57 ± 0.22

(a)

(b)

(c)

Figure 2. (a) Brain sections stained with triphenyltetrazolium chloride (TTC) 48 hours after neck surgery in the sham, Model and RIC group. Non-ischemic areas are colored deep red, ischemic areas are white. (n = 15). (b) Infarct area in the Model group (31.97% ± 2.5%) was significantly larger than in the RIC group (13.6% ± 1.3%). *P < 0.05. (c) Tissue swelling was reduced in the RIC group on the 2nd day after ischemia-reperfusion compared to the Model group. *P < 0.05.

in the RIC group 48 hours after ischemia-reperfusion (13.6% ± 1.3%) (*P < 0.05) (**Figure 2(b)**) Infarct area in the Model group (31.97% ± 2.5%) was significantly larger than in the RIC group (13.6% ± 1.3%)). A significant reduction in the area of infarction after RIC is likely due to its effect on the regulation of collateral blood flow in the ischemia area.

On the 2nd day after ischemia-reperfusion, tissue swelling was reduced in the RIC group (16% ± 2.1%) (*P < 0.05) compared to the Model group (47% ± 3.3%) (**Figure 2(c)**).

3.2. ELISA

Analysis of animal plasma samples after 30 min. of ischemia showed the following results of average concentration of annexin-5 molecules (ng/ml): 41.3 ± 0.9 (**P < 0.01) For animal plasma samples after 30 min. of ischemia and early remote ischemic conditioning, the result was (ng/ml): 42.1 ± 2.3 (**Figure 3(a)**).
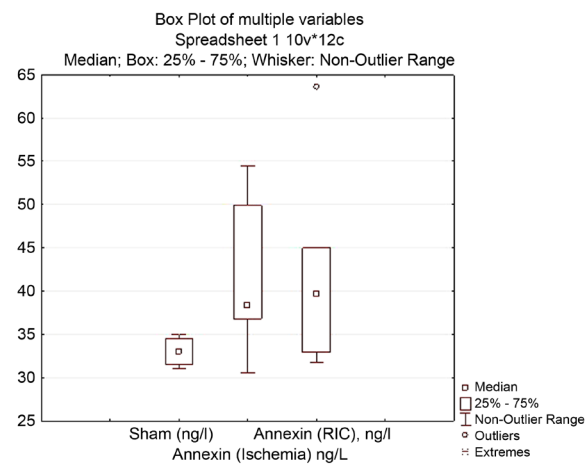
(a)
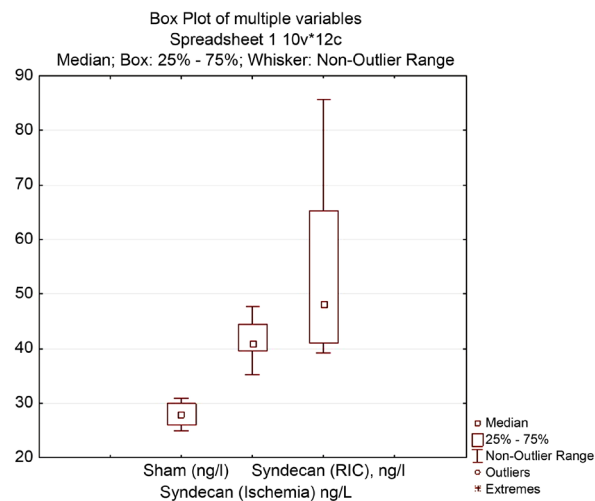
(b)

Figure 3. (a) Annexin-5 in animal plasma. Annexin-5 in the Model group (30 min. ischemia) and RIC group. (b) Syndecan-1 in animal plasma. Syndecan-1 in the Model group (30 min. ischemia) and RIC group.

Analysis of animal plasma samples after 30 min. of ischemia showed the following results of average concentration of syndecan-1 molecules (ng/ml): 41.4 ± 1.3. For animal plasma samples after 30 min. of ischemia and early remote ischemic conditioning, the concentration was (ng/ml): 54.9 ± 6.2 (**P < 0.01) (Figure 3(b)).

4. Discussion

As a result of remote ischemic conditioning, soluble factors appear in the blood, which are suspected to protect visceral cells from ischemic injury [18]. A large number of *in vivo* and *in vitro* studies suggest that there are several protective factors, including involvement protein kinase C, endothelial NO-synthase and other endothelial factors [19]. Evidences of peripheral release of adenosine in response to preconditioning stimulus and adenosine F2 receptor stimulation appear to exert inhibitory effect on platelet activation-aggregation [20].

Based on previous studies using the MCAO Model, transient focal brain ischemia was induced for 30 min. Focal transient ischemia in the middle cerebral artery basin can be accompanied by inflammatory process. We combined ischemia in the brain with remote ischemia in the low limb. Our findings revealed decrease of infarct area, brain swelling, neurological score. ELISA study detected syndecan-1 molecule increase in the RIC group. In the present study, we reveal the role of the Syndecan-1 molecule in remote ischemic conditioning effect. We anticipate that remote ischemic conditioning may trigger a short-term effect accompanied by a change in collateral blood flow and affect the Garcia test and infarct size. As a result of focal ischemia, the accumulation of free radicals leads to microvesicle shedding. Endothelial glycocalyx regulates not only vascular structure, but also the functions. Ischemia-reperfusion damages endothelial glycocalyx and initiates endothelial disruption. Cell membrane components that appear in plasma cause pro- and antithrombotic effects including syndecan family that has antithrombotic effect [21]. Syndecan-1, hyaluronic acid, heparan sulfate and other molecules of cell adhesion stimulate recruiting, activation and adhesion of leucocytes at the edges of inflammation region during ischemia-reperfusion [22]. Syndecan-1 molecule is a component of endothelial glycocalyx, and it is considered a marker of glycocalyx protection [23]. The presence of Annexin-5 in plasma is a sign of apoptosis [24] [25]. It has been demonstrated that glycocalyx at the apical surface of vascular endothelium is the first sensor of endothelial shear stress [26]. However, the results of other studies suggest that syndecan-1 is associated with adverse outcomes [27]. In addition, syndecan-1 is not only a biomarker of endothelial breakdown, but also a quantitative index for endotheliopathy of trauma [28]. Previous research of trauma confirms that RIC also induces shear stress and wall tension, which leads to endothelial breakdown in the remote organ. This causes endothelial dysfunction and, possibly, might become the source of coagulopathy and edema [28].

The present study has several limitations and caveats, such as small sample

size, which may preclude the possibility of arriving at a definitive conclusion. Additionally, because of the observational character of the study, it does not provide insight into cause and effect mechanisms, but simply points out associations that require additional research. Brain protection through RIC involves several mechanisms; using only syndecan-1 and annexin-5 represents a limitation in itself. However, both are biomarkers of endothelial cell injury, and several studies have demonstrated the role of syndecan-1 in endothelial damage and disruption, as well as successive downstream effects [29]. Tissue damage in the process of ischemia-reperfusion, when mass cell death occurs through necrosis and apoptosis in ischemic tissue, trigger cytoprotective mechanisms involving the synthesis of antiapoptotic and anti-inflammatory molecules by the components of histohematic barriers. In our experiment, short-term recurring episodes of distal limb ischemia were caused by significant increase of syndecan-1 in the blood plasma. The effects of endothelial responses of brain vessels in the ischemic area and of the vessels of remote organs are likely to sum up, which stimulates the establishment of intercellular contacts in the nervous tissue to a greater extent and eventually affects the size of the ischemic injury. Despite extensive research, exact mechanisms of capillary blood flow impairment triggered by microglial activity remain conjectural. Brain ischemia is followed by leaky endothelium with interstitial disturbances, vascular reactivity impairment, platelet clotting and capillary plugging with severe ischemia/hypoxia. We suggest that RIC effect on infarct size may be associated with reperfusion changes in the collateral blood flow.

5. Conclusions

1) Situations of ischemic-reperfusion tissue damage, when mass cell death occurs in ischemic tissue through necrosis and apoptosis, trigger cytoprotective mechanisms involving the synthesis of antiapoptotic and anti-inflammatory molecules by components of histohematic barriers. In our experiment, short-term recurring episodes of distal limb ischemia caused a significant increase of syndecan-1 in the blood plasma. Endothelial responses of brain vessels in the ischemic area and of the vessels of remote organs are likely to sum up, which stimulates the establishment of intercellular contacts in the nervous tissue to a greater extent and eventually affects the size of the ischemic injury.

2) Endogenous neuroplasticity mechanisms are the primary targets of post-stroke recovery treatment; the determination of the above components of nervous tissue is necessary to develop new methods of effective targeted stroke recovery therapy.

3) Syndecan-1, endothelial glycocalyx protein, might be the regulator which implements vascular control of interaction with inflammatory cell and is responsible for the mediate remote ischemic conditioning effect on the restriction of ischemia-reperfusion injury. In order to understand the neuroprotective properties of conditioning, further experimental studies are required.

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Ethical Consideration

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee.

Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

The following abbreviations are used in this article:

RIC: Remote Ischemic Conditioning

IP: Ischemic Postconditioning

MCAO: Middle Cerebral Artery Occlusion

TTC: Triphenyltetrazolium Chloride