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Research Report

Therapeutic potential of treatment with the flavonoid rutin after cortical focal ischemia in rats

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ABSTRACT

Flavonoids have known anti-inflammatory and antioxidative actions, and they have been described as neuroprotective and able to reduce damage in CNS diseases. We evaluated the action of the flavonoid rutin in an animal model of focal cortical ischemia induced by unilateral thermocoagulation of superficial blood vessels of motor (M1) and somatosensory (S1) primary cortices. Ischemic rats were submitted to daily injections (i.p.) for five days, starting immediately after induction of ischemia. We tested two doses: 50 mg/kg or 100 mg/kg of body weight. Sensorimotor tests were used to evaluate functional recovery. Bioavailability in plasma was done by chromatographic analysis. The effect of treatment in lesion volume and neurodegeneration was evaluated 48 h and 72 h after ischemia, respectively. We observed significant sensorimotor recovery induced by rutin, and the dose of 50 mg/kg had more pronounced effect. Thus, this dose was used in further analyses. Plasma availability of rutin was detected from 2 h to at least 8 h after ischemia. The treatment did not result in reduction of lesion volume but reduced the number of degenerated neurons at the periphery of the lesion. The results suggest rutin as an efficient drug to treat brain ischemia since it was able to promote significant recovery of sensorimotor loss, which was correlated to the reduction of neurodegeneration in the periphery of cortical injury. Increasing studies with rutin and other flavonoids might give support for further clinical trials with these drugs.

1. Introduction

Stroke is currently a critical public health problem and a major cause of death and disability in adults worldwide (Lloyd-Jones et al., 2009; Lotufo, 2005). Several pathophysiological events are triggered in brain tissue after an ischemic injury, including the inflammatory response and oxidative stress damage (Brouns and De Deyn, 2009; Deb et al., 2010). Thus, drugs with anti-inflammatory and antioxidative actions have been expected to have a protective effect in brain ischemia.

Abbreviations: ANOVA, Analysis of variance; FJC, Fluoro-Jade C; HPLC, High performance liquid chromatography; i.p., Intraperitoneal; MCAO, Middle cerebral artery occlusion; PID, Post-ischemic day; SEM, Standard error mean; TTC, 2,3,5-Triphenyltetrazolium chloride

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Polyphenols are natural substances found in plant products, as leaves and fruits, oils, wine and tea. They are divided into phenolic acids, flavonoids and non-flavonoid polyphenols (Ramassamy, 2006). Like beta-carotene and ascorbic acid, polyphenolic compounds are related to protective effects against cancer and cardiovascular disease (Heim et al., 2002). Flavonoids are part of this large group of polyphenolic compounds, and more than 2000 flavonoids have been identified (Ramassamy, 2006). The most important pharmacological properties of flavonoids are its anti-inflammatory and antioxidative actions (Benavente-García and Castillo, 2008; Formica and Regelson, 1995; Juurlink and Paterson, 1998; Procházková et al., 2011). The use of flavonoids has been proposed for pathologies of central nervous system, such as Parkinson's disease, Alzheimer's disease and stroke, due to such properties and to data from epidemiological studies (Ramassamy, 2006; Sun et al., 2008).

Rutin, also called as quercetin-3-O-rutinoside, is a flavonoid glycoside composed of the flavonoid quercetin and the disaccharide rutinose that have antioxidative, anti-inflammatory, antiallergic, anti-viral and anti-carcinogenic actions (Araújo et al., 2011). Few studies have evaluated the treatment with rutin in models of global and focal brain ischemia, showing positive effects (Gupta et al., 2003; Khan et al., 2009). Rutin administration has been evaluated in a model of focal brain ischemia, revealing protective action (Khan et al., 2009). However, only pre-ischemic administration was assessed (Khan et al., 2009).

Here, we studied the effect of treatment with rutin after induction of focal cortical ischemia. Thus, we aimed to analyze whether this flavonoid could be used as medicine to treat brain ischemia. We applied rutin into the acute phase of ischemia and evaluated its bioavailability and its effects on sensorimotor recovery and neurodegeneration.

2. Results

2.1. *Behavioral analyses*

To evaluate whether the administration of rutin after induction of cortical ischemia results in any functional recovery, ischemic animals were treated with rutin and their sensorimotor performance was measured.

In cylinder test, statistical analysis showed significant "treatment x day" interaction ($F=1.56$, $p<0.05$) and significant effects of treatment ($F=3.61$, $p<0.05$) and day ($F=16.5$, $p<0.0001$). Comparisons among groups showed more marked recovery in R50 group, and R100 showed discrete effect (Fig. 1). Thus, rutin promoted significant recovery of contralateral forelimb performance in support during vertical exploration. Similarly, in adhesive test, statistical analysis showed significant "treatment x day" interaction ($F=1.64$, $p<0.05$) and significant effects of treatment ($F=5.18$, $p<0.05$) and day ($F=30.19$, $p<0.0001$). Comparisons among groups also showed more marked recovery in R50 group than in R100 group (Fig. 2). Sham animals were also evaluated and showed no significant lost of function (Fig. 2). Thus, rutin promoted significant recovery of adhesive removal with contralateral forelimb after tactile stimulation.

Together, these results suggest that post-ischemic treatment with rutin is effective to recover sensorimotor function after cortical focal ischemia. Since the dose of

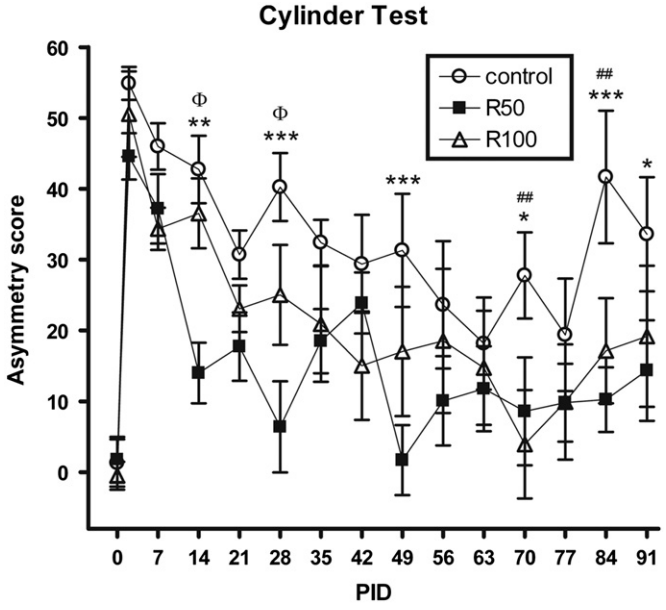

Fig. 1 – Recovery of the impaired forelimb in the cylinder test. Graph showing the accompaniment of the performance of control ($n=7$), R50 ($n=6$) and R100 ($n=7$) groups before ischemia and along post-ischemic weeks. In all groups, the greater asymmetry was observed at PID 2. R50 group was significantly different from the control group at PIDs 14, 28, 49, 70, 84 and 91, showing a higher level of recovery. R100 group showed recovery only at PIDs 70 and 84. * represents the comparison between control and R50 groups, # represents the comparison between control and R100 groups and Φ represents the comparison between R50 and R100 groups. (* or Φ=$p<0.05$; ##=$p<0.01$; ***=$p<0.001$; Tukey).

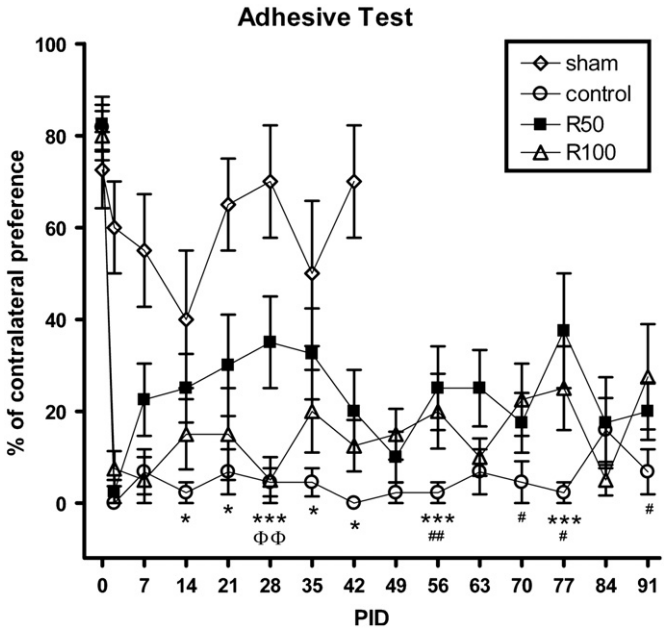

Fig. 2 – Recovery of the impaired forelimb in the adhesive test. Graph showing the accompaniment of the performance of control ($n=11$), R50 ($n=10$) and R100 ($n=10$) groups before ischemia and along post-ischemic weeks. Sham group ($n=5$) was accompanied until PID 42 and was shown to illustrate the lack of impairment. This group was not considered in statistical analysis. In all groups, the lower level of contralateral preference was observed at PID 2. R50 group was significantly different from the control group at PIDs 14, 21, 28, 35, 42, 56 and 77, showing a higher level of recovery. R100 group showed recovery at PIDs 56, 70, 77 and 91. Points in the graph represent mean ± SEM. * represents the comparison between control and R50 groups, # represents the comparison between control and R100 groups and Φ represents the comparison between R50 and R100 groups (* or # = $p<0.05$; ## or ΦΦ = $p<0.01$; *** = $p<0.001$; Tukey).

50 mg/kg showed better result, it was used in further experiments.

2.2. *Detection of rutin in plasma*

Experiments with HPLC showed the presence of rutin in plasma from 2 h to atleast 8 h after i.p. injection, with a peak at 2–4 h (Table 1, Fig. 3). Two equations showed a close fit for obtained data, and both statistic comparisons with F test (equation (1) as the null hypothesis, $F=0.09$, $p=0.77$) and Alkaike's Information Criteria (AlCc) (% equation (1)/% equation (2) = 17.24) indicated equation (1) (two phase exponential association) as the preferred model (Table 2, Fig. 3).

2.3. *Lesion volume*

As previously shown (Giraldi-Guimarães et al., 2009; Szele et al., 1995), the procedure of thermocoagulation induced a consistent ischemic lesion that included the six cortical layers, sparing the white matter as revealed by reaction with TTC (Fig. 4). Sham procedure induced no recognizable lesion (Fig. 4). Treatment with rutin promoted no significant reduction of ischemic lesion volume ($p=0.65$, Figs. 4 and 5).

2.4. *FJC staining*

As previously shown (Giraldi-Guimarães et al., 2009), the procedure of thermocoagulation induced large neurodegeneration, as revealed by FJC staining. The majority of FJC^+ cells were observed in the core of the lesion (not shown), but a significant number of stained cells was also observed in the periphery of the lesion (Fig. 6). The number of FJC^+ cells observed in the periphery of the lesion was higher in animals of the control group than in those of R50 group (Fig. 6). Quantification revealed that this difference was statistically significant (Fig. 7).

Table 1 – Values found for each animal in HPCL experiment.

Time (h)	Plasmatic concentration of rutin (μg/ml)	Mean ± SD
2	0.87	0.93 ± 0.54
	1.50	
	0.42	
4	1.28	0.99 ± 0.31
	1.02	
	0.67	
6	0.62	0.60 ± 0.07
	0.52	
	0.65	
8	0.79	0.45 ± 0.31
	0.37	
	0.19	

3. Discussion

In recent years, research on flavonoids is increasing. The interest in these compounds is due to the evidence of various

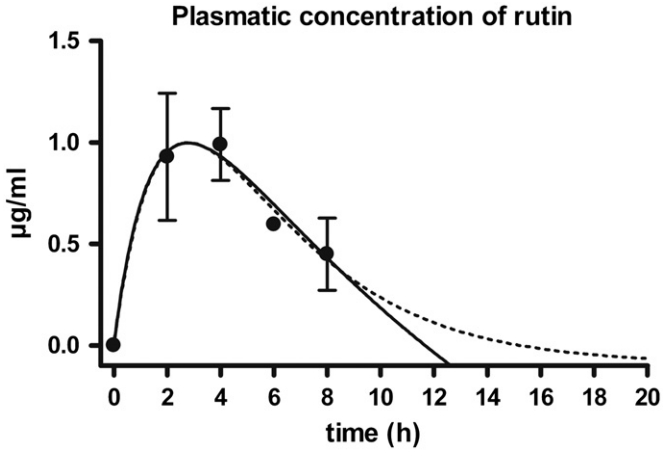

Fig. 3 – Plasmatic concentration of rutin. Graph showing the time course of plasmatic concentration of rutin after i.p. administration with a dose of 50 mg/kg. The measured times were 2, 4, 6 and 8 h (for each time, $n=3$). Time 0 h was plotted as 0 for regression analysis. Solid line represents the fit of nonlinear regression with equation (1), and dashed line represents the fit with equation (2) (see Table 2). Points in the graph represent mean ± SEM.

Table 2 – Equations of nonlinear regression.

Two phase exponential association.

$$Y=Ymax1\times(1-\exp(-K1\times X))+Ymax2\times(1-\exp(-K2\times X)) \quad (1)$$

Best-fit values	
*Ymax*1	−3.489
*K*1	0.06417
*Ymax*2	1.839
*K*2	0.6874

Two phase exponential decay.

$$Y=Span1\times\exp(-K1\times X)+Span2\times\exp(-K2\times X)+plateau \quad (2)$$

Best-fit values	
Span1	5.659
K1	0.2680
Span2	−5.573
K2	0.4495
Plateau	−0.08949

pharmacological properties and their presence in many human foods (Muzitano et al., 2008; Dajas et al., 2003). Furthermore, epidemiological studies have evaluated the correlation between reduced rates of cardiovascular disease and cytoprotection in neurological disorders in populations with diets rich in flavonoids (Bastianetto and Quirion, 2002 Esposito et al., 2002; Procházková et al., 2011). In fact, recent studies with flavonoids in models of brain ischemia, most of them with the flavonoid widely found in plant products, quercetin, have shown significant neuroprotection and promotion of functional outcome (Lee et al., 2011; Rivera et al., 2008).

A flavonoid with molecular structure similar to quercetin and putative neuroprotective action is rutin. Few previous studies with models of global brain ischemia have been conducted, showing protective effect of rutin when administrated in pre-ischemic stage (Abd-El-Fattah et al., 2010; Gupta et al., 2003; Pu et al., 2007) or after ischemia induction (Gupta et al., 2003). Regarding focal ischemia, a recent study evaluated rutin administration during 21 days before the induction of ischemia by middle cerebral artery occlusion (MCAO), revealing protective action (Khan et al., 2009). Here, we studied the therapeutic potential of rutin when administrated after induction of focal thermocoagulatory ischemic lesion in sensorimotor cortex, a model previously used by our research group to investigate therapeutic approaches (Giraldi-Guimarães et al., 2009). Administration of rutin from the beginning of ischemic process, by daily i.p. injections during the first five post-ischemic days, promoted sensorimotor recovery of impaired forelimb. Moreover, although no reduction of lesion volume was found, rutin reduced neurodegeneration in lesion periphery. Thus, the results indicate that rutin also has significant neuroprotective effect when administrated after the occurrence of a focal cortical ischemia, suggesting that this flavonoid might be used to treat ischemic damage in the acute phase of stroke.

We observed more sensorimotor recovery with the dose of 50 mg/kg than with 100 mg/kg. We are not able to explain this result, but previous reports about treatment of focal brain ischemia with quercetin, a structurally related flavonoid, have also shown better effects with lower than with higher doses (Pandey et al., 2011; Rivera et al., 2004). After *post-mortem* observation of the peritoneal cavity of our animals, we observed that those treated with 100 mg/kg had clusters of insoluble rutin, which was not observed in those treated with 50 mg/kg (data not shown). Some reasons for the instability of rutin solution in the peritoneal cavity are its aqueous environment and its range of pH values (7.46–8.10) (Noh, 2003), which turns flavonols less soluble in water when compared to neutral and acidic conditions. These peritoneal cavity features could lead to a precipitation of rutin when it is in higher concentrations, which might have some negative influence on the absorption by the blood vessels of the peritoneal membrane (e.g., reduction of membrane surface for absorption of the soluble rutin and inhibition by saturation of receptors involved in the absorption). Moreover, further toxicological studies about the possible deleterious effect of high doses of flavonoid are needed to help explain the better results of lower doses.

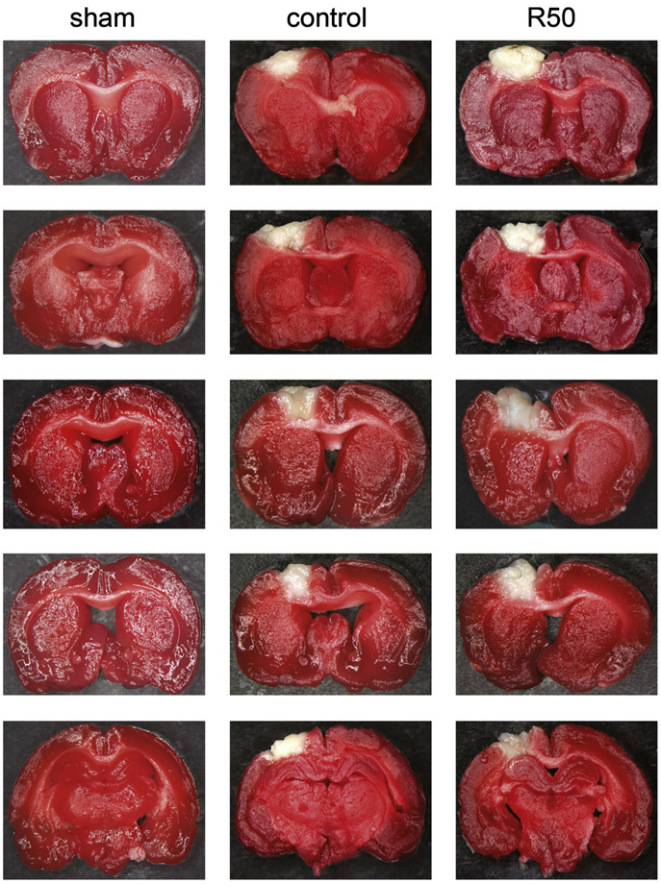

Fig. 4 – Extension of the ischemic lesion induced by thermocoagulation. Sequential images of coronal brain slices of a sham animal (representative of four sham animals), a control animal (representative of four control animals) and a R50 animal (representative of four R50 animals). All sections were reacted with TTC 48 h after surgery. From top to bottom, the images of each animal were placed in order from most rostral portion to most caudal portion of the ischemic lesion. In sham animals, the same regions were analyzed, but no lesion was observed. Each line represents the same stereotaxic region, and these five regions were standardized for lesion volume quantification.

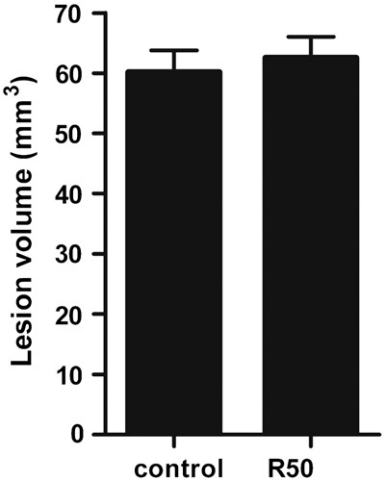

Fig. 5 – Evaluation of ischemic lesion volume. The dose of 50 mg/kg of rutin promoted no significant reduction in ischemic lesion volume. Control group, $n=4$; R50 group, $n=4$. Bars in the graph represent mean+SEM.

Similar to other flavonoids, the main expected mechanisms of action of rutin are its anti-inflammatory and antioxidative potential. In fact, anti-inflammatory action of rutin was demonstrated with reduction of inducible nitric oxide synthase expression in a model of Parkinson's disease (Khan et al., 2012). Neuroprotective effect of rutin was also correlated to its action as an antioxidant. Rutin has been described as a scavenger of superoxide radicals, which is highly formed during ischemic process (Khan et al., 2009). Pretreatment with rutin resulted in attenuation of the elevated levels of thiobarbituric acid reactive species, hydrogen peroxide and protein carbonyl induced by ischemia (Gupta et al., 2003; Khan et al., 2009). Moreover, its action also includes protection of biological antioxidative systems. Pretreatment with rutin resulted in protection against inhibition of antioxidant enzymes activity after MCAO (Khan et al., 2009). Indeed, beside these neuroprotective actions on already established ischemic injury, the therapeutic potential of rutin should be still higher. Rutin was recently found to be an inhibitor of

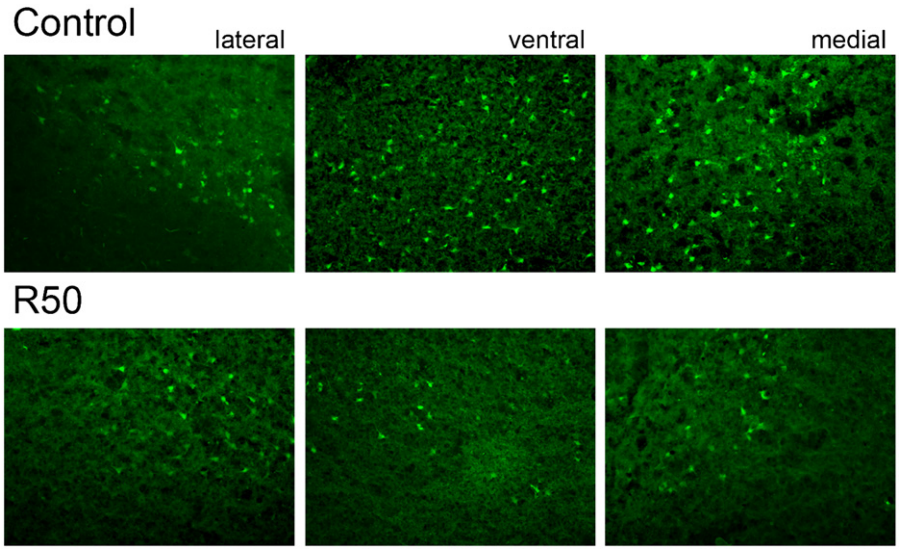

Fig. 6 – Evaluation of neurodegeneration after ischemia by FJC staining. Images of the three portions (lateral, ventral and medial) of the lesion periphery that were analyzed, taken from representative coronal sections of control and R50 animals. Note that the number of FJC$^+$ (degenerating) neurons was greater in control than in R50 animals.

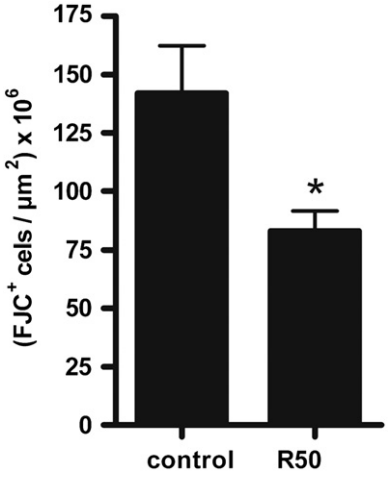

Fig. 7 – Quantification of degenerating neurons. The dose of 50 mg/kg of rutin promoted significant reduction in the number of FJC$^+$ neurons in the lesion periphery. Control group, $n=4$; R50 group, $n=4$. Bars in the graph represent mean+SEM. $^*=p<0.05$.

protein disulfide isomerase and this action potently blocks thrombus formation in mice, pointing to rutin as a preventive approach for cardiac ischemia and stroke (Jasuja et al., 2012).

In conclusion, the study contributes to suggest the flavonoid rutin as a putative candidate to treat stroke. Beside previous descriptions of the efficacy of pre-treatment in models of brain ischemia, the results suggest that its neuroprotective effect is also relevant to be used after the occurrence of stroke, in the acute phase of the disease. Thus, flavonoids might be suggested as another option in the arsenal of possible therapeutic approaches to treat stroke. Increasing studies about neuroprotective action of flavonoids in animal models of brain ischemia might support, soon, further clinical trials with this class of drugs.

4. Experimental procedures

4.1. *Animals*

The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of our institution. Male Wistar rats which were 2–3 months of age at the beginning of the experiment were used. All animals were housed in a colony room with controlled temperature, and with food and water available *ad libitum*. Before experimental procedures, animals were submitted to handling for five consecutive days to adapt to the experimenter and minimize stress.

4.2. *Surgery*

Thermocoagulation of the blood in the submeningeal blood vessels of the motor and sensorimotor cortices was used to induce ischemic lesion as previously described (Giraldi-Guimarães et al., 2009; Szele et al., 1995). Briefly, animals were anesthetized with ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg) and placed in a stereotaxic apparatus (Insight Ltda., Ribeirão Preto, SP, Brazil). Skull was exposed, and a craniotomy was performed, exposing the frontoparietal cortex contralateral to the preferred forelimb in the adhesive test (see Section 2.4.) (+2 to −6 mm A.P. from bregma; according to the atlas of Paxinos and Watson (2005). Blood was thermocoagulated transdurally by approximation of a hot probe to the dura mater. Sham

operated animals suffered only the craniotomy. After procedure, skin was sutured, and animals were kept warm under a hot lamp and returned to colony room after recovery from anesthesia.

4.3. *Rutin administration*

The flavonoid rutin was purchased commercially (Sigma-Aldrich, St. Louis, MO, USA). Rutin was diluted in propylene glycol. To facilitate the dissolution of rutin, the solution was made to stand for 15 min in a water bath at 50 °C for 10 min. Rutin solution or vehicle (propylene glycol) was administered by intraperitoneal (i.p.) injection. Ischemic animals were divided into three experimental groups: one that received vehicle (control group), one that received the dose of 50 mg of rutin/kg of body weight (R50 group) and one that received the dose of 100 mg/kg (R100 group). These doses were chosen from previous studies showing protective effect of rutin in models of global brain ischemia (Abd-El-Fattah et al., 2010; Pu et al., 2007). For behavioral analyses, all groups were used and the protocol of treatment was a daily injection during five consecutive days, starting just after the end of surgical procedure. In other analyses, as explained below, control and R50 groups were used with changes in protocol of treatment.

4.4. *Behavioral tests*

Functional recovery of the forelimb contralateral to the ischemic cortical hemisphere was evaluated using two sensorimotor tests: cylinder test and adhesive test (Schallert, 2006). Their effectiveness to assess sensorimotor function has been shown after thermocoagulatory cortical lesion (de Vasconcelos dos Santos et al., 2010, Giraldi-Guimarães et al., 2009). All animals were tested one day before ischemia and at post-ischemic day (PID) 2, and then weekly. Pre-ischemic day was plotted in graphs as PID 0. Tests were performed as previously described (de Vasconcelos dos Santos et al., 2010, Giraldi-Guimarães et al., 2009). Briefly, in the forelimb use asymmetry (cylinder) test, a trial consisted in placing the animal inside a glass cylinder (20 cm diameter X 30 cm height). Supports in the wall with ipsilateral (to the lesion) forelimb, contralateral forelimb or simultaneous support with both forelimbs were counted during vertical exploration. For each animal at each PID, percentage relative to the total number of uses (ipsilateral+contralateral+simultaneous) was calculated for ipsilateral (unimpaired) and contralateral (impaired) uses. An asymmetry score for each animal was calculated at each PID by the following formula: asymmetry score=(% of ipsilateral uses)−(% of contralateral uses). Animals with asymmetry score higher than 15 at PID 0 were discarded for statistical analysis.

In the adhesive removal patch test, a small round adhesive paper (13 mm diameter) was placed on the inner portion of each wrist of the animal. One trial consisted in placing the adhesive papers and their subsequent removal by the animal. Four trials were applied at each PID, and trials were always separated by at least 5 min. Preference was evaluated, and in each trial the first side (ipsilateral or contralateral to the lesion) of removal was recorded. For each animal at each PID, percentage of contralateral preference relative to the total number of removals (four) was calculated. Animals with preference to the right forelimb (more than 50% of first removal at pre-ischemic day) suffered focal ischemia in the left hemisphere (see Section 2.2.), and vice-versa. To check for lack of influence of whole experimental procedure in functional loss, untreated sham animals were also evaluated in adhesive test.

4.5. *Detection of rutin in plasma by high performance liquid chromatography (HPLC) coupled with a diode array detector*

To evaluate the plasmatic absorption of rutin after an i.p. injection, animals from R50 group were euthanized with CO_2 2, 4, 6 or 8 h after the injection. Animals from the control group were also evaluated. Blood was collected by cardiac puncture with heparin and the plasma obtained by centrifugation at 12,000 g for 10 min. Plasma was acidified to pH 4.0 with phosphoric acid. After acidification, methanol was added (1000 μl: 200 μl of plasma), and the sample was stirred for 1 min and centrifuged at 12,000 g for 10 min. Supernatant was collected, and the organic solvent was evaporated. Pellet was reconstituted with 200 μl of acidified water and analyzed using HPLC (LC-100, Shimadzu®) with reverse-phase column (RP-18, 5 μm, 4.0 × 250 mm^2, Merck®), detector (SPD-M20A, prominence diode array detector, Shimadzu®), loop injection of 20 μL, pump (LC 20 AT, prominence liquid chromatograph, Shimadzu®), injector (Rheodyne 7725i) and software LC Solution. The eluents were purified water adjusted to pH 3.2 with formic acid (A) and acetonitrile (B). The following solvent gradient was applied: from 100% A and 0% B to 80% A and 20% B within 10 min; from 80% A and 20% B to 75% A and 25% B within 5 min; from 75% A and 25% B to 70% A and 30% B within 10 min; from 70% A and 30% B to 50% A and 50% B within 10 min; and from 50% A and 50% B to 0% A and 100% B within 15 min (total analysis time: 45 min). Flow elution was 1 mL min^{-1}; 20 μL of plasma samples were injected. UV–vis spectra were recorded in the range 210–350 nm.

The flavonoid quantification was carried out using calibration graph with nine data points. Calibration graph for HPLC was recorded with rutin amounts ranging from 0.156 to 50.0 μg/mL. The relationship between peak areas (detector responses) and amount of rutin was linear (r^2=0.9953). To evaluate the repeatability of the injection integration, the rutin standard solution and all samples were injected three times and the relative standard deviation values were calculated. Identification was performed comparing the retention time (t_R) and UV spectrum of peaks in the samples of plasma with standard rutin: t_R=18.6 min (97.5% purity).

4.6. *Quantification of lesion volume*

To quantify the extension of lesion, animals from control and R50 groups suffered two injections, one just after the end of surgical procedure and other 24 h after ischemia. They were euthanized with CO_2 48 h after ischemia. Untreated sham animals were also evaluated to check for lack of cortical injury. Brains were rapidly removed from the skull and sectioned in the coronal plane at 2 mm thickness using a rat brain blocker/slicer (Insight Ltda.). The slices (five for each

animal) were immersed for 30 min into 2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution at 37 °C. Digital images from reacted slices were captured under conventional light illumination using a Nikon digital camera (Nikon Co., Tokyo, Japan) coupled to a dissecting microscope and a PC computer. Lesion areas of slices were measured from digital images using the ImageJ software (NIH). The lesion area of each slice was multiplied by its thickness (2 mm), obtaining the volume (mm^3). For each animal, the total lesion volume was calculated by summing the lesion volumes of its slices.

4.7. *Fluoro-Jade C (FJC) staining*

To analyze the effect of treatment with rutin in neurodegeneration, animals from control and R50 groups suffered three injections, one just after the end of surgical procedure, one 24 h and one 48 h after ischemia. They were euthanized with CO_2 72 h after ischemia and intracardially perfused with cold 0.9% NaCl solution followed by a solution of 4% paraformaldehyde, in 100 mM phosphate buffer (pH 7.4). Brains were removed and immersed in 100 mM phosphate buffer containing 20% sucrose for 24 h at 10 °C. Brains were sectioned in the coronal plane at 30 μm thickness at 20 °C on a CM 1850 cryostat (Leica Instruments GmbH, Heidelberg, Baden-Wurttemberg, Germany). Sections were subjected to FJC staining, in accordance with the manufacturer's instructions (Schmued et al., 2005). Briefly, they were immersed in a solution of 1% sodium hydroxide in 80% alcohol for 5 min, 70% alcohol for 2 min, distilled water for 2 min, and 0.06% potassium permanganate for 15 min. They were immersed into a solution of 0.0005% FJC (Histo-Chem Inc., Jefferson, AR, USA) in 0.1% acetic acid vehicle for staining for 30 min, rinsed three times in distilled water and allowed to dry at 45 °C for 20 min before mounting with DPX medium (Electron Microscopy Sciences, Hatfield, PA, USA).

FJC-positive (FJC^+) cell counting was done as previously described (Giraldi-Guimarães et al., 2009), but with some changes. Six sections located inside the rostro-caudal extension of the lesion were selected per animal. Stereotaxic positions of the selected sections were standardized for all animals. Cortical tissue surrounding the ischemic lesion was considered the periphery of the lesion, and only this region was considered for quantification. At coronal plane, cortical ischemic lesion has three well defined regions: lateral, ventral and medial. For each section, a digital image was captured from lesion periphery in each lesion region. Images were taken under fluorescent illumination (fluorescein filter) using a Zeiss AxioCam digital camera coupled to an Axioplan microscope (Carl Zeiss Inc., Germany) and a PC computer with Zeiss Axiovision 4.8 Software. FJC^+ cells were counted from each image (18 images per animal), and the area where cells were included was measured using the ImageJ software. The final value for each animal was Σ (cells counted per image)/Σ (area containing labeled cells per image, in μm^2).

4.8. *Statistical analyses*

Nonlinear regression was done with the HPLC data. F test and AlCc were used to compare and find the best curve fit (Table 2). Unpaired *t* test was performed for comparison among groups in lesion volume and FJC^+ cell counting analyses. For behavioral analyses, repeated measures two-way ANOVA ("treatment" × "PID"; PID as the matched factor) was used, followed by Tukey multiple comparisons post test. The level of significance was set at $p<0.05$.

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