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ORIGINAL ARTICLE

Effects of chronic guanosine treatment on hippocampal damage and cognitive impairment of rats submitted to chronic cerebral hypoperfusion

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Abstract Chronic cerebral hypoperfusion contributes to a cognitive decline related to brain disorders. Its experimental model in rats is a permanent bilateral common carotid artery occlusion (2VO). Overstimulation of the glutamatergic system excitotoxicity due to brain energetic disturbance in 2VO animals seems to play a pivotal role as a mechanism of cerebral damage. The nucleoside guanosine (GUO) exerts extracellular effects including antagonism of glutamatergic activity. Accordingly, our group demonstrated several neuroprotective effects of GUO against glutamatergic excitotoxicity. Therefore, in this study, we evaluated a chronic GUO treatment effects in rats submitted to 2VO. We evaluated the animals performance in the Morris water maze and hippocampal damage by neurons and astrocytes immunohistochemistry. In addition, we investigated the cerebrospinal fluid (CSF) brain derived neurotrophic factor (BDNF) and serum S100B levels. Additionally, the purine CSF and plasma levels were determined. GUO treatment did not prevent the cognitive impairment promoted by 2VO. However, none of the 2VO animals treated with GUO showed differences in the hippocampal regions compared to control, while 20% of 2VO rats not treated with GUO presented loss of pyramidal neurons and increased glial labeling cells in CA1 hippocampal region. In addition, we did not observe differences in CSF BDNF nor serum S100B levels among the groups. Of note, both the 2VO surgery and GUO treatment changed the purine CSF and plasma profile. In conclusion, GUO treatment did not prevent the cognitive impairment observed in 2VO animals, but our data suggest that GUO could be neuroprotective against hippocampal damage induced by 2VO.

Keywords Guanosine · Cerebral hypoperfusion · Cognitive impairment · Hippocampal damage · Neuroprotection

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Introduction

Vascular dementia (VD), which is a group of diseases with heterogeneous pathological conditions and physiopathological mechanisms, is the second most common cause of dementia associated with Alzheimer's disease and accounts for 10–50% of all dementias [1, 2]. Chronic cerebral hypoperfusion is considered a factor that contributes to

memory dysfunction in neurological diseases such as VD [3, 4]. Moreover, the degree of cerebral hypoperfusion has been suggested as a predictive biomarker of the gradual transition from a mild cognitive impairment to the Alzheimer's disease [5].

Permanent bilateral occlusion of the common carotid arteries (also denominated two vessels occlusion, 2VO) in rats is used as a chronic cerebral hypoperfusion model [6]. This procedure results in significant reduction of cerebral blood flow [6–9] and can cause progressive, long-lasting cognition deficits and neuronal damage resembling the effects observed in VD [9, 10]. 2VO in rats provides a useful model to understand the pathophysiology of chronic cerebral vascular disorders and to screen drugs with potential therapeutic value in VD.

The chain of events that eventually leads to neuronal cell death in chronic cerebral hypoperfusion begins with neuronal energy failure due to the blood flow reduction and the consequent oxygen and glucose deficiency [6, 11–13]. The disturbance of the energetic metabolism leads accumulation of extracellular glutamate [14, 15], the main excitatory neurotransmitter in the central nervous system, essential for the brain function [14, 16, 17]. However, overstimulation of the glutamatergic system (excitotoxicity), which increases the calcium influx triggers several intracellular processes, such as proteolytic hydrolysis, lipid peroxidation, and generation of reactive oxygen species, causing neuronal death [16, 18]. Thus, excitotoxicity has been proposed as a mechanism of neuronal damage impairing cellular energetics, as observed in hypoperfusion [19, 20].

The nucleoside guanosine (GUO) exerts various extracellular signaling effects, such as trophic effects on neural cells [21–26] and in vitro and in vivo antagonism of the glutamatergic system [27–29]. Accordingly, our group has demonstrated by several works that GUO is neuroprotective in different in vitro and in vivo experimental models of glutamatergic excitotoxicity [27, 28, 30–37], including ischemic insults [38, 39]. The GUO mechanism of action is not fully understood, however, our group demonstrated that GUO stimulates glutamate uptake by cultured astrocytes and brain slices [40–44], a physiological process that prevent glutamate toxicity.

Therefore, considering the involvement of excitotoxicity in the 2VO model and the neuroprotective potential of GUO, the aim of the present study was to investigate the effects of GUO in rats submitted to 2VO. For this purpose, we treated the animals during the first 6 weeks after the 2VO surgery (the period where the cerebral blood flow is highly reduced [12, 45]) with orally chronic GUO administration. After 6 weeks, some animals were sacrificed for purine cerebrospinal fluid (CSF) and plasma level analysis. Furthermore, we performed behavioral and/or histological analysis 6 months postoperative in another rats since significant time-dependent changes in the neural cell markers had been reported to occur until that period [6, 46]. Accordingly, we evaluated the spatial memory performance of the rats in the Morris water maze task and the hippocampal histology. Moreover, we also investigated the CSF brain derived neurotrophic factor (BDNF) levels and the serum S100B levels as possible markers of brain insult.

Materials and methods

Animals

Male adult Wistar rats (90–100 days old, weighing 300–350 g) were kept on a 12-h light/dark cycle (light on at 7:00 a.m.) at constant temperature of 22 ± 1°C. They were housed in plastic cages (5 per cage) with water and commercial food ad libitum. All behavioral tasks were conducted between 9:00 a.m. and 5:00 p.m. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE), and were approved by the Research Ethics Committee of Universidade Federal do Rio Grande do Sul.

Surgery procedure

Chronic cerebral hypoperfusion was performed (40 animals) by a modified protocol of permanent bilateral occlusion of the common carotid arteries (2VO) [47]; additionally, 40 animals served as sham-operated controls (SHAM). Rats were anesthetized with halothane. The common carotid arteries were exposed via a neck ventral midline incision, separated from their sheaths and vagal nerves. Rats were submitted to the modified 2VO protocol: carotids were permanently occluded with 5-0 silk suture with a 1-week interval between interventions, the right common carotid being the first to be assessed and the left one being occluded 1 week later. Sham-operated controls received the same surgical procedures without carotid artery occlusion.

Treatment and groups

Rats received water (for control group) or GUO solution (0.5 mg/ml) for 6 weeks ad libitum in the bottle water immediately from the 2VO surgery. The GUO dose was chosen based on a previously study, which demonstrated that it was neuroprotective against seizures induced by quinolinic acid (Vinadé et al. 2003). The water consumption and body weight were monitored during the period of

treatment every 2 days. The animals were randomly assigned to four different groups: sham-operated animals receiving water (SHAM-CT) or GUO (SHAM-GUO) and 2VO operated animals receiving water (2VO-CT) or GUO (2VO-GUO). The purine CSF and plasma level were evaluated after the treatment. The behavioral, histological and proteins analysis were performed 6 months postoperative.

CSF, serum and plasma sampling

CSF and blood (plasma or serum) were collected in two different times after the surgery for different parameters analysis.

Six weeks postoperative, 10 animals per group were sacrificed for purine CSF and plasma level measurements. The rats were anesthetized with sodium thiopental (40 mg/kg, 1 mL/kg, i.p.), and whole blood was obtained through a retrobulbar venous plexus puncture using a capillary tube. Plasma was separated by centrifugation at 3,000*g* for 10 min RT in a sodium-citrate tube. After, the animals were then positioned in a stereotaxic holder for CSF collection from the cisterna magna. The puncture was performed using an insulin syringe (27 gauge 9 1/200 length). The CSF were then centrifugated at 3,000*g* for 10 min 4°C to obtain a CSF cellfree supernatants. The CSF and plasma samples were frozen (−80°C) until analysis.

One day after the conclusion of the behavioral study (approximately 6 months postoperative), the CSF and serum of 40 animals (10 per group) were obtained for BDNF and/or S100B quantification, respectively. First, the rats were anesthetized and the CSF were collected accordingly reported above. After, rats were then removed from the stereotaxic apparatus and placed in a flat place; whole blood was obtained through an intracardiac puncture using a 0.37-mm diameter needle that was inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3,000*g* for 10 min RT. CSF and serum samples were frozen (−20°C) until analysis.

HPLC procedure

High-performance liquid chromatography (HPLC) was performed to measure the concentration of purines. The measurement was done according to previously determined guidelines [28]. It measured the CSF concentrations of the following purines: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine (INO), hypoxanthine (HIPOX), xanthine (XAN), and uric acid (UA). Analyses were performed with the Shimadzu Class-VP chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF autoinjector valve with 50 mL loop and a UV detector (Shimadzu, Kyoto, Japan). Separations were achieved on a Supelco C18 250 ± 4.6 mm, 5-mm particle size column (Supelco, St Louis, MO, USA). The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24°C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 mL were injected into the injection valve loop. Absorbance was read at 254 nm. CSF and plasma concentrations of purines are expressed as mean ± SEM in micromoles.

Morris water maze

Cognitive function was evaluated in the Morris water maze task [48]. The maze consisted of a black circular pool (200 cm diameter, 60 cm height) filled to a depth of 40 cm with water (22 ± 1°C). A circular platform (10 cm diameter) was submerged 2 cm below the surface of the water and hidden from the rat's view. Four points, equally spaced along the circumference of the pool, were arbitrarily assigned as North, South, East and West. The pool, therefore, was divided into four quadrants (Northeast, Southeast, Southwest, and Northwest). These points served as the starting positions where the rats were gently lowered into the water, with its head facing the wall of the water maze. We submitted the rats to a reference memory protocol for spatial memory analyzes.

Reference memory protocol

In this protocol, 10 rats per group received 5 training sessions (one session/day) and a probe trial in the 6th day. Each session consisted of four trials with a 15-min intertrial interval. The location of the escape platform was fixed throughout training in the middle of the NE quadrant, 30 cm from the wall. A trial began when the rat was placed in the water at one of the 4 starting positions, randomly chosen, facing the wall. The order of the starting position varied in each trial and any given sequence was not repeated. A trial ended when the rat escaped onto the platform, and the escape latency for each trial was recorded. When the animal did not succeed, it was gently guided to the platform and left on it for 10 s and the escape latency was recorded as 60 s. Rats were dried and returned to their

home cages after each trial. The mean escape latency of daily trials was then calculated.

Probe trial

In this 1-day test, each rat was subjected to a probe trial (60 s) without the platform. The latency to reach the original platform position, the number of crossings over that place and the time spent on the target quadrants were measured.

Sessions were recorded with a video acquisition system. Videotapes were used by a trained observer using dedicated software (ANY-maze®). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

Quantification of CSF BDNF

BDNF levels in CSF were measured by anti-BDNF sandwich-ELISA, according to the datasheet from DuoSet kit (R&D Systems, Inc, USA) in a Spectra Max M5 molecular Devices (USA). Microtiter plates (96-well flat-bottom) were coated 2 h with samples ($n = 10$ per group) and reference curve standards (ranging from 23 to 1,500 pg/mL BNDF). The plates were then washed 4 times with PBS + Tween 0.05% and a diluted biotinylated mouse anti-human BDNF monoclonal (1:1,000) was added to each well and incubated for 2 h at room temperature. After this time, the plates were washed 4 times and then a diluted streptavidin-horseradish peroxidase (HRP) conjugate solution was added to each well and incubated at room temperature for 20 min. Wells were then washed four times before adding TMB chromogen (Tetramethylbenzidine) and maintained at room temperature for 20 min before the addition of stop solution. The amount of BDNF was determined at 450 nm and expressed as ng/mL.

Quantification of serum S100B

Serum S100B concentrations were measured using an enzyme linked immunosorbent assay (Diasorin® S100 ELISA Kit) in a Spectra Max M5 molecular Devices (USA). Calibrators and serum samples (100 μL—$n = 10$ per group) were incubated in a plate previously coated with anti-S100B antibody. The S100 ELISA was a two-site, one-step, enzyme linked immunosorbent assay. In the assay calibrators, controls and serum samples react simultaneously with two solid phase capture antibodies and a detector antibody conjugated with HRP during the incubation in the microtiter wells for 2 h. After a washing step (with PBS + Tween 0.05%) a TMB chromogen was added and the reaction was allowed to proceed for 15 min. The enzyme reaction was stopped by adding stop solution and the absorbance was measured at 450 nm. S100B concentrations were derived by comparison with the calibration curve based on the total absorbance for each given calibrator provided with the assay. All determinations were carried out within the same experiment. The S100B calibration curve is cubic spline up to 5 μg/L, and the CVs for duplicates across the entire concentration range for the calibrators and samples were 5%. The detection limit of the assay is 0.03 μg/L. The results are expressed as ng/mL.

Histological analysis

After CSF and serum sampling, the animals were injected with 400 IU of heparin ($n = 10$ per group). They were then submitted to transcardiac perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The brains were removed and post-fixed in the same solution at room temperature for 4 h. For the morphological analysis, brains were cryoprotected with a 30% sucrose solution for 2 days. Coronal sections (50 μm) were obtained using a Vibratome (Leica, Germany).

One section in six random series was collected for immunohistochemistry. Immunohistochemical detection was carried out in the sections with all fields of the hippocampus (an area of the hippocampus extending between the Bregma ±2.30 and ±3.80, Plate 29–34, according to Paxinos & Wattson Atlas). The sections were incubated for 48 h at 4°C with polyclonal rabbit anti-GFAP antiserum (Dako, UK, 1:500) and polyclonal mouse anti-NeuN (Chemicon, 1:250) in tris buffered (pH 7.4) saline antiserum (TBS) containing 0.2% of Triton-X 100 and 10% of bovine serum. After being washed several times with TBS, the sections were incubated with 594 alexa-conjugated goat antirabbit and 488 alexa-conjugated rabbit antimouse antibodies for 2 h at room temperature. The sections were mounted on slides coated with 2% gelatin with chromium and potassium sulfate, using Vectashield mounting medium (Vector Laboratories, São Paulo, Brazil). All sections were photographed with a confocal microscope (Olympus, Japan).

Astrocytic immunohistochemical analyzes

The number of GFAP-stained astrocytes/mm^2 in the hippocampus—CA1 was estimated according to previously reported [49]. All lighting conditions and magnifications were kept constant. The images were captured and a square region of interest (ROI) was created considering the minor size of the organ/layer. The ROI square was overlaid in all sections of stratum radiatum of CA1, with blood vessels and artifacts being avoided. Eight images were analyzed

per field from a total of five animals per group. GFAP-reactive astrocytes located inside this square or intersected by the lower and/or right edges of the square were counted. Astrocytes intersected by the upper and/or left edges of the square were not counted [50]. Morphological analysis was performed by three separate observers who were blind to the experimental groups, and results were averaged in the final results.

Statistical analysis

The data obtained are expressed as mean ± SEM of mean. As the variances of the data were homogenous (analyzed by the Kolmogorov–Smirnov test), behavioral performance in reference memory task was analyzed by two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni posttest when indicated. Mean escape latency was the dependent variable, day was the within-subjects variable, and the four groups were the between-subjects variables. Remaining data were analyzed by two-way ANOVA (factors were surgery and treatment). In all statistical comparisons, $P < 0.05$ was used as the criterion for significance.

Results

Behavioral and treatment parameters

Chronic cerebral hypoperfusion had no effect on liquid and food consumption or body weight during 6 months (data not shown). In addition, ad libitum 0.5 mg/mL GUO oral treatment did not affect the parameters mentioned above.

Purine CSF and plasma level

As shown in Table 1a, 6 weeks after chronic cerebral hypoperfusion, the 2VO-CT animals presented a significant higher UA and a lower INO CSF level compared to SHAM-CT animals ($P < 0.05$). No other purine level significantly differed between these two groups. In addition, 6 weeks of orally chronic GUO administration significant increased the CSF level of XAN compared to control groups ($P < 0.05$) but did not significantly changed the GUO nor another CSF purine level investigated here.

As shown in Table 1b, the plasma level of several purines investigated were changed after 6 weeks of chronic cerebral hypoperfusion. The levels of GMP, XAN,

Table 1 Purine CSF (A) and plasma (B) concentration

Treatment	SHAM-CT	SHAM-GUO	2VO-CT	2VO-GUO
(a) Purine CSF concentration (μM)				
ATP	0.09 ± 0.05	0.06 ± 0.05	0.15 ± 0.05	0.25 ± 0.06
ADP	0.79 ± 0.22	0.51 ± 0.09	0.60 ± 0.08	0.60 ± 0.06
AMP	0.86 ± 0.18	1.09 ± 0.13	1.00 ± 0.17	0.85 ± 0.11
ADO	0.10 ± 0.01	0.12 ± 0.01	0.10 ± 0.02	0.09 ± 0.03
GTP	1.38 ± 0.03	1.44 ± 0.04	1.48 ± 0.06	1.47 ± 0.03
GDP	0.15 ± 0.04	0.12 ± 0.03	0.17 ± 0.02	0.15 ± 0.03
GMP	1.13 ± 0.05	1.05 ± 0.07	1.50 ± 0.16	1.09 ± 0.04
GUO	0.10 ± 0.01	0.10 ± 0.02	0.10 ± 0.1	0.12 ± 0.03
INO	0.32 ± 0.07	0.32 ± 0.10	0.21 ± 0.09*	0.17 ± 0.04*
HIPOX	2.02 ± 0.15	1.51 ± 0.15	1.85 ± 0.25	1.96 ± 0.37
XAN	2.26 ± 0.13	2.86 ± 0.13#	2.60 ± 0.14	2.76 ± 0.06#
UA	2.22 ± 0.11	2.49 ± 0.10	4.23 ± 0.60*	3.03 ± 0.21*
(b) Purine plasma concentration (μM)				
ATP	n.d.	n.d.	n.d.	n.d.
ADP	n.d.	n.d.	n.d.	n.d.
AMP	0.09 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
ADO	0.03 ± 0.01	0.07 ± 0.01#	0.09 ± 0.01*	0.09 ± 0.01#
GTP	0.18 ± 0.02	0.20 ± 0.03	0.23 ± 0.04	0.12 ± 0.10
GDP	0.17 ± 0.02	0.16 ± 0.02	0.22 ± 0.04	0.21 ± 0.04
GMP	0.74 ± 0.03	0.70 ± 0.05	0.92 ± 0.09*	0.95 ± 0.10*
GUO	0.44 ± 0.06	0.32 ± 0.06	0.33 ± 0.05	0.33 ± 0.03
INO	0.61 ± 0.10	0.66 ± 0.24	0.96 ± 0.14	0.73 ± 0.10
HIPOX	0.09 ± 0.01	0.07 ± 0.01#	0.11 ± 0.02	0.06 ± 0.01#
XAN	0.49 ± 0.01	0.59 ± 0.02#	0.73 ± 0.03*	0.65 ± 0.04#
UA	9.71 ± 0.65	13.99 ± 1.81	22.65 ± 2.24*	11.96 ± 1.13

* Statistical difference from respectively sham groups (2VO-CT compared to SHAM-CT, 2VO-GUO compared to SHAM-GUO)

Statistical differences from SHAM-CT ($P < 0.05$). $N = 10$ per group

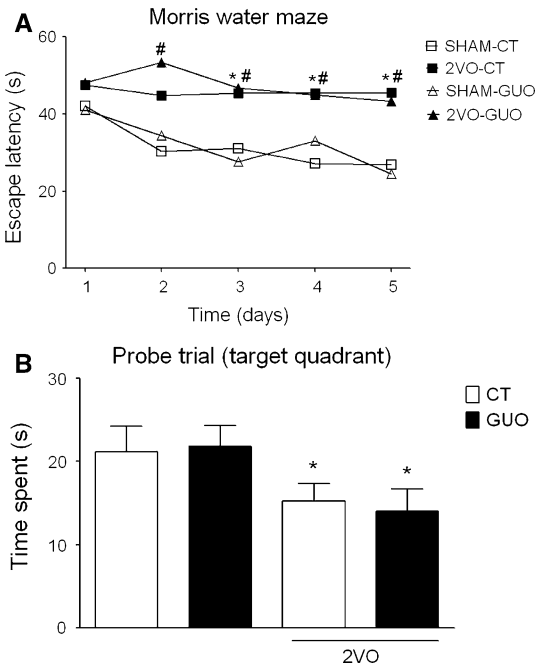

Fig. 1 Performance of rats in the water maze task submitted to chronic cerebral hypoperfusion evaluated. **a** Curves in the Morris water maze performance in the reference memory protocol. 2VO groups showed significantly longer escape latencies than SHAM groups, respectively ($^{*}P < 0.05$, $^{\#}P < 0.05$). **b** Memory time spent in the target quadrant showing memory impairment of 2VO-CT and 2VO-GUO compared to SHAM-CT and SHAM-GUO groups ($^{*}P < 0.05$). $N = 10$ animals per group

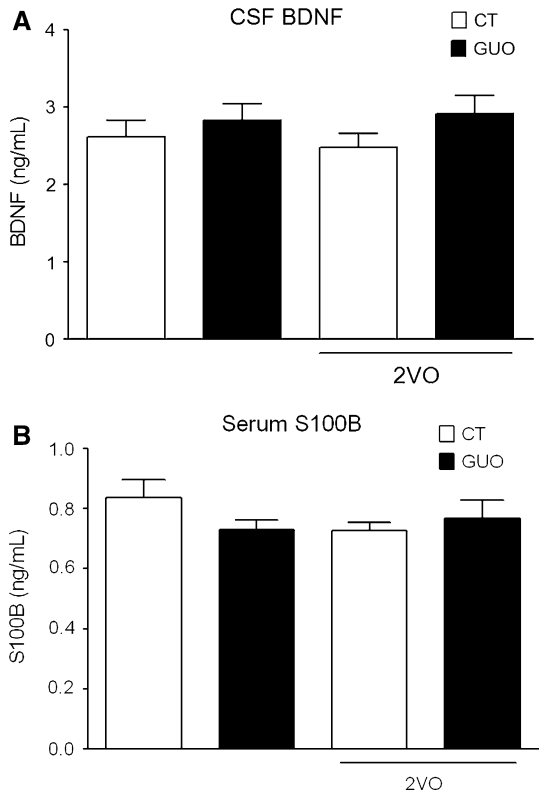

Fig. 2 CSF BDNF and serum S100B levels. No differences were observed in CSF BDNF concentration (**a**) and in serum S100B (**b**) ($P > 0.05$). $N = 10$ animals per group

UA and ADO were significant higher in the plasma of 2VO-CT compared to SHAM-CT animals ($P < 0.05$). Regarding GUO treatment, GUO administration significantly increased XAN and ADO levels while decreased HIPOX level compared to SHAM-CT ($P < 0.05$). In addition, Two Way ANOVA of variances analysis pointed that there was interaction between the surgery and the treatment on plasma GUO and ADO levels [$F(1.28) = 5.06$, $P = 0.03$].

Morris water maze learning

All rats showed similar escape latencies on training day 1, a two-way ANOVA with one between factor (4 groups) versus five repeated measures (escape latencies) indicated a significant difference among groups [$F(3, 144) = 6.36$, $P < 0.05$], and a significant difference over training days [$F(4, 133) = 7.64$, $P < 0.01$] (Fig. 1a). The escape latency of both sham groups (CT and GUO) decreased from the 1st to the 5th day of the task, while both 2VO groups (CT and GUO) did not present this performance, indicating that 2VO procedure impaired the learning of this task and that GUO did not interfere with this impairment.

In the probe test, both sham groups (CT and GUO) spent more time in the former platform quadrant (Fig. 1b) than both 2VO groups (CT and GUO) [by two-way ANOVA, $F(1, 35) = 5.20$, $P < 0.01$]. Again, GUO had no effect on this performance. In addition, it was not observed any motor deficit: the mean swimming speed was 0.15 m/s for control group and 0.17 m/s for 2VO group.

Brain insult markers

As shown in Fig. 2, CSF BDNF (A) and serum S100B (B) levels were not affected by 2VO procedure and/or by GUO administration, by two way ANOVA ($P > 0.05$).

Hippocampal damage

Immunohistochemistry was carried out using NeuN and GFAP for labeling neurons and astrocytes, respectively. The hippocampal damage was evaluated 6 months after 2VO-surgery. Evident loss of CA1 pyramidal neurons and increased reactive glial labeling cells was observed in 2 of 10 animals in the 2VO-CT group (Fig. 3). No difference was detected in the NeuN or GFAP labeling in CA3 or DG (Dentate Gyrus) regions. Representative images of

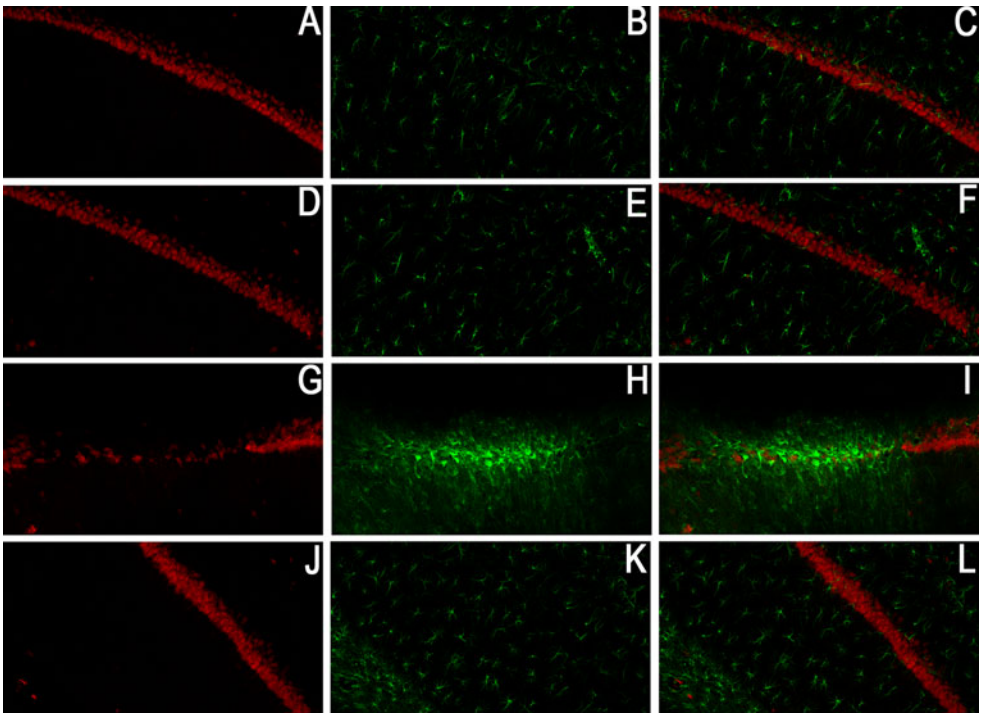

Fig. 3 Pyramidal neurons and GFAP immunoreactivity in the CA1 hippocampal region of 2VO-CT rats six month after chronic cerebral hypoperfusion. Immunofluorescence showing NeuN (**a, d, g, j**) and GFAP (**b, e, h, k**) positive cells in SHAM-CT (**a–c**), SHAM-GUO (**d–f**), 2VO-CT (**g–i**) and 2VO-GUO (**j–l**) groups. ×20, *scale bar* 100 μm

hippocampal CA1, CA3 and DG from rats of the four groups are shown in Figs. 3, 4 and 5, respectively.

Astrocytic cell bodies and processes were identified in the CA1 *stratum radiatum* of all animals excepting the two rats displaying pronounced reactive glial labeling. There was no significant difference in the number and/or in the morphology of astrocytes among the groups (data not shown).

Discussion

The results of the present study demonstrate that chronic cerebral hypoperfusion produced by 2VO, impaired spatial learning and memory evaluated by Morris water maze task and caused severe hippocampal damage in 20% of the animals. The behavioral effect was not affected by GUO treatment. However, no hippocampal damage was observed in the 2VO animals treated with GUO. In addition, no alteration was observed in neither the CSF BDNF levels nor in the serum S100B concentration caused by 2VO surgery and/or by GUO treatment.

Studies involving the use of the 2VO model have demonstrated that an impaired spatial learning function is associated with hippocampal damage [6, 10, 12]. In our study, we did not observe this correlation: animals presenting behavioral alteration did not present cell hippocampal damage. We cannot exclude that other cell damage not investigated here may be present in the 2VO group. In fact, the relationship between chronic cerebral hypoperfusion produced by 2VO and impaired cognitive function has not been completely elucidated. Other studies also found almost no correlation between the loss of CA1 neurons and the Morris maze performance [51–53]. The pyramidal and granular cells in the hippocampus are not the exclusive cells involved in spatial memory. Other regions of the hippocampal formation such as the entorhinal cortex, the parahippocampal gyrus, and the rhinal and cingular gyri are also involved in spatial memory [54, 55]. Moreover, the memory deficit correlates with the white matter damage in 2VO rats [6, 56–58]. Thus, a direct link cannot be established between 2VO-induced memory failure and a specific appearance of neuronal damage in the hippocampus.

It is noteworthy that variable hippocampal damage has been reported by others [6, 59–61]. As in most vascular models, individual anatomical differences in the cerebrovascular anatomy at the circle of Willis of rodents can influence experimental variability [62]. Differences in the focal lesions outcome after 2VO in different laboratories may be explained possibly by differences in rat strain, animal age at the time of occlusion or anesthesia.

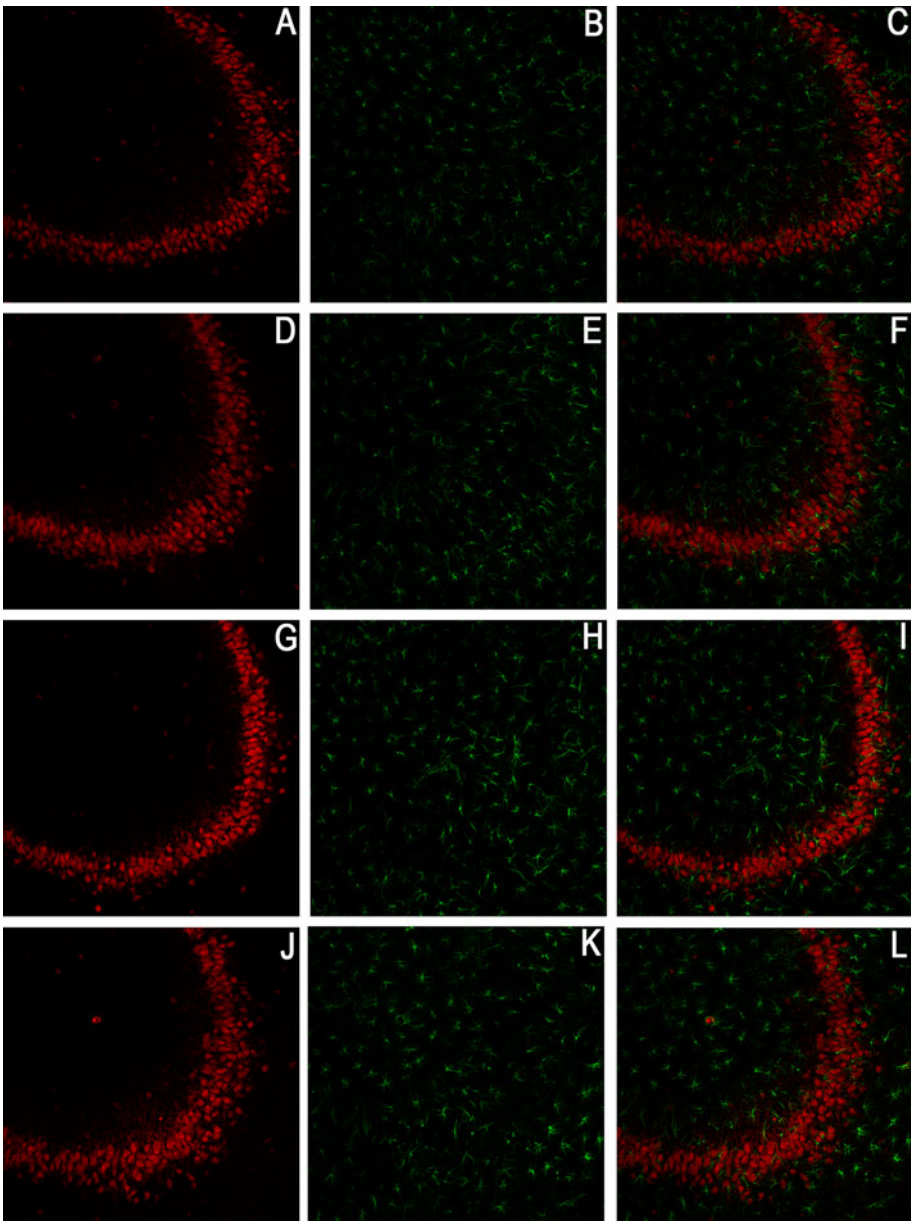

Fig. 4 Pyramidal neurons and GFAP immunoreactivity in the CA3 hippocampal region of rats. Immunofluorescence showing NeuN (**a, d, g, j**) and GFAP (**b, e, h, k**) positive cells in SHAM-CT (**a–c**), SHAM-GUO (**d–f**), 2VO-CT (**g–i**) and 2VO-GUO (**j–l**) groups. ×20, *scale bar* 100 μm

The hippocampal damage here observed in two animals was evidenced by a massive neuronal loss in the CA1 region that was accompanied by an intense increase in GFAP labeling, indicating an astrogliosis process. No alteration in the CA3 or DG areas was observed. This is consistent with the selective vulnerability of CA1 neurons that has been observed in others studies using the 2VO model and with various acute ischemia models [6, 8, 63]. GFAP is commonly used as a marker for changes in astroglial cells during brain development and injury [64]. In fact, CNS injury, as consequence of brain diseases as trauma, ischemia, genetic disorders, neurodegenerative disorders or chemical insult causes astrocytes to become reactive, a condition accompanied by an increase in GFAP levels [65].

In our study, 2VO procedure did not affect the CSF BDNF level and the serum S100B concentration in rats. BDNF and S100B are two proteins commonly used as biomarkers for brain diseases, including vascular and Alzheimer's diseases [66–73]. BDNF in the hippocampus is critical for the acquisition and/or consolidation of spatial memory [74–76]. Previous studies showed that chronic cerebral hypoperfusion induced down-regulation of hippocampal BDNF [77]. In addition, 2VO rats presenting an increase in hippocampal BDNF levels also presented better performance in the water maze task [77]. S100B is a calcium-binding protein found in brain tissue, predominantly in astrocytes. This protein has putative intra and extracellular functions. Intracellular roles include regulation of protein phosphorylation, cytoskeleton components and

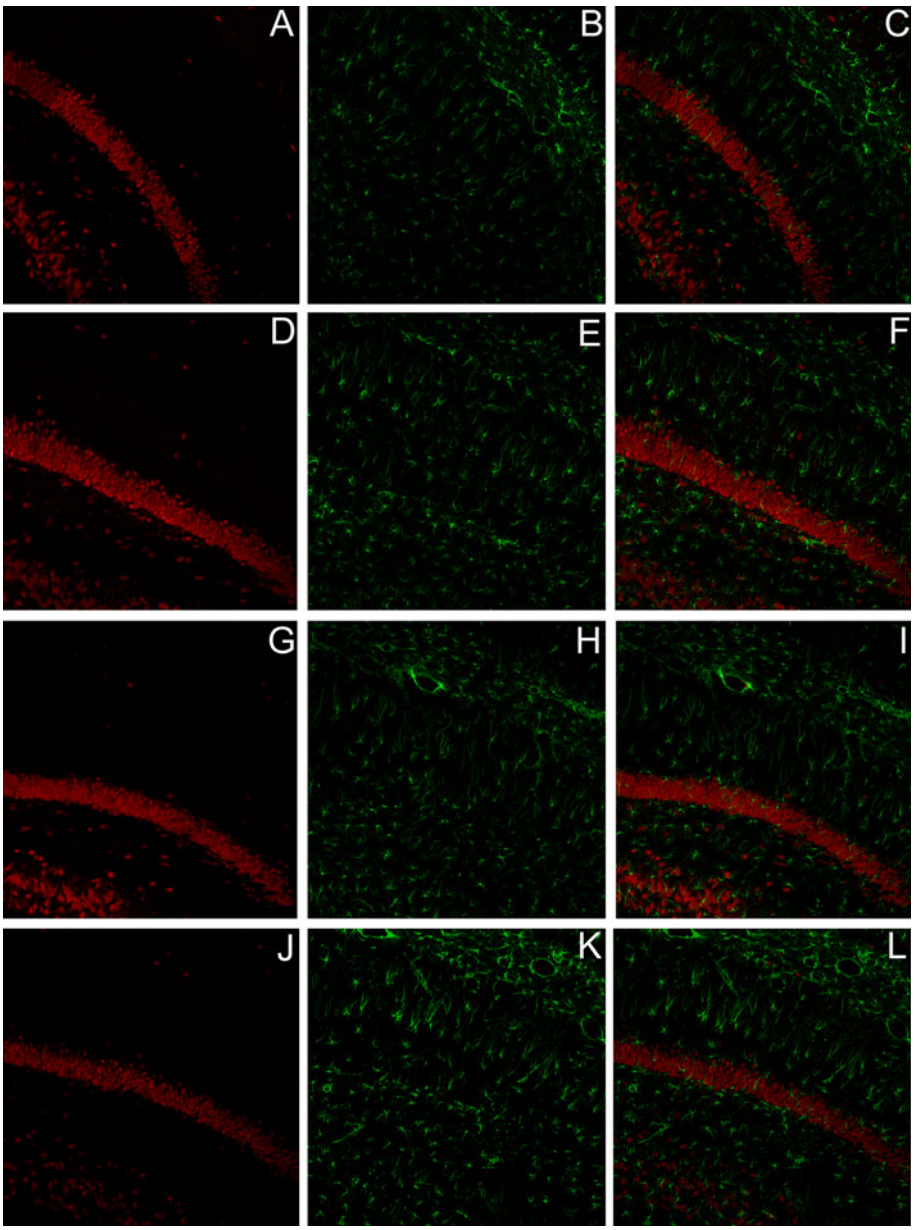

Fig. 5 Pyramidal neurons and GFAP immunoreactivity in the DG hippocampal region. Immunofluorescence showing NeuN (**a, d, g, j**) and GFAP (**b, e, h, k**) positive cells in SHAM-CT (**a–c**), SHAM-GUO (**d–f**), 2VO-CT (**g–i**) and 2VO-GUO (**j–l**) groups. ×20, *scale bar* 100 μm

transcriptional factors [78, 79]; extracellularly, S100B plays trophic roles on neuronal and glial cells, but elevated levels of this protein could induce apoptosis in neural cells [80]. In a previous work, we demonstrated that the hippocampal S100B levels increased, while CSF S100B levels decreased, after the 2VO surgery [48]. To note, previous works evaluated the BDNF and S100B proteins 8 and 10 weeks after the 2VO surgery, respectively [48, 77]. In the present work, we investigated the 2VO effects 6 months after the surgery. Therefore, we cannot rule out that alteration in the CSF BDNF level or serum S100B concentration may be altered in other periods after 2VO surgery.

Glutamatergic excitotoxicity is suggested to play a pivotal role in the behavioral alterations and neuronal damage observed in ischemic insult, including hypoperfusion [19, 20, 48]. In a previous work, we demonstrated that 2VO animals presented a decrease of hippocampal glutamate uptake, indicating a higher susceptibility of these animals to excitotoxicity [48]. A correlation between hippocampal glutamate uptake and Morris water maze performance was also evidenced [48]. Our group has demonstrated that acute in vitro administration of GUO stimulates glutamate uptake [40, 41, 44], and in vivo administration prevents the glutamate uptake decrease after excitotoxic stimuli [38, 39, 81, 82]. Therefore, we expected that GUO treatment would be able to prevent the behavioral alterations observed in 2VO rats, which did not occur in this study. Of note, none of the animals that received GUO treatment after the 2VO surgery showed neuronal injury.

GUO was given orally for 6 weeks immediately from the 2VO surgery. We chose to treat the animals with chronic orally treatment because we previously demonstrated that GUO chronic administration was neuroprotective against excitotoxicity insults [37, 82]. However, no other study, to our knowledge, administrated GUO for such a long period as 6 weeks as we did it here. Surprising, we could not observe any difference in the CSF or plasma GUO level after the treatment (Table 1). Of note, we observed that the level of XAN (one direct product of GUO degradation) is increased at CSF and plasma. Additionally, GUO administration increased the plasma ADO and decreased HIPOX levels, suggesting some change in the homeostasis of the purinergic system. Importantly, previous works already demonstrated that GUO can be metabolized both in plasma [83] and CSF [84]. As we already have demonstrated that 2 weeks of orally chronic GUO treatment increased CSF GUO levels [82], we can speculate that the long period of GUO treatment used here (6 weeks) could, at somehow, accelerate GUO degradation. In fact, it is important to mention that both the CSF and plasma levels of most of the purines evaluated here showed a higher variability between the GUO treated rats in contrast to the control animals. In this way, other protocols using GUO treatment with different administration schedule should be design to investigate the GUO effects in 2VO animals.

Noteworthy, the plasma level of several purines (GMP, XAN, UA and ADO) were higher after 6 weeks of chronic cerebral hypoperfusion compared to control animals (Table 1b). Regarding CSF, only UA level was higher and INO level was lower in the 2VO-CT animals compared to SHAM-CT group. Although the meanings of these results are difficult to explain here, it could suggest that the purinergic system could play an important role in the pathophysiology of chronic cerebral hypopefusion.

In summary, we did not observe correlation between the cognitive impairment and hippocampal cell damage in the 2VO animals. In addition, CSF BDNF and serum S100B levels were not brain injury markers, at least 6 months after the 2VO surgery. Although the GUO neuroprotective potential in experimental models involving excitotoxicity events has been well demonstrated, in the present work GUO chronic orally treatment did not prevent the behavioral alterations observed in 2VO animals. We are continuing to characterize the GUO effects on the behavioral and histological alterations observed by chronic cerebral hypoperfusion.

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