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Neuroprotective Effect of Pharmacological Preconditioning with Dicholine Succinate in Experimental Ischemic Stroke in Rats

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Summary

Ischemic stroke is currently considered as one of the most pressing public health issues. Despite the differences in underlying mechanisms of ischemic and ischemic-reperfusion damage to the nervous tissue, the ultimate percentage of disability depends on intervention effects on the penumbra zone. Dicholine succinate (DCS), a neuronal insulin-sensitizer, is a promising pharmacological agent for management and prevention of stroke consequences.

The aim of the study was to investigate the effect of pharmacological preconditioning with DCS on brain cell death in experimental ischemic stroke in rats.

Materials and methods. Ischemic stroke in rats (*N*=16) was modeled by injecting the vasoconstrictor endothelin-1 (ET-1) into the striatum. The effect of pharmacological preconditioning with DCS as the active substance was evaluated by measuring the area of brain infarction in brain sections stained with cresyl violet. The effect of DCS on glycolysis and oxidative phosphorylation in primary cultures of rat cerebellum cells was assessed by measuring the rate of extracellular acidification and the rate of oxygen uptake, respectively.

Results. DCS administration in the preconditioning mode for 7 days, once a day orally, at a dose of 50 mg/kg, reduces the maximum area of the brain infarction zone by 34% ($P<0.05$) compared to the control in the subsequent experimental ischemic stroke induced by ET-1 administration. Three-day incubation of rat cerebellum primary culture with 50 μM DCS does not affect the basal levels of glycolysis ($P=0.916$) and cellular respiration ($P=0.8346$), but increases cellular glycolytic reserve by 70.0% ($P<0.0001$) compared to the control.

Conclusion. For the first time, the neuroprotective effect of pharmacological preconditioning with the neuronal insulin-sensitizer DCS in ischemic stroke has been shown. Mechanism of DCS action associates with an increase in the glycolytic reserve of brain cells, i.e., with increased ability of preconditioned cells to produce ATP and lactate via glycolysis in case of acutely compromised oxidative phosphorylation.

Keywords: ischemic stroke; pharmacological preconditioning; dicholine succinate; neuroprotection; endothelin-1; rats; glycolysis; oxidative phosphorylation

Conflict of interest. The authors declare no conflict of interest.

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Introduction

Ischemic stroke is the main cause of disability in Russia and remains the third leading cause of death and disability worldwide [1]. Compromised blood supply due to embolism, thrombosis, or constriction of cerebral arteries leads to hypoxia, decrease of ATP and phosphocreatine levels, anoxic depolarization of neuronal membranes, release of glutamate, and development of glutamate excitotoxicity. Increased production of reactive oxygen species and inflammatory response of microglia after clot dissolution causes brain cells death and expansion of necrotic zone [2]. Even though the primacy of protecting neurons in the acute phase of stroke is evident, there is still no pharmacological solution to this problem, and successful preclinical trials of more than 1,000 candidate substances failed to prove reliable neuroprotection in clinical practice [3, 4]. Some success in this area was achieved after discovery of ischemic preconditioning, demonstrating reduced cell death in ischemic incident after a brief episode of non-lethal ischemia [5, 6].

The protective effect of ischemic preconditioning includes adaptation of cell metabolism to hypoxia, in particular, switching to increased anaerobic glycolysis as a source of ATP and partial shutdown of energy-consuming processes [7]. Activation of hypoxia-inducible factor 1 (HIF-1), which regulates the transcription of more than 700 genes, including genes for erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes, and the glucose transporter GLUT1, plays a central role in adaptation to ischemia [8, 9]. Insulin is a known alternative activator of HIF-1 under normoxic conditions [10]. Both insulin and hypoxia induce the transcription of common target genes that collectively promote adaptation to hypoxia/ischemia, including EPO, VEGF, GLUT1 genes, and glycolytic enzymes [11–16]. Additionally, there is limited evidence of insulin preconditioning effect. For example, intracerebroventricular insulin administration reduced hippocampal CA3 neuronal death in Mongolian gerbils in a subsequent episode of transient cerebral ischemia compared to placebo [17].

Based on these findings, preconditioning with agents that improve insulin signaling in the brain may represent a new approach to protecting neurons in ischemic stroke.

Dicholin succinate (DCS) is a salt of choline and succinic acid (Fig. 1, *a*) displaying features of neuronal insulin-sensitizer with the ability to increase phosphorylation of the insulin receptor in neurons in response to low suboptimal concentrations of insulin [18]. DCS prevents cognitive decline in rats under experimental chronic cerebral hypo-perfusion [18, 19].

Pretreatment with DCS significantly reduces the depletion rate of ATP macroergic bonds and phosphocreatine in the brain during subsequent episodes of global ischemia, as demonstrated using ^{31}P NMR *in vivo* [20]. However, the question of whether neuronal preconditioning with DCS can reduce brain cell death in ischemic stroke remains open.

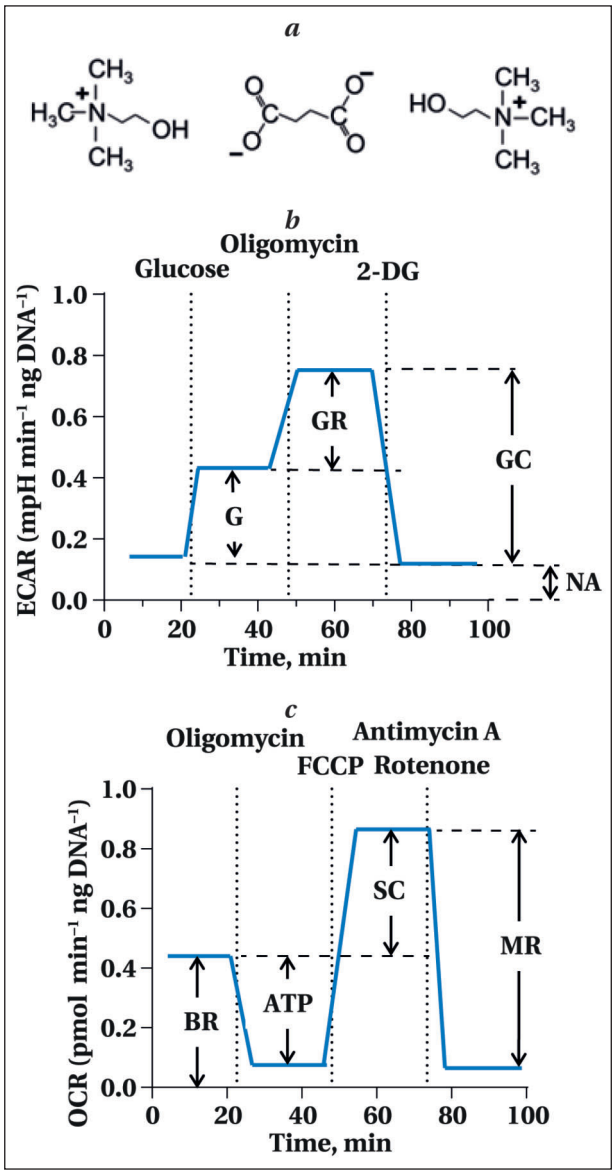

Fig. 1. Scheme for assessing the effect of DCS (*a*); on glycolysis indicators in the glycolytic stress test (*b*); and on oxidative metabolism indicators in the mitochondrial stress test (*c*).

The aim of this study was to investigate the effect of pharmacological preconditioning with DCS on brain cell death in experimental ischemic stroke in rats.

Material and Methods

Sigma-Aldrich (Merck, USA) materials were used in the study.

Modeling of ischemic stroke. Experiments were performed on male Wistar rats, weighing 200–250 g, obtained from the «Stolbovaya» branch

of the Federal State Budgetary Scientific Institution National Center for Biomedical Technologies (FSBSI NCBT) of the Federal Medical and Biological Agency (FMBA) of Russia, Moscow region. The animals were fed with standard granulated laboratory animal complete feed (extruded) PC-120 in accordance with GOST R 51849-2001 R.5. Tap water was provided ad libitum to all animals. The animals were kept in a controlled environment with air temperature of 18–22°C, a relative humidity of 60–70%, and indoor lighting with a 12/12 cycle.

The experiments were conducted in accordance with the «Principles of Good Laboratory Practice (GLP)», decree No. 202 of the Board of the Eurasian Economic Commission dated November 26, 2019, «On Approval of the Guidelines for Preclinical Safety Studies for Clinical Research and Drug Registration». All experiments were approved by the Bioethics Commission of the FSBSI NCBT, FMBA of Russia (Protocol No. 8 dated February 6, 2024).

16 animals were divided into two groups. Group 1 received normal saline (control), and Group 2 — DCS orally at a dose of 50 mg/kg in 1 ml of water through a gastric tube for 7 days before stroke induction. One day after the last administration of saline or DCS, rats were anesthetized with 2.0% isoflurane in a 70/30 volume ratio of nitrous oxide/oxygen, placed on a stereotactic frame, and maintained under 1–1.5% isoflurane anesthesia for the remainder of the procedure. The body temperature was monitored by a rectal probe, and normothermia was maintained with a heated blanket. A small hole was drilled in the skull. To induce ischemic brain damage, 1 µL of saline containing 25 pmol of endothelin-1 (ET-1) was injected into the left striatum for 2 minutes using a glass capillary needle (tip <50 µm). The following coordinates were used for stereotactic injections: anteriorly from the Bregma +1.0 mm, laterally +3.0 mm and +4.5 mm deep from the brain surface. 24 hours after ET-1 administration, rats were subjected to deep anesthesia and transcardial perfusion with heparinized saline followed by fixation with formalin solution. The brain was quickly extracted and frozen in isopentane cooled on dry ice. The striatum was divided into sections on a cryostat (20 µm thick sections) at 80 µm intervals. All sections were stained with cresyl violet. The area of the largest infarction zone was recorded.

Cerebellum cell culture. The cerebellum of 5–7 day-old Wistar rats was placed in a cold HBSS solution v/v with Ca^{2+}/Mg^{2+} and 1 mM sodium pyruvate, 10 mM Hepes, and was minced and incubated in a 0.5% trypsin-HBSS solution v/v Ca^{2+}/Mg^{2+} at 37°C for 15 minutes. The resulting cell suspension was washed twice with a cold HBSS solution and centrifuged at 1700 rpm for 3 minutes at +4°C. The precipitate was resuspended in standard Neurobasal Medium supplemented with B-27 Supplement (50X), 2 mM GlutaMax, 20 mM KCl, 100 units/mL of penicillin, and 100 µ/mL of streptomycin. Cells were seeded at a concentration of 1×10^5 cells per well in a standard 24-well Seahorse XF 24 plate pre-coated with polyethyleneimine, and cultivated at 37°C in the presence of 5% CO_2. Starting from the 7th day of incubation, 50 µM DCS or buffer (control) was added to the samples of the study group once a day for three consecutive days. 24 hours after the last DCS or buffer supplementation, cell metabolic activity was analyzed using the Seahorse XF 24 analyzer (Agilent Technologies) according to the manufacturer's instructions.

Assessment of metabolic activity. A standard protocol for the Seahorse glycolytic stress test (Aglient technologies) was used to assess the glycolytic activity of cells. The cells were previously washed twice with 500 µl Ng buffer (pH 7.4, 0.4 mM NaH_2PO_4, 3.5 mM KCl, 120 mM NaCl, 5 mM HEPES, GlutaMAX, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM sodium pyruvate), then incubated with 500 µl Ng buffer for 40 minutes in a thermostat at 37°C without CO_2, after which the extracellular acidification rate (ECAR) was measured in accordance with the manufacturer's instructions. The basal level of glycolysis (G), glycolytic capacity (GC), glycolytic reserve (GR), and non-glycolytic acidification (NA) were measured by the sequential addition of glucose, oligomycin, and 2-deoxyglucose (2-DG), as shown in Fig. 1, *b*.

To assess oxidative phosphorylation, we used the standard mitochondrial stress test protocol (Seahorse Mito-stress test, Agilent technologies). The cells were previously washed twice with 500 µl Nm buffer (pH 7.4, 0.4 mM NaH_2PO_4, 3.5 mM KCl, 120 mM NaCl, 5 mM HEPES, GlutaMAX, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose), then incubated with 500 µl Nm buffer for 40 minutes in a thermostat at 37°C without CO_2, after which the oxygen consumption rate (OCR) was measured in accordance with the manufacturer's instructions. The basal respiratory rate (BR, basal respiration), maximal respiratory rate (MR, maximal respiration), ATP production (ATP), and spare capacity (SC) were measured by the sequential addition of oligomycin, protonophore FCCP, and antimycin A with rotenone, as shown in Fig. 1, *c*.

OCR and ECAR data were normalized by DNA. To do this, DNA was isolated from cells using the standard protocol the ReliaPrep™ gDNA Tissue (Promega) and quantified using the QuantiFluor® dsDNA fluorescent staining kit (Promega).

Statistical analysis was performed using the Student's unpaired two-way *t*-test, two-way analysis of variance (two-way ANOVA) with a posteriori Sidak's test for multiple comparisons between groups using GraphPad Prism v.8.3.0 software (San Diego, USA). The Shapiro–Wilk test was used to select parametric or nonparametric methods of statistical

analysis. The following symbols were used: *M* — mean, *m* — standard error, *n* — sample size, and *P* — achieved significance level. Differences were considered statistically significant at $P<0.05$.

Results

In order to find out whether pharmacological preconditioning using DCS as an active substance could affect the size of ischemic lesion in the brain after a subsequent episode of ischemia, male Wistar rats were administered DCS or saline orally once a day for 7 days. Ischemic stroke was induced by injection of a vasoconstrictor ET-1 into the striatum in 24 hours after the last administration of solutions. The infarct/ischemic lesion size was measured in brain sections obtained 24 hours after stroke induction (Fig. 2). Comparison of average values of greatest infarct sizes showed its' significant reduction by 34% after preconditioning with DCS ($P<0.05$) compared to the control.

Taken together, these results suggest that preconditioning with DCS may reduce brain cell death during subsequent episodes of ischemia.

In order to clarify whether the DCS neuroprotective effect is related to its effect on metabolism and, in particular, to glycolysis in brain cells, the primary culture of rat cerebellum cells was incubated for 3 days with added DCS or without it (control), after which extracellular acidification rate (ECAR) was measured in the presence of different additives (Fig. 3, *a*).

Glycolysis indicators expressed in ECAR units, such as the basic level of glycolysis (G), glycolytic capacity (GC), glycolytic reserve (GR) and non-glycolytic acidification of the medium (NA) are shown in Fig. 3, *b*. Two-way ANOVA revealed the presence of statistically significant differences between the groups by «glycolysis index» factor ($F_{3.157}=157.0$; $P<0.0001$), and the «DCS/control» factor ($F_{1.157}=52.28$; $P<0.0001$). A posteriori Sidak's test showed that DCS significantly increased glycolytic cell capacity by 50.5% ($P<0.0001$) and glycolytic reserve by 70.0% ($P<0.0001$) compared to the control, but did not affect basal glycolysis levels ($P=0.916$) or non-glycolytic acidification ($P=0.699$).

In order to find out whether DCS affects oxidative phosphorylation in brain cells, a primary culture of rat cerebellum cells was incubated for 3 days in the presence of DCS or without it (control), and then the oxygen consumption rate (OCR) was measured in the presence of the additives (Fig. 3, *b*). The oxidative metabolism parameters, such as the basal respiratory rate (BR), maximum respiratory rate (MR), ATP production (ATP), and spare respiratory capacity (SC), expressed in terms of OCR, are presented in Fig. 3, *c*. Two-way ANOVA revealed statistically significant differences between the groups in terms of the «oxidative metabolism index» ($F_{3,208}=249.0$; $P<0.0001$) and the «DCS/control» ($F_{1,208}=17.28$; $P<0.0001$). A posteriori Sidak's test showed that DCS significantly reduced the maximum respiratory rate by 19.4% ($P=0.0006$) and the spare respiratory capacity by 18.7% ($P=0.014$) compared to the control, but did

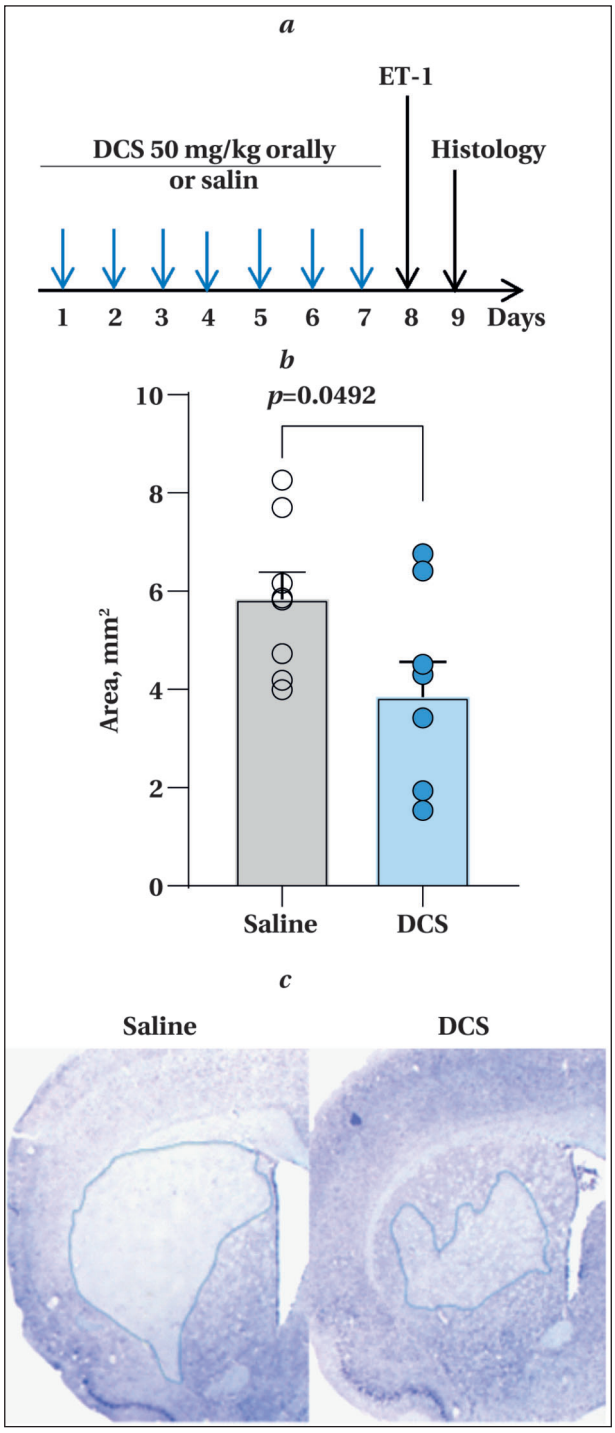

Fig. 2. The effects of pharmacological preconditioning using DCS as an active substance on the area and volume of the brain infarction zone in rats with stroke induced by ET-1 vasoconstrictor.

Note: *a* — experiment schedule; *b* — average areas of the maximum infarct zone, mm²; *c* — representative images of histological samples, stained with cresyl violet. The results were presented as *M*±*m* (*N*=8). * — $P<0.05$.

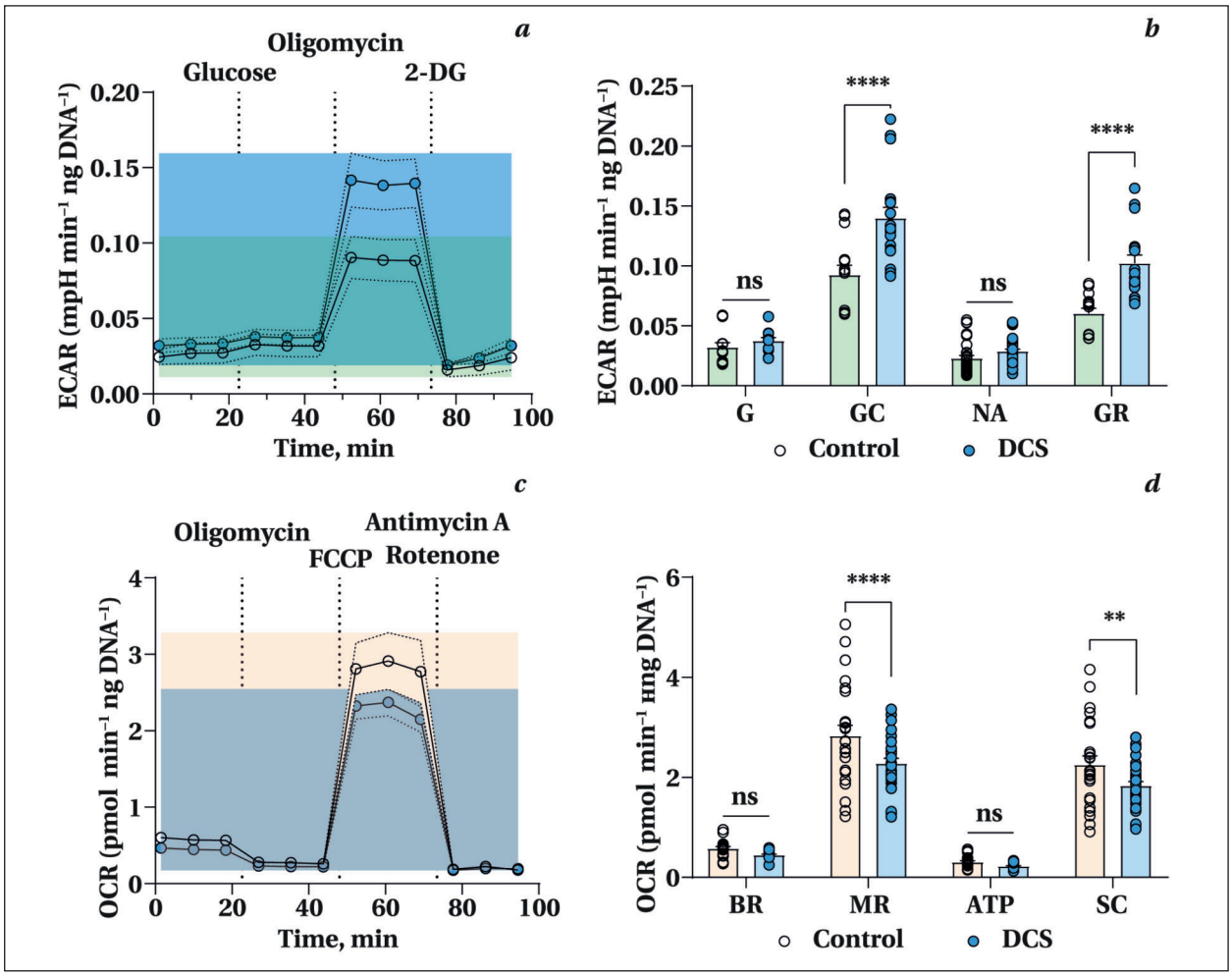

Fig. 3. DCS effect on glycolysis and oxidative metabolism in primary cultures of rat cerebellum cells, as analyzed by Seahorse.
Note. *a* — extracellular acidification rate (ECAR) in the presence of glucose, oligomycin, and 2-deoxyglucose (2-DG) additives; *b* — indicators of glycolysis: basal glycolysis level (G), glycolytic capacity (GC), glycolytic reserve (GR), and non-glycolytic acidification of the medium (NA); *c* — oxygen consumption rate (OCR) of cells in the presence of oligomycin, FCCP protonophore, and also rotenone and antimycin A, inhibitors of mitochondrial complexes I and III, respectively; *d* — indicators of oxidative metabolism – basal respiratory rate (BR), maximal respiratory rate (MR), ATP production (ATP) and spare capacity (SC). The results were presented as $M \pm m$ (N=15–30). ** — $P<0.01$; **** — $P<0.0001$; ns — not significant.

not affect the basal respiratory rate (P=0.8346) or ATP production (P=0.9596).

Taken together, these results show that pharmacological preconditioning using DCS builds up brain cells capacity to increase the rate of anaerobic conversion of glucose to lactate in milieu of acute oxidative phosphorylation deficiency, without affecting the baseline levels of glycolysis and oxidative metabolism.

Discussion

Pharmacological preconditioning is considered as an alternative to hypoxic preconditioning approach to protect brain cells under conditions of ischemia. As candidates for the role of pharmacological agents with a similar effect, compounds of different classes were investigated, including erythropoietin growth factor, volatile anesthetics (isoflurane), mitochondrial ATP-sensitive selective potassium channel opener diazoxide, iron chelator deferoxamine, opioids [21] and insulin [17]. In this study, it was shown that agents that improve insulin sensitivity (insulin sensitizers) can also be considered as potential neuroprotectors when used for preconditioning.

Dicholine succinate, a neuronal insulin sensitizer, is used as an active ingredient in a drug for treatment of ischemic stroke in the early recovery period [22, 23]. In this study, we demonstrated for the first time, that DCS administration to healthy animals for preconditioning was an effective way to reduce the size of brain infarct after subsequent episode of acute cerebrovascular accident.

The mechanism of DCS's preconditioning outcomes may be related to its metabolic effects. Although DCS did not affect the baseline levels of glycolysis and oxidative phosphorylation in the primary cell culture, it significantly increased the gly-

colytic reserve by 70%, which is the ability of cells to produce ATP after abrupt decrease in oxidative metabolism.

This effect appears to underlie the neuroprotective action of DCS and explains the results of one early study, in which administration of DCS to Wistar rats in the same preconditioning regimen significantly slowed down the decline rate of ATP and phosphocreatine levels in the brain during a subsequent episode of global ischemia induced by cardiac arrest [19]. In addition, DCS protective effect may be related to increased ability of cells to produce lactate, as lactate has neuroprotective properties in cerebral ischemia [24–27].

The decrease in maximum respiratory rates in preconditioned brain cells by an average of 19.4% can also be attributed to the protective effects of DCS, as the emergence of active oxygen metabolites, recognized as damaging factors in stroke [28], is directly related to increased oxygen consumption during the reperfusion phase.

Conclusion

For the first time, the results demonstrated neuroprotective effect of pharmacological preconditioning with the neural insulin-sensitizer dicholine succinate in ischemic stroke. Data show that DCS, when administered preventively, reduces the size of brain infarct in rats in a subsequent episode of ischemia, provoked by injection of vasoconstrictor endothelin-1 into the striatum. Mechanism of DCS action associates with an increase in the glycolytic reserve of brain cells, i. e., an increase in the ability of DCS-preconditioned cells to enhance production of ATP and lactate via glycolysis after abrupt reduction of oxidative phosphorylation.

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