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Research Article

Activity of ROS-induced processes in the combined preconditioning with amtizol before and after cerebral ischemia in rats

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Abstract

Introduction: The dose-dependent effect of reactive oxygen species (ROS) in tissues in preconditioning (PreC) and oxidative stress, as well as NO-synthase participation in mitochondrial ROS production determined the study aim – to assess the impact of the neuroprotective method of combined preconditioning (CPreC) on free radical reactions (FRRs) in brain in normoxia and in cerebral ischemia, including in NO-synthase blockade.

Materials and methods: The intensity of FRR by iron-induced chemiluminescence (CL), the content of lipid peroxidation products and antioxidant enzyme activity were investigated 1 hr (early period) and 48 hrs (delayed period) after CPreC (amtizol and hypobaric hypoxia) in Wistar rat brain. Some animal groups were operated (common carotid artery bilateral ligation) 1 hr and 48 hrs after CPreC, as well as with preliminary introduction of L-NAME and aminoguanidine.

Results and discussion: In normoxia, CPreC led to increase the CL maximum level (Fmax) in the delayed PreC period. The amount of thiobarbituric acid reactive products (TBA-RP), activity of superoxide dismutase (SOD) and catalase in mitochondrial fraction of rat brain did not change in comparison with the intact control in both PreC periods. In cerebral ischemia, oxidative stress was observed. The CPreC use before ischemia caused a decrease in CL parameters and TBA-RP in brain, the maintenance of SOD and high catalase activity. NO-synthase inhibitors partially abolished the antioxidant effect of CPreC in ischemia.

Conclusion: CPreC had no influence on FRRs in brain tissue in normoxia, but prevented their excessive activation after ischemia, especially in the delayed period. NO-synthase was involved in the CPreC neuroprotection.

Keywords

reactive oxygen species (ROS), amtizol, hypoxic preconditioning, chemiluminescence, TBA-RP, superoxide dismutase (SOD), catalase, NO-synthase inhibitors.

Introduction

Cerebral ischemia continues to be the leading cause of disability and mortality among the human population. Preconditioning (PreC) is considered as one of the promising neuroprotective approaches to increase an organism resistance to ischemia (Koch and Gonzalez 2013). PreC is a potent mobilization of the adaptive abilities of

the organism pre-exposed to such factors as short-term and repeated ischemia or hypoxia episodes, which can be combined with pharmacological agents (Danilenko et al. 2015, Shabelnikova et al. 2016, Yang et al. 2017, Korokin et al. 2019, Novikov et al. 2020a). It is important that there are two protective periods in PreC with differing mechanisms of their development: the early PreC period (from several min to 2 hrs) and the delayed PreC period (occurs after 24 hrs, lasts 48–72 hrs) (Shlyakhto et al. 2012). Reactive oxygen species (ROS) play an important role in the neuroprotection, including in PreC.

ROS in tissues are dose-dependent and can play the role of triggers and signaling molecules in the PreC phenomenon development or, conversely, can cause oxidative stress (OxS) in ischemic damage (Pozhilova et al. 2015, Krylatov et al. 2018). In low doses, ROS can perform a signaling function in biochemical cascades and promote the activation of the certain signaling pathways for the PreC development (Lukyanova and Kirova 2015, Sementsov et al. 2019). Low doses of ROS produced in mitochondria under the influence of PreC can suppress prolylhydroxylase activity and stabilize HIF-1α, a subunit of the transcription factor HIF-1, the main factor of the cellular response to hypoxia (Shlyakhto et al. 2012, Li et al. 2019, Mesa-Ciller et al. 2019, Semenza 2020). Complete inhibition of ROS production blocks the formation of tolerance to ischemia and hypoxia. The influence of PreC on the activity of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, is possible (Shlyakhto et al. 2012, Rybnikova and Samoilov 2015, Chang et al. 2019, Palma et al. 2020).

High doses of ROS, including those formed in mitochondrial enzyme complexes (MECs) of the respiratory chain, play a key role in the development of ischemic damage, structural and functional disorder of cell membranes and apoptosis initiation (Dröse 2013, Kalogeris et al. 2014). OxS is one of the most important pathophysiological mechanisms of brain cell damage and represents a degree of ischemic damage (Devyatov et al. 2017, Silachev et al. 2018, Rodriguez et al. 2021).

In previous studies, we already demonstrated the neuroprotective effect of combined PreC, where amtizol potentiated the hypoxic action of PreC (Levchenkova et al. 2016a, Novikov et al. 2019). Amtizol is known for its ability to increase an organism resistance to oxygen deficiency and induce adaptive changes, similar to immediate adaptation to hypoxia. In addition, it is characterized by the antioxidant activity. Amtizol demonstrated an antiradical activity in *in vitro* tests: it inhibited adrenaline autooxidation, supressed ascorbate-dependent lipid peroxidation in the metabolizing model system in brain and liposomes (Zarubina and Shabanov 2004). The antioxidant activity is obviously useful in the formation of an organism resistance to subsequent ischemia (Silachev et al. 2018), but in the context of PreC, on the contrary, it can invalidate one of the pathways of the PreC development mechanism (Galagudza et al. 2016).

The activity of the redox-sensitive signal transducer involved in the PreC development – nitric oxide synthase (NOS) – is associated with the ROS production (Barua et al. 2010, Chen et al. 2017, Tsibulnikov et al. 2019, Wang et al. 2020). Neuronal nNOS was revealed among NOS isoforms in the inner mitochondrial membrane. It is represented most in the cerebral mitochondria and is involved in reversible inhibition of MEC IV, functionally associated with MEC I of the respiratory chain (O-Uchi et al. 2014, Sakamuri et al. 2020).

The study was aimed at the investigation of the influence of the combined PreC with amtizol on the iron-induced chemiluminescence, content of thiobarbituric acid reactive products (TBA-RP), activity of antioxidant enzymes – superoxide dismutase and catalase in the cerebral mitochondrial fraction in normoxia and after ischemia, and to evaluate the effect of the combined PreC in ischemia in case of non-selective and selective NOS blockade.

Materials and methods

Animals

All the experiments were conducted with 150 white Wistar male rats, weighing 190–250 g each, obtained from the Stolbovaya Laboratory Animal Nursery (Moscow region, Russia). The rats were allowed free access to water and standard rat chow, with natural dark-light conditions. All the studies were performed in accordance with the rules and international recommendations of The European Convention for the Protection of Vertebrate Animals Used in experimental Studies (1997) and the "Rules of Good Laboratory Practice" (The Order of the Ministry of Health of RF №199n dated 01.04.2016).

Preconditioning method

The combined PreC was modeled by the alternate use of amtizol and moderate hypobaric hypoxia (HBH) for 6 days (Levchenkova et al. 2016a). On the first, third and fifth day of the experiment, the animals were injected intraperitoneally with amtizol (3,5-diamino-1,2,4-thiadiazole) in a dose of 25 mg/kg (the substance was synthesized in the Department of Pharmacology of the Military Medical Academy named after S.M. Kirov, St. Petersburg). On the second, fourth, and sixth day, moderate hypobaric hypoxia was performed by putting the rats under a glass dome, from where the air was pumped out through a Komovsky pump to the level of to 410 mm Hg, which was equal to the altitude of 5.000 meters (HBH-5000), the exposure was 60 min (Fig. 1).

Study design

The brains were removed after immediate decapitation of the rats. It was performed 1 hr and 48 hrs after the last episode of PreC in the experimental groups without ischemia

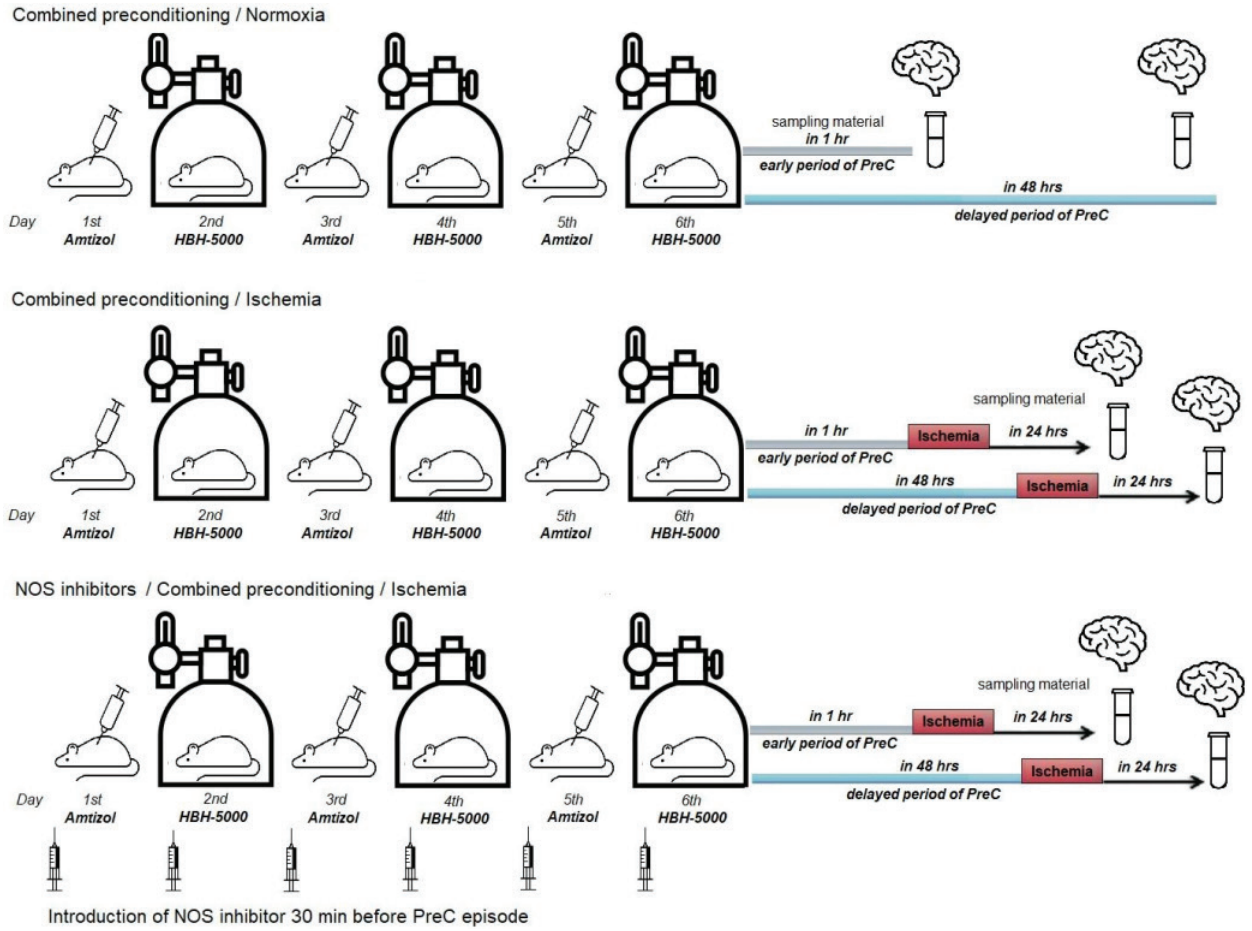

Figure 1. Schematic representation of experimental groups (experiment design).

and one day after the surgery in groups with ischemia (Novikov et al. 2020b).

Cerebral ischemia in rats was modeled by one-stage bilateral common carotid artery ligation (CCAL) under anesthesia (8% chloral hydrate solution in a dose of 400 mg/kg intraperitoneally). As the control for ischemic animals, the sham-operated rats were used, which had undergone all the stages of the operation without CCAL. The surgery was modeled 1 hr (early period) and 48 hrs (delayed period) after the cessation of combined PreC.

Activity of ROS-induced processes was evaluated in the rat brain homogenate supernatant by the iron-induced chemiluminescence (CL) on a Dialog 3606 luminometer, using standard CL3603 software. The following values were registered: Fmax, the chemiluminescence peak amplitude induced by hydrogen peroxide, characterizing the content of the substrate ready to enter immediately into free radical reactions, i.e. the intensity of FRRs in the the test sample; and the sum of luminescence light (the area under the CL curve), showing the ratio of the intensity of ROS production and the participation of the antioxidant system in the substrate (Levchenkova et al. 2016b). Lipid peroxidation (LPO) activity was assessed by the content of thiobarbituric acid reactive products (TBA-RP) in the mitochondrial fraction obtained from brain tissue by differential centrifugation, for which we used diagnostic kits for quantitative determination of TBA-RP manufactured by Agat-Med Company (Russia). Optical density units were calculated according to the manufacturer's formula, then expressed in terms of the protein amount in the sample of the mitochondrial suspension.

The total activity of superoxide dismutase (SOD) was measured using the method for assessing a degree of inhibiting the quercetin oxidation reaction (Tyurenkov et al. 2017). The SOD activity was determined based on a degree of quercetin oxidation inhibition (%) when constructing the calibration curve. Then the SOD activity in the mitochondrial fraction was expressed in terms of the protein amount in the sample, determined by the Lowry method.

Catalase was evaluated by the method based on the formation of a color complex of hydrogen peroxide (H_2O_2) and ammonium molybdate. The optical density of the samples was determined spectrophotometrically at a wavelength of $\lambda = 410$ nm in cuvets with an optical path length of 10 mm against water. The catalase activity in the cerebral mitochondrial fraction was expressed in nmol of H_2O_2/min/mg of protein, determining the protein in the samples by the Lowry method (Tyurenkov et al. 2017).

Besides, there was a series of experiments conducted in which the contents of TBA-RP, SOD and catalase were estimated with prior inhibiting NOS (Fig. 1). The blockade of NO-synthases was carried out using

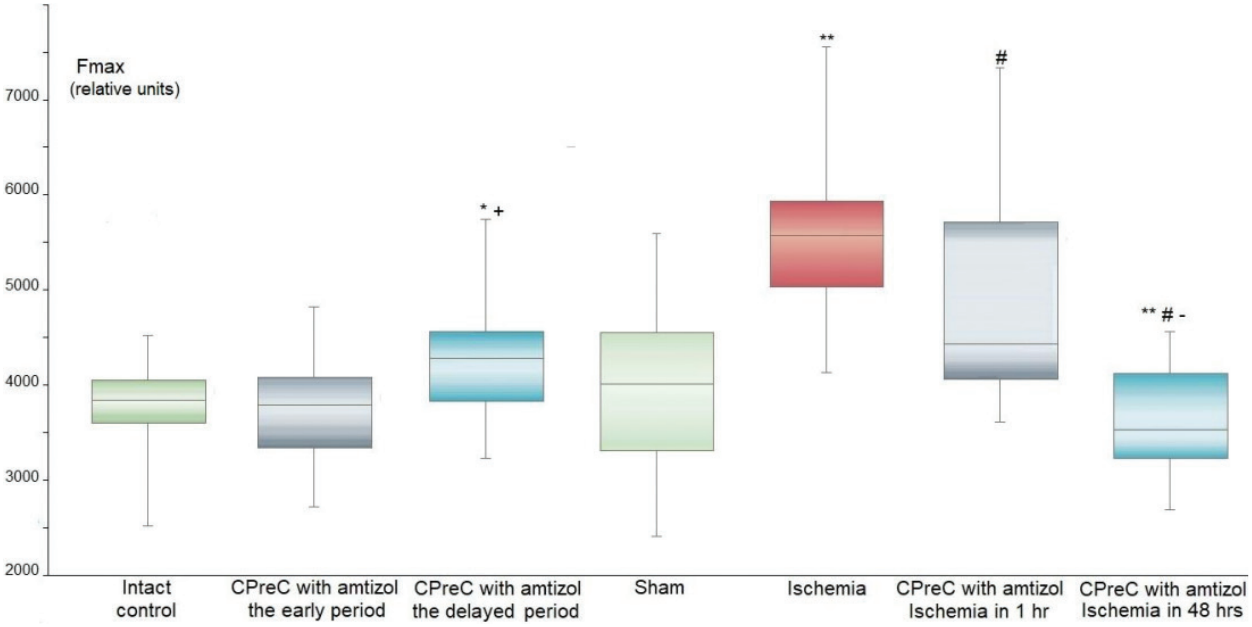

Figure 2. The hydrogen-peroxide-induced chemiluminescence peak amplitude under the influence of the combined preconditioning before and after cerebral ischemia in rats (n = 56). **Note:** The data are presented as: median, Q1-Q3 quartiles (box and whisker plot) with min and max whiskers. The differences were significant ($p \leq 0.05$, Mann-Whitney U-test) in comparison with: * – intact group, ** – sham-operated animals (Sham), # – Ischemia, +/- – significantly more or less compared to the appropriate CPreC group in the early period.

intraperitoneal injection 30 min before each preconditioning factor: a nonselective inhibitor of NOS – methyl ester of N-nitro-L-arginine (L-NAME, Sigma, USA) in dose of 10 mg/kg or the selective inhibitor of inducible NOS – aminoguanidine (Sigma, USA) in dose of 50 mg/kg (Tyurenkov et al. 2017).

Statistical analysis

The statistical analysis of the results obtained was performed using a Stat Plus Pro 7.0.1.0 software package. The differences among the groups were statistically tested by the Mann-Whitney U-test. Statistical significance was assumed at $p \leq 0.05$. The data were presented as a median (Me) with Q1 and Q3 quartiles (interquartile range).

Results and discussion

The results of activity of ROS-induced processes according to the iron-induced CL data in the brain tissue in case of using the combined PreC are presented in Figures 2 and 3. It follows from Fig. 2 that in normoxia, the value of CL peak (Fmax) in the combined PreC group with amtizol in the early period of PreC did not differ from that of the intact control (p=0.828; U=298). There were no differences in the value of the light sum (p=0.544; U=61.5) between this group and the intact control (Fig. 3).

In the combined PreC group with amtizol in the delayed period of PreC, Fmax was significantly higher in comparison with that in the intact control (p=0.007; U=157), as well as in comparison with that in the early period of PreC (p=0.046; U=37.5), which indicates an increase in the lipid substrates ready to enter into free radical reactions under the CPreC action (especially in the delayed period). At the same time, there were no differences between this group and the intact control in terms of the light sum value (p=0.260; U=52.5) in the brain tissue that is indicative of the balance between the pro- and antioxidant systems in the brain tissue (Fig. 3).

In the shame-operated animals in comparison with the intact control, there were no differences in the Fmax values (p=0.116; U=128) and the light sum values (p=0.942; U=130), which indicates that there were no signs of oxidative stress a day after the shame operation. The pronounced intensification of ROS-induced processes in brain tissue in form of an increase in Fmax value ($p < 0.001$; U = 1490) and the light sum ($p < 0.001$; U = 283) in comparison with sham-operated animals was observed in case of performing CCAL.

The positive effect on ROS-induced processes was noted in the experimental groups with preliminary combined PreC before modeling cerebral ischemia. Fmax was lower in the group in which ischemia had been simulated in the early PreC period in comparison with the Ischemia control group ($p = 0.019$; U = 294), showing no difference from the values of the Sham animals ($p = 0.136$; U = 241). At the same time, the light sum value did not reach that of the Sham group ($p = 0.027$; U = 193), but was significantly lower in comparison with that in the Ischemia group ($p = 0.001$; U = 19). In the group of the delayed PreC period, Fmax was lower than both the values of the Ischemia group ($p < 0.001$; U = 25.5) and the values of the Sham group ($p = 0.049$; U = 365), as well as the group of the early PreC period ($p < 0.001$; U = 493.5). In addition, the light sum value was lower than that in the Ischemia group

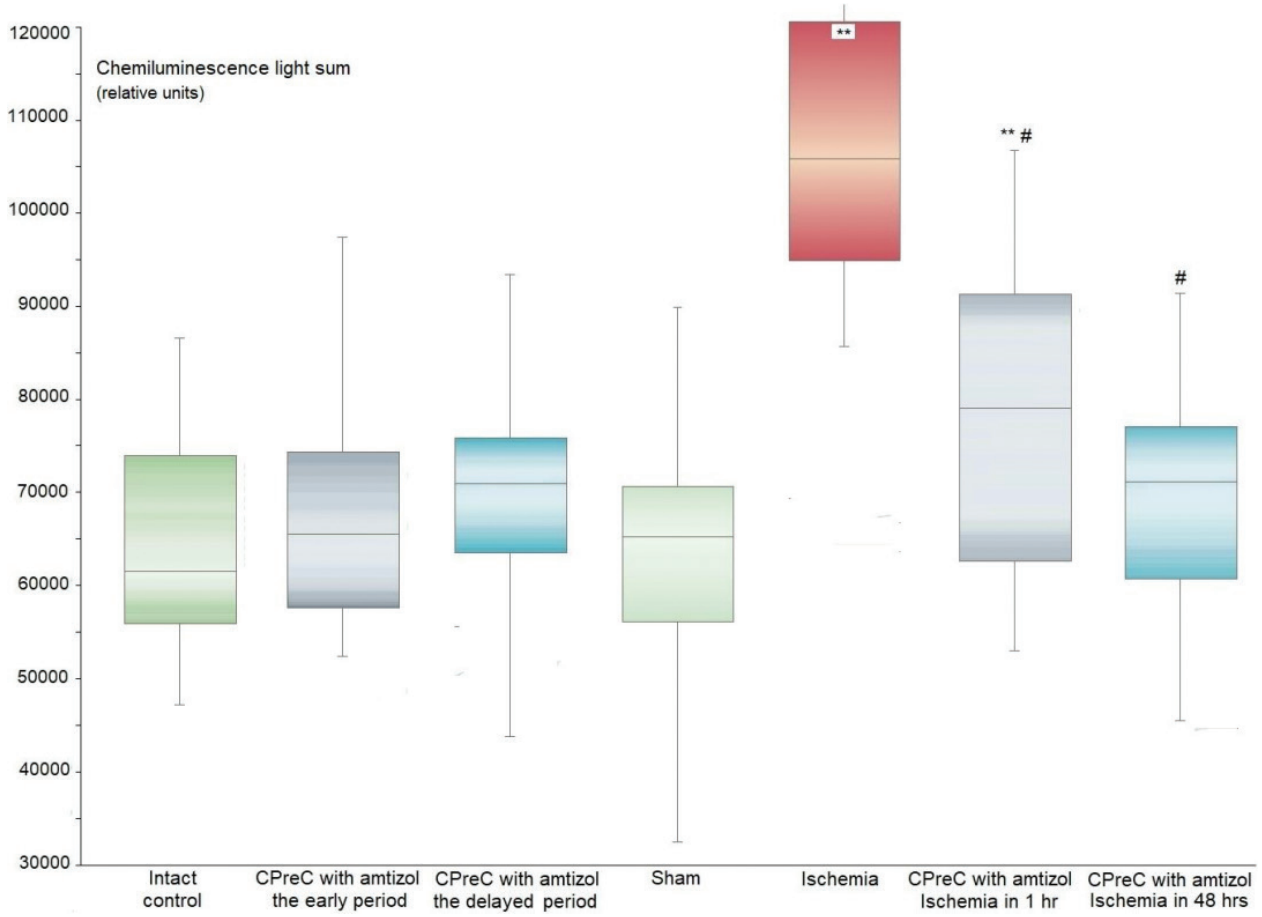

Figure 3. The chemiluminescence light sum under influence of the combined preconditioning before and after cerebral ischemia in rats (n = 56). **Note:** The data are presented as: median, Q1-Q3 quartiles (box and whisker plot) with min and max whiskers. The differences were significant ($p \leq 0.05$, Mann-Whitney U-test) in comparison with: ** – sham-operated animals (Sham), # – Ischemia.

($p < 0.001$; $U = 3$), while showing no difference from the values of the Sham group ($p = 0.159$; $U = 171$) and the group of the early PreC ($p = 0.225$; $U = 93$).

The levels of TBA-RP content under the combined PreC action with amtizol in normoxia and after modeling ischemia are shown in Table 1. In the mitochondrial fraction of rat brain in the groups of combined PreC with amtizol with normoxia, both in the early and delayed periods of PreC, the content of TBA-RP did not significantly differ from the values of the intact control ($p = 0.521$; $U = 14$ and $p = 0.200$; $U = 10$, respectively), nor were there any differences in the content of TBA-RP between the intact control and the Sham animals ($p = 0.200$; $U = 10$). Cerebral ischemia led to the activation of LPO processes, which was expressed in a 61.5% increase in the TBA-RP content in the mitochondrial fraction of the animal brain ($p = 0.001$; $U = 0$) a day after the surgery compared to that in the Sham. When using the CPreC with amtizol before ischemia, both in the early and delayed periods of PreC, the TBA-RP content was significantly lower than that in the Ischemia by 29.4% and 34.8%, respectively ($p = 0.001$; $U = 0$ for both periods), showing no difference from those in the Sham values ($p = 0.200$; $U = 10$ for the early period of PreC and $p = 0.872$; $U = 17$ for the delayed period of PreC).

Table 1. Effect of the Combined Preconditioning on TBA-RP and the Activity of Antioxidant Enzymes in the Brain Mitochondrial Fraction in Normoxia and Cerebral Ischemia

Experimental groups (n = 60)	Brain mitochondria		
	TBA-RP mcmol/ mg of protein	SOD units/mg of protein	Catalase nmol of H_2O_2/min/ mg of protein
1. Intact control (n = 8)	50.2 (49; 58.5)	29.05 (27.08; 32.49)	2.46 (2.27; 2.83)
2. 1 hr after CPreC with amtizol (n = 8)	58.8 (54.3; 60.2) [1](17.1%)	30.67 (27.56; 32.23) [1](5.5%)	2.66 (2.5; 2.9) [1](8.1%)
3. 48 hrs after CPreC with amtizol (n = 8)	61.4 (54.3; 68.1) [1](22.3%)	31.65 (27.66; 32.97) [1](8.9%)	2.84 (2.48; 3.05) [1](15.4%)
4. Sham (n = 10)	55.9 (52.4; 62.7) [1](11.3%)	28.41 (26.47; 30.73) [1](-2.3%)	2.32 (2.15; 2.51) [1](-5.7%)
5. Ischemia control (n = 10)	90.3** (82.6; 97.8) [4](61.5%)	19.44 ** (17.1; 23.51) [4](-31.5%)	3.26 ** (2.81; 3.54) [4](40.5%)
6. Ischemia 1 hr after CPreC with amtizol (n = 8)	63.8# (59.6; 68.4) [5](-29.4%)	25.56 ** # (23.66; 27.7) [5](31.4%)	3.59 ** [+(2)] (3.21; 3.74) [5](10.1%)
7. Ischemia 48 hrs after CPreC with amtizol (n = 8)	58.9# (52.3; 64.8) [5](-34.8%)	27.85 # (25.51; 29.71) [5](43.2%)	3.65 ** [+(3)] (3.07; 3.88) [5](11.9%)

Note: The data are presented as: median (Q1; Q3 quartiles). The differences are significant ($p \leq 0.05$, U-Mann-Whitney test) compared to: ** – Sham (sham-operated animals), # – Ischemia. Changes in percentages are shown relative to the group number indicated before the brackets.

The total activity of superoxide dismutase (SOD) in the mitochondrial fraction of the intact control brain tissue was 29 (27; 32.49) units/mg of protein (Table 1). In the group of combined PreC with amtizol in the early and delayed periods of PreC, the activity of SOD did not change in comparison with the intact control ($p = 0.644$; $U = 64$ and $p = 0.326$; $U = 55$, respectively). In the Sham operated animals, the activity of SOD did not differ from the activity of the enzyme of the intact control ($p = 0.544$; $U = 82.5$). A day after ischemia, SOD activity decreased sharply and was 31.5% lower than the Sham values ($p < 0.001$; $U = 121$). When modeling ischemia in animals, 1 hr after the last session of CPreC, the SOD activity did not reach the values of the Sham animals ($p = 0.037$; $U = 108$), but was 31.4% higher than in the Ischemia group ($p = 0.011$; $U = 28$). In the group of animals in which ischemia was modeled 48 hrs after the last session of CPreC with amtizol, the SOD activity was 43.3% higher than in the Ischemia group ($p = 0.003$; $U = 21.5$), reaching the Sham values ($p = 0.452$; $U = 85$). However, there were no differences in the total activity of SOD between the two experimental groups of CPreC with amtizol (early and delayed periods of PreC) ($p = 0.248$; $U = 52$).

The total activity of superoxide dismutase (SOD) in the mitochondrial fraction of the intact control brain tissue was 29 (27; 32.49) conv.units/mg protein (Table 1). The activity of SOD did not change in the group of combined PreC with amtizol in the early and delayed periods of PreC, in comparison with the intact control ($p = 0.644$; $U = 64$ and $p = 0.326$; $U = 55$, respectively). In the Sham animals, the SOD activity did not differ from the activity of the enzyme of the intact control ($p = 0.544$; $U = 82.5$). A day after ischemia, SOD activity decreased sharply and was 31.5% lower than the Sham values ($p < 0.001$; $U = 121$). When modeling ischemia in animals 1 hr after the last session of CPreC, SOD activity did not reach the values of the Sham animals ($p = 0.037$; $U = 108$), but was 31.4% higher than the Ischemia ($p = 0.011$; $U = 28$). In the group of animals that were modeled for ischemia 48 hrs after the last session of CPreC with amtizol, the SOD activity was 43.3% higher than the Ischemia ($p = 0.003$; $U = 21.5$), reaching the Sham animal values ($p = 0.452$; $U = 85$). However, there were no differences in the total activity of SOD between the two experimental groups of CPreC with amtizol (early and delayed periods of PreC) ($p = 0.248$; $U = 52$).

The catalase activity in the intact control group was 2.46 (2.27; 2.83) nmol of H_2O_2/min/mg of protein. No statistically significant changes in the catalase activity were detected under normoxia, either 1 hr or 48 hrs after the final session of combined PreC with amtizol, in comparison with that in the intact control ($p = 0.644$; $U = 64$ and $p = 0.326$; $U = 55$, respectively). The data obtained are consistent with some literature data, which do not confirm the role of ROS in the PreC initiation. For example, there was no increase in ROS-induced processes, LPO product content, antioxidant enzymes SOD and catalase on the 1st day after hypoxic PreC in the cerebral cortex of the animals. Besides, an increase in HIF-1α expression in the early period of PreC can be both against the background of high and low free radical activity (Lukyanova and Kirova 2015, Lukyanova 2019). Moreover, it should be noted that changes in the balance of the pro-/antioxidant system, which is part of the adaptive response, are often difficult to register. This may be due to the time interval taken to evaluate the ROS-induced processes. For example, in Kislin et al (2013), it was shown that immediately after hypoxic PreC in the brain tissue, there was an activation of free radical reactions, but 3 hrs and 24 hrs later, on the contrary, they were inhibited, resulting in a decrease in SOD expression.

In the group of Sham animals, the catalase activity did not differ from the values of the intact control ($p = 0.355$; $U = 88$). In the Ischemia group, the catalase activity measured a day after the surgery was 40.5% higher than the Sham values ($p = 0.003$; $U = 22$). The use of combined PreC with amtizol before ischemia in both periods of PreC maintained the catalase activity at a high level. In the group of animals with ischemia simulated 1 hr after the last session of CPreC with amtizol, the catalase activity was higher than the Sham values ($p < 0.001$; $U = 7$), while showing no difference from the values of the Ischemia group ($p = 0.272$; $U = 53$). The similar results were obtained in the group of animals with ischemia simulated 48 hrs after the last CPreC session, where the catalase activity was also higher than the Sham values ($p < 0.001$; $U = 7.5$), without being significantly higher than the values of the Ischemia group ($p = 0.193$; $U = 49.5$). There were no differences in the effect on the catalase activity between the two experimental groups of CPreC with amtizol ($p = 0.603$; $U = 64$).

The use of agents with antioxidant properties for correcting the ischemic condition is considered justified, since oxidative stress plays the role of an active mechanism in the brain cell damage during its ischemia (Pozhilova et al. 2015, Silachev et al. 2018, Rodriguez et al. 2021). Our study confirmed it: ischemia in the preconditioned animals was not accompanied by any signs of oxidative stress, nor was there any pronounced activation of ROS-induced processes as observed in the control animals with Ischemia. The application of compounds with an antioxidant activity in the CPreC protocol may lead to the weakening of the cytoprotective effect induced by preconditioning due to the inhibition of ROS, which play a signaling role in the mechanism of organism adapting to hypoxia (Kislin et al. 2013). In our study, this was not observed. The result obtained by us may indicate that ROS do not have a key role in the realization of the protective action of the studied PreC or are not the main triggers of preconditioning. It is possible that amtizol does not act on those ROS, the inactivation of which could weaken the PreC development.

The results of studying the ROS-induced processes in cerebral ischemia after CPreC under the conditions of preliminary administration of NO-synthase blockers are

Table 2. The content of TBA-RP and the Antioxidant Enzyme Activities Under the Combined Preconditioning After Cerebral Ischemia in Rats in NO-synthase Blockade (n = 68)

Experimental groups	Brain mitochondria		
	TBA-RP mcmol/ mg of protein	SOD units/mg of protein	Catalase nmol of H_2O_2/min/ mg of protein
1. Sham (n = 10)	55.9 (52.4; 62.7)	28.41 (26.47; 30.73)	2.32 (2.15; 2.51)
2. Ischemia control (n = 10)	90.3* (82.6; 97.8) [1](61.5%)	19.44* (17.1; 23.51) [1](-31.6%)	3.26* (2.81; 3.54) [1](40.5%)
3. Ischemia 1 hr after CPreC with amtizol (n = 8)	63.8 # (59.6; 68.4) [2](-29.4%)	25.56* # (23.66; 27.7) [2](31.5%)	3.59* (3.21; 3.74) [2](10.1%)
4. Ischemia 48 hrs after CPreC with amtizol (n = 8)	58.9 # (52.3; 64.8) [2](-34.8%)	27.85 # (25.51; 29.71) [2](43.2%)	3.65* (3.07; 3.88) [2](11.9%)
5. L-NAME + CPreC with amtizol Ischemia 1 hr afterwards (n = 8)	84.95* +(3) (73; 96.3) [3](33%)	20.05* -(3) (18; 22.57) [3](-21.5%)	2.56 # -(3) (2.22; 2.76) [3](-28.7%)
6. L-NAME + CPreC with amtizol Ischemia 48 hrs afterwards (n = 8)	94.7* +(4) (86.82; 100.7) [4](60.7%)	21.1* -(4) (19.2; 25.8) [4](-24.3%)	2.17 # -(4) (2.13; 2.47) [4](-40.5%)
7. Aminoguanidine + CPreC with amtizol. Ischemia 1 hr afterwards (n = 8)	71.15* # ^(3) (68.6; 73.4) [3](11.5%)	24* (19.65; 25.58) [3](-6.1%)	2.95* -(3) +(5) (2.8; 3.18) [3](-17.8%)
8. Aminoguanidine + CPreC with amtizol. Ischemia 48 hrs aftwerwards (n = 8)	80.2* ^(2) +(4) ^(6) (75.5; 83.6) [4](36.1%)	22.75* (21.65; 27.25) [4](-18.3%)	2.81 ^(1) ^(2) -(4) (2.45; 2.92) [4](-23%)

Note: The data are presented as: median (Q1; Q3 quartiles). The differences are significant ($p \leq 0.05$, U-Mann-Whitney test) compared to: * – Sham (sham-operated animals), # – Ischemia. +/- – significantly more or less compared to the appropriate CPreC group in the early period. ^ – $p \leq 0.1$ – differences at the level of the statistical trend compared to the group number indicated in brackets. Changes in percentages are shown relative to the group number indicated before the brackets.

presented in Table 2. The introduction of L-NAME in the CPreC group in the early period of PreC led to that the TBA-RP content in this case did not differ from the Ishemia values (p = 0.438; U = 18) and was significantly higher than that in the similar group of PreC, which had not been administered L-NAME (p = 0.016; U = 3). Under L-NAME influence, some changes in the antioxidant enzyme activity were observed: the SOD activity was at the level of that in the Ischemia group and was lower than in the similar group without L-NAME (p = 0.002; U = 9), whereas the catalase activity was lower than that in the similar group without NOS blockade (p = 0.002; U = 8).

The changes observed with the preliminary L-NAME use in the CPreC group with amtizol in the delayed PreC period were similar to those in the early period: the TBA-RP content was at the level of that in the Ischemia group (p = 0.561; U = 28.5), but significantly higher than that in the similar group without L-NAME (p = 0.003; U = 0). Though in the delayed PreC period, the TBA-RP level was 60.7% higher in comparison with that in the similar group of PreC without L-NAME, in the early PreC group – only 33% higher; no significant differences between the groups of early and delayed PreC periods could be found (p = 0.262; U = 11). The SOD activity did not differ from the that in the Ischemia group (p = 0.396; U = 59), but was lower than that in the similar group of PreC without L-NAME (p = 0.033; U = 20.5). The catalase activity was lower than when L-NAME was not used (p = 0.002; U = 8).

The use of more selective iNOS blocker aminoguanidine in the CPreC group with amtizol in the early PreC period led to less pronounced changes than when using L-NAME. For example, the TBA-RP content in this group was lower than that in the Ischemia group (p = 0.004; U = 2), but higher than in the Sham animals (p = 0.016; U = 3). The activity of SOD did not differ from that in the Ischemia group. The catalase activity was lower than that in the similar group of PreC without NOS blockers (p = 0.02; U = 18).

In the CPreC group with amtizol in the delayed PreC period with the preliminary use of aminoguanidine, the TBA-RP content was higher than that in the similar PreC group without NOS blockers (p = 0.006; U = 2) and lower at the statistical trend level in comparison with the Ischemia group (p = 0.093; U = 11) and in comparison with the similar group where L-NAME was used as a NOS blocker (p = 0.054; U = 30). There was a decrease in the SOD total activity in comparison with the Sham animals (p = 0.02; U = 18). The catalase activity was lower than that in the PreC group without NOS blockers (p = 0.012; U = 15.5).

Thus, there was no decrease in the LPO processes in terms of the TBA-RP content in the mitochondrial fraction of cerebral ischemic rats when we used the nonselective NOS blocker– L-NAME before any of the studied preconditioning sessions. The values in the groups did not differ from those in the Ischemia control, but were higher than those in the sham-operated animals. In the case of a more selective blockade of the inducible NOS isoform by aminoguanidine, the changes were less pronounced, i.e. aminoguanidine did not completely abolish the effect of the studied PreC methods on TBA-RP and the activities of SOD and catalase, which makes it possible to assume, in addition to inducible NOS, the participation of other NOS isoforms in the CPreC antioxidant action.

Conclusion

In normoxia conditions, the combined PreC with amtizol did not cause significant changes in free radical oxidation processes in the early (after 1 hr) and delayed (after 48 hrs) periods of PreC. An increase in peak amplitude of H_2O_2-induced CL (Fmax) was observed only in the delayed PreC period. No changes were revealed in the TBA-RP content, total activities of superoxide dismutase and catalase in the cerebral mitochondrial fraction, either in the early or delayed periods of PreC.

The use of combined PreC with amtizol prevented the intensification of ROS-induced processes both in the early and delayed periods of cerebral ischemia performance. There was a decrease in CL indices (Fmax and light sum) in brain tissue in comparison with ischemia control, a decrease in the TBA-RP content in the cerebral mitochondrial

fraction, an increase in the total activity of SOD, and maintenance of a high level of catalase activity. These results indicate that there is the weakening of oxidative stress caused by the cerebral ischemia in the mechanism of the combined PreC neuroprotective action. The CPreC effect was more pronounced in the delayed than in the early period.

There was the stimulation of NOS (not so much of the inducible isoform as other NOS isoforms, in particular, the neuronal mitochondria localized nNOS) in the antioxidant effect development of the studied PreC method during ischemia.

Conflict of interests

The authors declare no conflict of interests.

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