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Pharmacological induction of ischemic tolerance in hippocampal slices by sarcosine preconditioning

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ABSTRACT

Brain ischemic tolerance is a protective mechanism induced by a preconditioning stimulus, which prepare the tissue against harmful insults. Preconditioning with *N*-methyl-D-aspartate (NMDA) agonists induces brain tolerance and protects it against glutamate excitotoxicity. Recently, the glycine transporters type 1 (GlyT-1) have been shown to potentiate glutamate neurotransmission through NMDA receptors suggesting an alternative strategy to protect against glutamate excitotoxicity. Here, we evaluated the preconditioning effect of sarcosine pre-treatment, a GlyT-1 inhibitor, in rat hippocampal slices exposed to ischemic insult. Sarcosine (300 mg/kg per day, i.p.) was administered during seven consecutive days before induction of ischemia in hippocampus by oxygen/glucose deprivation (OGD). To access the damage caused by an ischemic insult, we evaluated cells viability, glutamate release, nitric oxide (NO) production, lactate dehydrogenase (LDH) levels, production of reactive oxygen species (ROS), and antioxidant enzymes as well as the impact of oxidative stress in the tissue. We observed that sarcosine reduced cell death in hippocampus submitted to OGD, which was confirmed by reduction on LDH levels in the supernatant. Cell death, glutamate release, LDH levels and NO production were reduced in sarcosine hippocampal slices submitted to OGD when compared to OGD controls (without sarcosine). ROS production was reduced in sarcosine hippocampal slices exposed to OGD, although no changes were found in antioxidant enzymes activities. This study demonstrates that preconditioning with sarcosine induces ischemic tolerance in rat hippocampal slices submitted to OGD.

1. Introduction

Ischemic tolerance is referred to brain protection induced by its pre-exposing to several stimuli that reduces neuronal vulnerability to a subsequent ischemic insult (Gidday, 2006). Initial evidences of cerebral preconditioning were based on the observation that brief periods of anoxia were capable to increase the survival time of animals exposed to a prolonged period of anoxia (Dahl and Balfour, 1964; Schurr et al., 1986), indicating that ischemic tolerance is a phenomenon that occurs in order to protect the neural system (Gidday, 2006).

Neurons produce reactive oxygen species (ROS) by oxidative metabolism during excitotoxic damage and hypoxia (Liu et al., 2009; Perez-Pinzon et al., 2005). However, there are strong evidences that brain preconditioning involves the generation of ROS (Puisieux et al., 2004; Mori et al., 2000). Superoxide anion (O_2^-) is a product of a large number of reactions involving some enzymes like NADPH oxidase, monooxygenases and NADH dehydrogenase. O_2^- is dangerous to cell and is rapidly scavenged by superoxide dismutase, which converts two molecules of O_2^- into a molecule of hydrogen peroxide (H_2O_2) and one of oxygen (O_2) (Puisieux et al., 2004). In turn, cellular levels of H_2O_2 are controlled by catalase (CAT) and glutathione peroxidase (GPX), which convert two molecules of H_2O_2 into a molecule of H_2O and one of O_2. During the excitotoxic process induced by glutamate, there is an increase on O_2^- and H_2O_2 production, which contributes to ischemic brain

Abbreviations: ACSF, artificial cerebrospinal fluid; CAT, catalase; DAN, 2,3-diaminonaphthalene; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DNPH, 2,4-Dinitrophenylhydrazine; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; GlyT-1, glycine transporters type 1; GPX, glutathione peroxidase; GST, glutathione S-transferase; GR, glutathione reductase; MCAO, middle cerebral artery occlusion; MDA, malonaldehyde; NMDA, *N*-methyl-D-aspartate; NO, nitric Oxide; NOS, oxide nitric synthase; OGD, oxygen/glucose deprivation; ROS, reactive oxygen species; SRG, sarcosine group; SLG, saline group; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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injury. In addition, activation of *N*-methyl-D-aspartate (NMDA) receptors induces the production of nitric oxide (NO) by neuronal nitric oxide synthase (NOS), which reacts with O_2 to produce reactive nitrogen species such as peroxynitrite that can damage a wide array of molecules in cells. Nevertheless, H_2O_2, O_2 and NO play a role in the development of hypoxic and ischemic tolerance, since the inhibition of NOS or the scavenging of NO and O_2 during the preconditioning period significantly attenuates the process of tolerance (Cho et al., 2005; Furuichi et al., 2005).

Programmed cell survival is a complex mechanism of cerebral plasticity involving protein phosphorylation and post-translational modifications closely related to the excitatory effect of glutamate by the activation of NMDA receptors (Chu et al., 2007; Turovskaya et al., 2011). These receptors are activated by glutamate and its co-agonists (D-serine or glycine) promoting influx of Ca^{2+} into the cells. A single dose of NMDA produces brain preconditioning and neuroprotective effects against O_2 and glucose deprivation, neuronal death induced by quinolinic acid and kainate-induced toxicity (Ogita et al., 2003; Boeck et al., 2004; Miao et al., 2005). This neuroprotective effect is characterized by a decrease on necrotic and apoptotic cell death and is achieved by the attenuation of injury-inducing excitotoxicity, oxidative and nitrosative stress, metabolic dysfunction and inflammation (Gidday, 2006).

Over the past years, new compounds that potentiate glutamatergic neurotransmission by increasing the concentration of glycine in the synaptic cleft have been developed (Lim et al., 2004). These compounds are selective inhibitors of glycine transporter type 1 (GlyT-1) that mediate uptake of glycine through the binding and co-transport of Na^+ and Cl^- ions, being the Na^+ gradient generated and maintained by the plasma membrane Na^+/K^+-ATPase (Aragón and López-Corcuera, 2003). GlyT-1 can be found in areas such as diencephalon, retina, olfactory bulb and brain hemispheres, being closely associated with NMDA receptors, where it plays a role on glycine uptake (Cubelos et al., 2005; Zafra et al., 1995a,b). GlyT-1 is expressed in neurons and glial cells and it was demonstrated its distribution on the plasma membrane of PC12 cells line (Geerlings et al., 2002). The uptake of glycine by GlyT-1 is down-regulated by protein kinase C and up-regulated by protein kinase G-I, being closely related to NO formation by neuronal NOS and activation of NMDAr (Vargas-Medrano et al., 2011; Jimenez et al., 2011). Inhibition of GlyT-1 induces a pronounced antipsychotic effect and also enhances social memory in rats, which was attributed to an increase on glutamatergic signaling (Harsing et al., 2003; Shimazaki et al., 2010). Recent studies in humans showed that patients who took daily 2 g of sarcosine, a selective inhibitor of GlyT-1, had a reduction in positive and negative symptoms (Hsien-Yuan et al., 2008; Tsai et al., 2004).

Besides the antipsychotic activity, the effects of inhibitors of GlyT-1 on neuromodulation and neuronal survival remain unclear (Hsien-Yuan et al., 2008). Acute blockade of GlyT-1 did not elicit significant neuroprotection of brief oxygen/glucose deprivation (OGD) in the CA1 region of mouse hippocampal slices, but enhanced the amplitude of the NMDA component of a glutamatergic excitatory postsynaptic current in hippocampal pyramidal neurons (Tanabe et al., 2010; Bergeron et al., 1998). Therefore, the aim of the present study was to evaluate if the repetitive administration of sarcosine in rats promotes a neuroprotective phenotype and induces chronic tolerance to OGD insult through the modulation of glutamatergic neurotransmission.

2. Materials and methods

2.1. Animals and treatment

After approval of the experimental protocol by Ethics Committee for Animal Experimentation (Protocol No. 042/11), adult male Wistar rats, weighing 150–200 g, were maintained on a 12-h dark-light cycle, at 25 °C controlled room, with free access to water and food.

Sarcosine (methylglycine) was obtained from Sigma–Aldrich (Wien, Austria) and was dissolved in physiological saline. In order to define the dose of sarcosine for the following experiments, different doses of the drug (30, 100 or 300 mg/kg) were administered i.p. once a day during seven days. Control animals received saline injections during the same period.

2.2. OGD insult

All animals were sacrificed by decapitation twenty-four hours after the last administration of sarcosine or saline. The brain were carefully removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) containing: 127 mM NaCl, 2 mM KCl, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose, bubbled with carbogenic mixture (95% O_2 and 5% CO_2). Hippocampus were dissected on ice and sliced on 400 μm with a Mcllwain Tissue Chopper (Brinkman Instruments, UK). As described by Pinheiro et al. (2009), after dissection, the slices were transferred to individual chambers of a superfusion system (SF-12; Brandel, Gaithersburg, MD, USA) and superfused (37 °C) at a constant rate of 0.5 mL/min with ACSF (95% O_2 and 5% CO_2) during 90 min for recovery from the dissection trauma.

OGD was performed by perfusing the ischemic chambers with ACSF containing glucose 4 mM and bubbled with a hypoxic mixture (95% N_2 and 5% CO_2) during 20 min. Paired control slices were maintained on oxygenated ACSF solution containing glucose 10 mM. After the ischemic period, the slices were reperfused with oxygenated ACSF solution with glucose 10 mM during 4 h. Thereafter, hippocampal slices were processed to experimental evaluation. The supernatant from the ischemic period and reperfusion period was collected to additional analysis.

2.3. Homogenization of hippocampal slices

Hippocampal slices were transferred to 1.5 mL tubes, weighed and homogenized (1:10; w/v) in 50 mM of phosphate buffer saline, containing 140 mM of KCl at 4 °C (pH 7.4). The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was collected in 1.0 mL tubes and was frozen at −70 °C. The protein content was measured according to the Bradfords method (Bradford, 1976) using bovine serum albumin as standard (1 mg/mL).

2.4. Evaluation of hippocampal cells viability

Hippocampus slices submitted to OGD were stained with 6 μM ethidium homodimer (Molecular Probes, Inc.) in oxygenated ACSF/glucose solution for 30 min and then washed for 15 min in oxygenated ACSF/glucose solution at room temperature and protected from light. The cells were excited by red fluorescence (568 nm) and dead cells were evidenced by nucleic acid bound with ethidium homodimer. Fluorescence images were collected and used to quantify the ischemia-induced by OGD. Images were acquired using a fluorescence microscope Axiovert 200 M using the ApoTome system (Carl Zeiss, Germany) to obtain optical sections of the tissue. The Carl Zeiss Axiovision 4.8 software was used to acquire the images.

To improve the quantitative analysis of the images they were processed using the median filter. In the current approach, nucleus was defined as connected pixels that were above the threshold calculated using the image histogram. Regions in the CA1 area of hippocampus were selected from the threshold images and analyzed using the Image J software to calculate the percentage of threshold area in the image, which reflects the number of dead cells.

Additional evaluation of cell death was performed through analysis of lactate dehydrogenase (LDH) content in the incubation media after the period of ischemia/reperfusion (I/R). LDH activity was measured using a colorimetric method through an assay kit (Labtest, Brazil). The results were normalized by protein content and expressed as a percentage of the ischemia control.

2.5. Evaluation of glutamate release

Supernatant from hippocampal slices on the OGD period was collected and centrifuged (1000*g* at 4 °C for 5 min). Glutamate release was assayed by following the increase on fluorescence due to NADPH production in the presence of NADP and glutamate dehydrogenase as previously described (Nicholls et al., 1987). The concentration of glutamate in the supernatant was determined using a standard concentration and the results were normalized by the protein content. The data were expressed as percentage of the ischemia control.

2.6. Measurement of free radical content and antioxidant enzyme activities

Nitrite measurements were performed by using 2,3-diaminonaphthalene (DAN), (Sigma–Aldrich, Wien, Austria), a fluorescent method previously described (Misko et al., 1993). Briefly, 15 μL of 3.2 mM DAN were added to 200 μL of incubation media, collected immediately after OGD period. After 10 min of incubation at 20 °C and protected from light, the reaction was stopped by adding 15 μL of 2.8 M NaOH. The formation of fluorescent product was measured using a fluorescent plate reader (Cary Eclipse, Varian, USA) with excitation at 360 nm and emission at 440 nm. The results were normalized by protein content and expressed as a percentage of the ischemia control.

ROS measurements were performed using 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Sigma–Aldrich, Wien, Austria), a fluorescent probe (Siqueira et al., 2004). Briefly, 20 μL of the sample was incubated with 80 μL of DCF-DA (125 μM) at 37 °C for 30 min and protected from light. Formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, in a fluorescent plate reader (Cary Eclipse, Varian, USA). The results were normalized by protein content and expressed as a percentage of the ischemia control.

CAT activity was assayed based on the decomposition of H_2O_2 by the enzyme (Shangari and O'Brien, 2006). Briefly, the reaction was started by the addition of supernatant to 7.5 mM of H_2O_2 prepared in 50 mM of potassium phosphate buffer (pH 7.0) in a final volume of 1 mL. The rate of decomposition of H_2O_2 was measured by spectrophotometry and the optical density was noted in 15 s intervals during 1.5 min, on absorbance at 240 nm (25 °C). The results were normalized by protein content and expressed as a percentage of the ischemia control.

Superoxide dismutase (SOD) activity was evaluated using a spectrophotometric method previously described (Marklund and Marklund, 1974). The hippocampal homogenate was incubated in a solution containing 100 mM potassium phosphate buffer and 50 mM EDTA, pH 7.4. Reaction was initiated by the addition of 2 mM pyrogallol. Oxidation of pyrogallol was measured at 420 nm (UV/visible U-200L Spectrophotometer, Hitachinaka, Japan) for 5 min, at intervals of 30 s. A 50% inhibition was defined as one unit (U) of SOD, and the results were normalized by protein content and expressed as a percentage of the ischemia control.

2.7. Evaluation of glutathione system

Activity of glutathione peroxidase (GPX) was determined according to Paglia and Valentine (1967). Briefly, reaction solution were prepared in 580 μL of phosphate buffer (100 mM; pH 7.0) containing EDTA 5 mM, 100 μL of NADPH 8.4 mM, 10 μL of glutathione reductase (100 IU/mg protein/mL), 10 μL of NaN3 1.125 M; 100 μL of reduced glutathione 0.15 M and 100 μL of the sample. Enzymatic reaction was started by adding 250 μL of 2.2 mM H_2O_2. Conversion of NADPH to NADP was measured by in a spectrophotometer (Hitachi, model U-2001, Hitachinaka City, Japan) during 4 min. Enzyme unit was determined by the oxidation of 1 mol of NADPH per minute and was calculated based on the absorbance of NADPH at 340 nm. The results were normalized by protein of sample and expressed as a percentage of the ischemia control.

Glutathione reductase (GR) was determined according to Carlberg and Mannervik (1975). Briefly, 100 μL of sample were added to 900 μL of 0.10 M potassium phosphate buffer and 0.5 mM EDTA with 67 μM of NADPH and 133 μM of oxidized glutathione (pH 7.6). Enzyme unit was determined by the oxidation of 1 mol of NADPH per minute and was calculated based on the molar absorptive of NADPH at 340 nm. The results were normalized by protein of sample and expressed as a percentage of the ischemia control.

Activity of glutathione S-transferase (GST) was performed according to Habig et al. (1974). Briefly, 50 μL of sample was added to 850 μL of phosphate buffer 0.1 M and EDTA 1.0 mM (pH 6.5) with GSH (1.06 mM) and 1-chloro-2,4-dinitrobenzene (1.06 mM) (Sigma–Aldrich). The reagents were placed directly into buckets and the readings were made in a spectrophotometer (Hitachi, model U-2001, Hitachinaka City, Japan) at 345 nm.

Glutathione reduced (GSH) content was determined according to Tietze (1969). Briefly, 25 μL of supernatant was added in 96 wells, containing 165 μL of phosphate buffer 0.1 M and EDTA 1.0 mM (pH 8.0) and 10 μL of 100 mM of dinitrobisnitrobenzoic acid were added. The plate were incubated for 30 min and the absorbance was read after 5 min at 412 nm. The results were normalized by protein of sample and expressed as a percentage of the ischemia control.

2.8. Lipid peroxidation

Lipid peroxidation was determined by measuring the accumulation of thiobarbituric acid reactive substances (TBARS) in homogenates and expressed as malonaldehyde (MDA) content, which was measured at 532 nm (UV/visible U-200L Spectrophotometer, Hitachinaka, Japan), as described by Ohkawa et al. (1979). The results were expressed as percentage of increase on TBARS (nmol of MDA/mg protein) relative to their respective controls.

2.9. Data analysis

Experiments of cell viability were analyzed with one-way ANOVA followed by Bonferroni test. The other experiments were analyzed by two-way ANOVA followed by Bonferroni test. Results were expressed as mean ± SEM from at least five independent experiments. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sarcosine preconditioning induced ischemic tolerance in hippocampal slices submitted to OGD

Hippocampal slices from rats pre-treated with different doses of sarcosine (30, 100 and 300 mg/kg) or saline daily during 7 days were submitted to 20 min of OGD and 4 h of reperfusion (Fig. 1). Sarcosine 30 mg/kg did not promote neuroprotection ($p > 0.05$, One-Way ANOVA, followed by Bonferroni post-test). Nevertheless, sarcosine 100 mg/kg had a neuroprotective effect as cell death

reduced to 55.05 ± 6.26% ($p < 0.05$, One-Way ANOVA, followed by Bonferroni post-test). The animals treated with 300 mg/kg of sarcosine showed 38.00 ± 3.33% of cell death ($p < 0.05$, One-Way ANOVA, followed by Bonferroni post-test), a value close to that found in the negative control (29.28 ± 2.47% of cell death).

To confirm the ischemic tolerance induced by sarcosine we evaluated the effect of sarcosine 300 mg/kg on the content of LDH in the supernatant. LDH release from hippocampal slices exposed to OGD was lower in the sarcosine group (SRG) (106.0 ± 3.45%) in comparison with the saline group (SLG) (154.3 ± 11.57%) after I/R injury (Fig. 1C; $p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). In addition, LDH release from SRG hippocampal slices exposed to OGD was similar to the SRG without OGD injury (104.4 ± 12.09%) (Fig. 1C). Taken together, these data indicate that preconditioning with sarcosine produced ischemic tolerance in hippocampal slices exposed to OGD.

3.2. Sarcosine preconditioning reduced the release of excitotoxic signals in hippocampal slices submitted to OGD

To investigate the excitotoxic signals in hippocampal slices submitted to OGD, we evaluated the release of glutamate and NO production in the supernatant after 20 min of ischemic insult. Fig. 2A shows that the content of glutamate on the supernatant of SLG hippocampal slices increased by 206.1 ± 39.51% after 20 min of OGD ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). In constrast, SRG animals submitted to OGD showed a lower release of glutamate in the supernatant (112.5 ± 14.27%) when compared to SLG ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). Fig. 2B shows that the SRG animals submitted to OGD presented a decrease in NO production (54.83 ± 15.82%) when compared to SLG (171.8 ± 24.48%) after 20 min of ischemia ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). These data suggest that preconditioning with sarcosine reduced main signals of excitotoxicity induced by ischemic insult.

3.3. Sarcosine preconditioning reduced oxidative damage in hippocampal slices submitted to OGD

To access cells damage induced by OGD, we firstly performed the evaluation of the content of ROS in hippocampal slices. Fig. 3A shows that OGD insult increased the levels of ROS in SLG hippocampus (337.4 ± 55.33%) after I/R injury ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). However, the levels of ROS in SRG hippocampus (61.12 ± 15.35%) were lower when compared to SLG hippocampus ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test), indicating that sarcosine treatment reduced the production of ROS after I/R process.

In accordance with this data, lipoperoxidation of SLG hippocampus submitted to OGD increased by 144.6 ± 16.34% after I/R injury (Fig. 3B). Preconditioning with sarcosine reduced lipoperoxidation induced by OGD (72.92 ± 4.956%) when compared with SLG ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). These data suggests that oxidative damage was reduced by the treatment with sarcosine.

3.4. Effect of sarcosine preconditioning on the activity of antioxidant enzymes in hippocampal slices submitted to OGD

In order to evaluate the involvement of antioxidant enzymes on the reduction of oxidative stress in hippocampal slices from SRG submitted to OGD, we performed the biochemical evaluation of CAT and SOD activity. As shown in Fig. 4A, we observed a reduction on SOD activity in SLG submitted to OGD (77.97 ± 11.29%) ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). In addition, SOD activity of SRG was similar in control and ischemia groups after I/R injury. Sarcosine also reduced SOD activity of hippocampal slices not exposed to OGD, (63.19 ± 6.07%). In contrast with SOD, CAT activity increased in SLG submitted to OGD (473.3 ± 72.83%) when compared with the control group ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). Moreover, as observed in Fig. 4B, SRG presented lower CAT activity

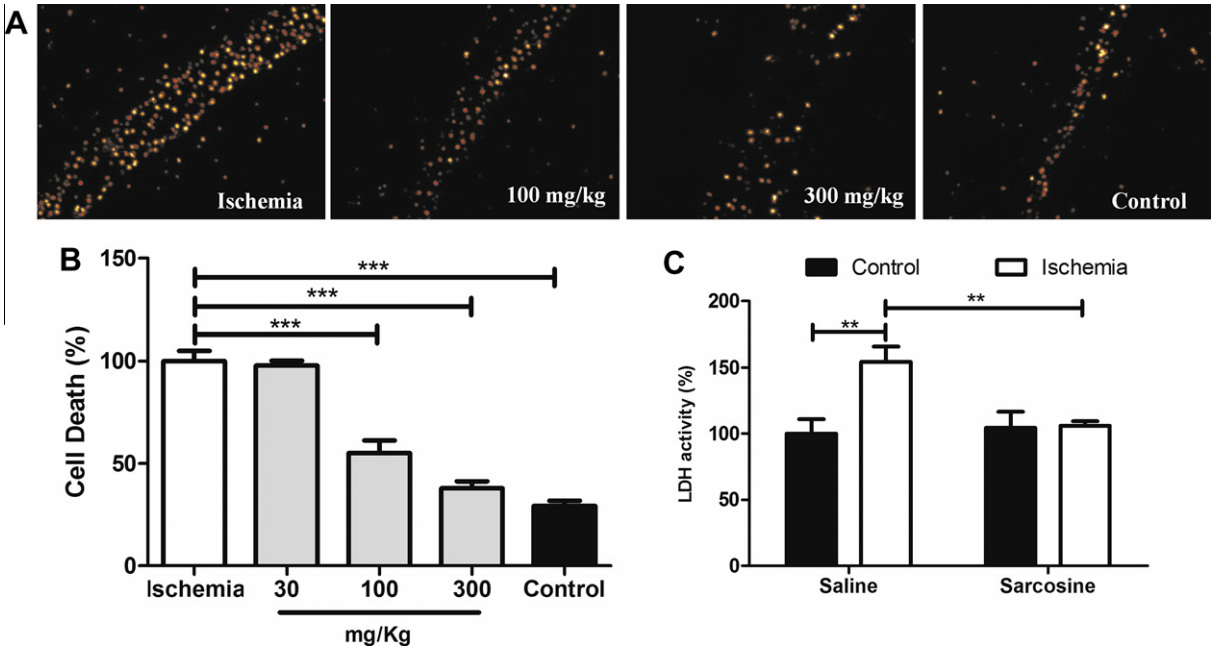

Fig. 1. Preconditioning with sarcosine induced tolerance in hippocampal slices submitted to OGD. (A) Representative hippocampal slices of control and sarcosine groups after I/R process. (B) Cell death assessed by ethidium homodimer stained after I/R process (***$p < 0.001$, One-Way ANOVA, followed by Bonferroni post-test). (C) LDH activity in hippocampal slice medium after I/R process (**$p < 0.01$, Two-Way ANOVA, followed by Bonferroni post-test). The results express the mean ± SEM of cell death from at least five different animals.

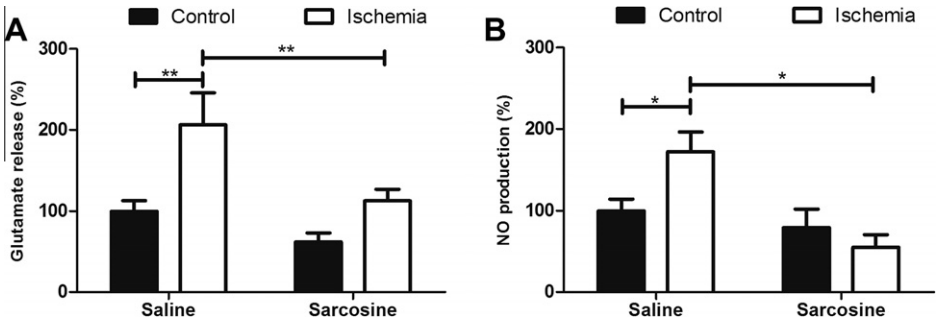

Fig. 2. Preconditioning with sarcosine reduced the release of excitotoxic mediators in hippocampal slices submitted to OGD. (A) Glutamate release from hippocampal slices after 20 min of OGD insult. (B) NO production from hippocampal slices after 20 min of OGD insult. The results express the mean ± SEM of each group from at least five different animals ($^{*}p < 0.05$ and $^{**}p < 0.01$, Two-Way ANOVA, followed by Bonferroni post-test).

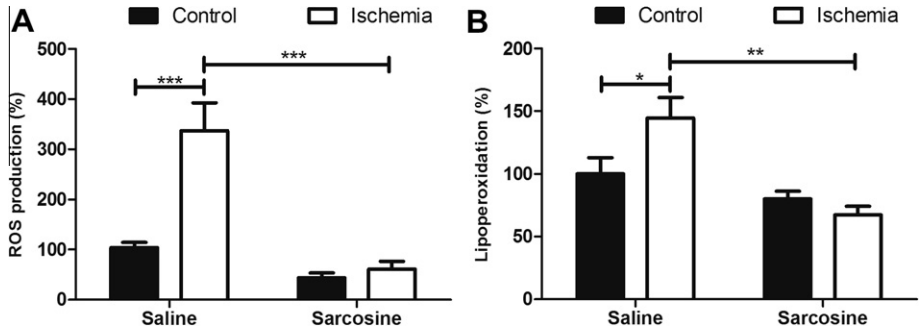

Fig. 3. Preconditioning with sarcosine reduced oxidative stress in hippocampal slices submitted to OGD. (A) ROS production from hippocampal slices after I/R process. (B) Lipoperoxidation in hippocampal slices after I/R process. The results express the mean ± SEM of each group from at least five different animals ($^{*}p < 0.05$ and $^{***}p < 0.001$, Two-Way ANOVA, followed by Bonferroni post-test).

in hippocampal slices exposed to OGD (97.32 ± 30.63%) when compared with SLG ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test). These data suggest that antioxidant enzymes activities were maintained after I/R process in hippocampus of animals treated with sarcosine.

3.5. Effect of sarcosine preconditioning on glutathione system in hippocampal slices submitted to OGD

As shown in Fig. 5A, there was a significant decrease on total glutathione content in SLG after OGD (71.75 ± 5.03%) ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test), which was not observed in hippocampal slices of SRG. GPX activity of SLG was also reduced (72.39 ± 6.51%) after I/R process ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test), which was not observed in SRG (Fig. 5B). On the other hand, GR activity increased in SLG after OGD (315.2 ± 82.0%) ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test), an effect that was not observed in SRG exposed to OGD, despite a slight reduction on basal activity when comparing SLG with SRG (Fig. 5C). Similarly, GST activity increased in SLG exposed to OGD (256.7 ± 51.10%) (Fig. 5D) ($p < 0.05$, Two-Way ANOVA, followed by Bonferroni post-test), which was not observed in SRG. We also observed a slight reduction in GST basal activity when comparing SLG with SRG. Taken together, these data indicate that the activity of glutathione system was maintained after I/R process in hippocampus from animals preconditioned with sarcosine.

4. Discussion

The present work demonstrated that pre-treatment with sarcosine can be used as preconditioning stimulus in hippocampal

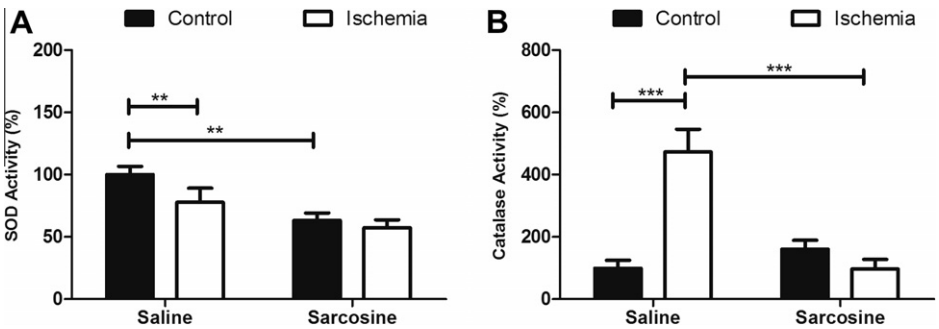

Fig. 4. Preconditioning with sarcosine maintain antioxidant enzymatic activity in hippocampal slices submitted to oxygen-glucose deprivation. (A) SOD activity of hippocampal slices after I/R process. (B) Catalase activity of hippocampal slices after I/R process. The results express the mean ± SEM of each group from at least five different animals ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, Two-Way ANOVA, followed by Bonferroni post-test).

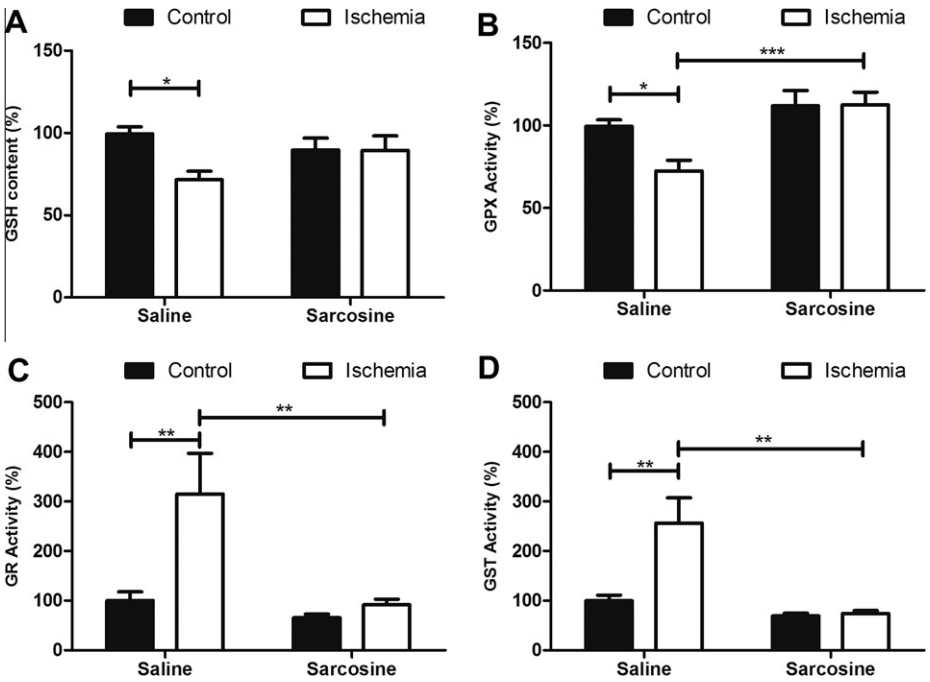

Fig. 5. Preconditioning with sarcosine maintain glutathione system capacity in hippocampal slices submitted to oxygen–glucose deprivation. (A) Glutathione content of hippocampal slices after I/R process. (B) Glutathione peroxidase activity of hippocampal slices after I/R process. (C) Glutathione reductase activity of hippocampal slices after I/R process. (D) Glutathione-s-transferase activity of hippocampal slices after I/R process. The results express the mean ± SEM of each group from at least five different animals ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, Two-Way ANOVA, followed by Bonferroni post-test).

slices, which promote an ischemic tolerance against OGD insult. Sarcosine is a competitive inhibitor of GlyT-1 (IC_{50}: 40–150 μM), a co-agonist of NMDA receptor (IC_{50}: 26 μM) and a weak agonist of glycine receptors (IC_{50}: 3.0 mM) (Zhang et al., 2009). It is well know that GlyT-1 inhibitors potentiate NMDA receptors response in glutamatergic neurotransmission through an increase of the co-agonist glycine in the synapse cleft (Chen et al., 2003; Lim et al., 2004; Kinney et al., 2003). In addition, the stimulation of presynaptic glycine receptors by high concentrations of glycine enhanced the release of glutamate (Waseem and Fedorovich, 2010), which can also activate NMDA receptors. Therefore, the neuroprotection observed with sarcosine is closely related to the modulation of glutamatergic neurotransmission and seems to be related to NMDA receptors.

Indeed, chronic modulation of NMDA receptors is intimately related to tolerance against excitotoxicity. Chronic administration of NMDA antagonist to rodents, as phencyclidine and MK-801, has been associated with an increase on the susceptibility to excitotoxicity, apoptotic cell death and an up-regulation of NMDA receptor (NR1 subunit) (Rujescu et al., 2006; Wang et al., 2000; Wang et al., 1999). On the other hand, NMDA activation with non-convulsivant doses induces tolerance to excitotoxicity in several experimental models (Gidday, 2006; Chu et al., 2007; Turovskaya et al., 2011; Ogita et al., 2003; Boeck et al., 2004; Miao et al., 2005). Sarcosine treatment is capable to potentiate NMDA activation and its effect on brain ischemic tolerance seems to be related to an interference on glutamatergic neurotransmission.

In agreement with this idea, we observed a reduction on glutamate release during I/R injury and a trend to reduction of glutamate release under basal conditions in the sarcosine group. Recent studies have shown that some drugs are capable to induce neuroprotection by a similar mechanism. The treatment with resveratrol (30 mg/kg) during 7 days had a neuroprotective effect in a middle cerebral artery occlusion (MCAO) model in rats (Li et al., 2010). This effect was related to a decrease on the release of excitatory neurotransmitters (glutamate, aspartate and D-serine) during the I/R period and an increase on basal levels of inhibitory neurotransmitters (GABA, glycine and taurine). It was also described the neuroprotective effect of ceftriaxone and beta-lactam antibiotics in *in vitro* OGD model and *in vivo* MCAO model of brain ischemia after 5 days of treatment, being this phenomenon related to an interference on glutamate uptake by the glutamate transporter EAAT2 (Lipski et al., 2007; Chu et al., 2007; Rothstein et al., 2005).

We also observed that sarcosine treatment decreased NO release from hippocampal slices after OGD. It is well know that NMDA receptors present a physical binding to nNOS, which corroborate to NO production during glutamate activation of neurons (Jimenez et al., 2011). Taken together this data indicate that cell death was reduced by a decrease of excitotoxic mediators, indicating that ischemic tolerance achieved by sarcosine is related to the classic preconditioning effect observed with NMDA agonists, which involves the reduction of glutamate release and also the excitotoxic signals during OGD.

We observed a reduction on the production of ROS from hippocampal slices of the animals treated with sarcosine, which is in agreement with the decrease on glutamate and NO signaling. The reduction of ROS production during the ischemic insult is related to the low lipoperoxidation in hippocampal slices of sarcosine treated animals submitted to OGD. On the other hand, the oxidative stress is an important step of the ischemic tolerance induced by a preconditioning stimulus (Obrenovitch, 2008). In fact, the administration of antioxidant compounds can suppress the ischemic preconditioning in MCAO model (Mori et al., 2000). The preconditioning treatment with sarcosine did not change the basal activity of SOD or CAT, as well the activity during the ischemic insult. Some studies using hypoxic condition as a preconditioning agent reported an increase on the expression and activity of SOD enzymes at basal condition (Arthur et al., 2004; Danielisová et al., 2005). In contrast, this effect did not occur in a MCAO model of preconditioning (Puisieux et al., 2004). In this model, CAT activ-

ity increased after 5 min of four-vessel occlusion precondition. Although it does not affect the activity of these enzymes, other antioxidant system may be involved.

The glutathione system is involved in the control of peroxides by brain cells and protection against ROS (Ralf, 2000). Primary cultures of rat cortical neurons subjected to hypoxic preconditioning presents an increase in GPX and GR activities, which is in accordance with the role of an antioxidant mechanism during the preconditioning (Arthur et al., 2004). Nevertheless, ischemic tolerance induced by MCAO in rats did not change the activity and expression of GPX (Puisieux et al., 2004). Our results showed that preconditioning stimulus with sarcosine was able to maintain the activity of glutathione system after the OGD insult with no increase on the antioxidant capacity.

In conclusion, brain ischemic tolerance induced by a preconditioning treatment with sarcosine seems to be related to a reduction on glutamate release during the OGD insult. The decrease on glutamate release reduced the production of NO and ROS and consequently, decreased cell damage that was not associated with an increase on the activity of antioxidant enzymes. Brain ischemic tolerance induced by sarcosine preconditioning might be related to protein expression in membrane or/and with mitochondrial function. Further studies using *in vitro* and *in vivo* approaches are necessary to investigate the preconditioning effect of sarcosine in other brain structures, the functional activity of animals exposed to ischemic insult, as well as, the glutamatergic activity after neuromodulation.

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