Neuroscience International

Original Research Paper

Green Tea Treatment Attenuates Oxidative Damage and Neuromotor Deficit Induced by an Experimental Model of Intracerebral Hemorrhage in Rats

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Abstract: The present study shows the neuroprotective effect of green tea supplementation (GT; *Camellia sinensis*) in a model of Intracerebral Hemorrhage (ICH) in rats. ICH was induced by intra-striatum collagenase infusion in male Wistar rats. GT (400 mg Kg^{-1}) was administered via gavage for 10 days after ICH. We assessed the effect of GT on neuromotor recovery and oxidative damage. Our results show that ICH causes neuromotor deficits observed by Neurological Deficit Scale (NDS), Open Field (OF) and Rotarod (RR). GT treatment attenuates this deficit on day 3 for NDS and OF and on day 7 for RR. Also, ICH increases Reactive Oxygen Species (ROS) production and lipid peroxidation, what is not observed in ICH + GT group. Currently, there are no effective pharmacologic or non-pharmacologic neuroprotective treatments for ICH. Nutritional interventions that help on its outcome are important, since they are generally accessible and have few side effects.

Keywords: Hemorrhagic Stroke, Green Tea, Nutritional Intervention, Neuroprotection, Neurological Deficit, Oxidative Imbalance

Introduction

Hemorrhagic stroke caused by spontaneous ICH represents 15 to 20% of all strokes (Flower and Smith, 2011). ICH is the more severe type of stroke and has serious development (Hwang *et al*., 2011). Most patients either die or are left with significant neurological morbidity (Flower and Smith, 2011). Moreover, the incidence of ICH is expected to grow, considering the increase on life expectancy of population.

The primary damage caused by ICH starts soon after the bleeding onset and is mainly due to hematoma formation, which compresses the surrounding brain tissue, thus destroying it (Xi *et al*., 2006). The progressive brain tissue deterioration suggests that secondary brain damage after ICH plays a fundamental role in neurological impairment (Babu *et al*., 2012), even without any signs of hematoma expansion.

Neurochemical alterations induced by secondary events cascade after ICH have not been well delineated, but they may represent important therapeutic targets to attenuate further brain damage. As a result of hemorrhage, hemoglobin and its breakdown products (iron, biliverdin and carbon monoxide) get in touch with brain parenchyma, activating cytotoxic, oxidative and inflammatory cascades (Xi *et al*., 2006; Aronowski and Zhao, 2011; Hu *et al*., 2016). Iron and iron-related compounds, including hemoglobin, increase hydroxyl radical production and oxidation of lipids (Sadrzadeh and Eaton, 1988), which expose the brain to higher levels of ROS. There is increasing evidence that oxidative stress contributes to ICH-induced secondary brain injury via generation of ROS (Hu *et al*., 2016). Despite the advances in research and care, very limited options of treatments are available and the recovery of patients with ICH is still poor. There is a compelling need for more options of therapeutic and adjuvant therapies to aid this critical population. Potential nutritional interventions targeting secondary brain injury are arousing a great deal of interest, especially those with an antioxidant component.

Green tea (*Camellia sinensis*) has been reckoned as a possible source of antioxidants available through diet (Wu *et al*., 2012; Xu *et al*., 2010). Catechins, a group of flavonoids, constitute around 30-45% of the solid GT extract (reviewed in (Mak, 2012)). The beneficial effects of tea consumption are probably due to its bioactive components (catechins and their derivatives), demonstrated to act directly as radical scavengers and to exert indirect antioxidant effects through activation of transcription factors and phase II antioxidant defense, thus modulating the cellular redox state (see review: Mandel *et al*., (2006). The Epigallocatechin Gallate (EGCG) is a major component of GT and the administration of both GT extract and EGCG has shown to be a neuroprotective compound in experimental models of ischemic stroke, protecting the brain against oxidative stress, neuromotor deficit and lesion volume in both rats and mice (Wu *et al*., 2012; Chang *et al*., 2014; Shah *et al*., 2010; Schimidt *et al*., 2014).

Although ischemia and ICH share many secondary events cascade, fundamental physiopathological differences remain (Shah *et al*., 2010).

Thus, it is important to test potential treatments in ICH models instead of rely on research findings in ischemia models. To study the effects of a popular drink such as GT could be a useful tool for the treatment of ICH. Besides, this is a non-invasive low cost nutritional intervention. Therefore, our aim is to investigate whether green tea treatment can protect against oxidative damage and neuromotor deficit induced by ICH in rats.

Materials and Methods

Animals and Reagents

All procedures followed the "Principles of laboratory animal care" (NIH publication No. 80-23, revised 1996) and the guidelines established by the Institutional Animal Care and Use Committee of the Local Institution (Approval n. 014/2014). We used 41 male Wistar rats (250-300 g, ~3 months old) randomly assigned to 4 groups with blinded assessment. Rats were grouped 4 per cage and maintained under controlled environmental conditions (12 h light/12 h dark cycle at temperature 23±2°C and humidity 50±10%) with food and water *ad libitum*.

Green tea was purchased from Madrugada Co. All the other drugs were purchased from Sigma-Aldrich.

Experimental Procedures

Surgery and Experimental Groups

Surgical procedures were performed aseptically. Rats were anesthetized with ketamine and xylazine (i.p., 75 and 10 mg kg^{-1}, respectively). Body temperature was maintained at 37°C during anesthesia with a heating pad. After placing the animals in a stereotaxic frame a hole was drilled 3.5 mm right and 0.5 mm anterior to Bregma. A 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV, USA) was inserted 6.5 mm unilaterally into the right striatum to infuse 1 μL of sterile saline, containing 0.2 U bacterial collagenase (Type IV) in the case of ICH groups, over 5 min. The needle was left in place for 5 min and then slowly removed. The hole was sealed with a metal screw, clips were used to close the wound and lidocaine was used.

Animals were randomly divided into four groups: Sham (n = 10); ICH (n = 10); Sham + GT (n = 11); ICH + GT (n = 10).

Green Tea Supplementation

Animals treated with GT had the first oral administration by gavage (400 mg Kg^{-1}) (Xu *et al*., 2010) four hours after surgery and for the following 10 days (Chang *et al*., 2014). GT infusion of 400 mg kg^{-1} per day for rats is equivalent to 400 mL day^{-1} for humans (Xu *et al*., 2010).

Green tea infusion was freshly prepared daily and administered at ambient temperature. Control groups received the same volume of tap water. Green tea samples from Madrugada Co. used in this study were purchased from standard markets and analyzed by High Performance Liquid Chromatography (HPLC), which ensured the presence of epicatechin (concentration of 1.3 mg mL^{-1}), epigalocatechin gallate EGCG (3.7 mg mL^{-1}) and Epicatechin Gallate (ECG) (1.8 mg mL^{-1}).

Behavioral Assessment

All animals were subjected to behavioral assessment followed by biochemical analyses at 11-day survival (Fig. 1).

Neurological Deficit Scale (NDS)

This battery of tests sensitive to striatal damage (Del Bigio *et al*., 1996) was used on post-surgery days 1, 3 and 7 (vs. days before surgery). Briefly, rats were assessed on spontaneous circling, hind limb retraction, bilateral forepaw grasp, contralateral forelimb flexion and beam walking. A maximum score of 14 denotes greatest neurological impairment.

Open Field

The open field test was carried out in a wooden box (50×60×60 cm) divided into 16 regular square areas. Each rat was set in the center of the box at the beginning of the session and was allowed to explore the arena for 5 min. Locomotion (number of squares crossed with the four paws), was assessed. About 70% ethanol was used to clean the arena between each rat.

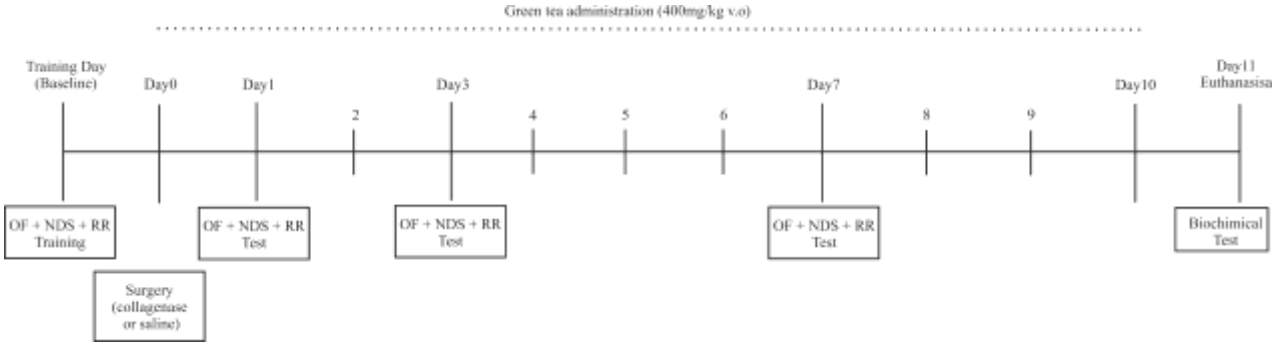


Fig. 1. Representation of experimental design. One day before surgery, animals were trained in Open Field task (OF), Neurological Deficit Scale (NDS) and Rota Rod (RR) for baseline values. At day 0 the surgery procedure was performed to induce hemorrhage injury (collagenase) or sham injury (saline). Green tea treatment started four hours after injury and lasted 10 days. The animals were tested on OF, NDS and RR at day 1, 3 and 7 after surgery

Rotarod

Rotarod is a test that assesses motor coordination, balance and muscle strength. The apparatus consists of a cylinder with a diameter of 7.62 cm; suspended 20 cm from the device surface, run by a motor that keeps a constant speed.

Before the training session, rats were habituated to stay on the stationary drum for 3 min. Habituation was repeated every day (days 1, 3 and 7) for 1 min just before the testing session. On the training day, the speed used was of 16 rotations per minute and on testing days, it was of 25 rotations per minute.

Rats were placed on the cylinder and the first fall was timed to a maximum of 360 sec as well as the number of falls up to five times in one session (Shiotsuki *et al*., 2010; Stroobants *et al*., 2013).

Biochemical Assays

Tissue Preparation

Rats of all groups were euthanized 24h after the last GT administration. Their brains were removed and ipisilateral striatum was quickly dissected out and homogenized in 50 mM TrisHCl, pH 7.4, (1/10, w/v). Afterwards, samples were centrifuged at 2400g for 10 min and supernatants (S1) were used for assay.

Reactive Oxygen Species (ROS)

Spectrofluorimetric method using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe was used to evaluate ROS content (Ali *et al*., 1992). The sample (S1) was incubated in the dark with 5 µL DCFH-DA (1 mM). Detection of intracellular ROS was given by DCHF-DA oxidation to fluorescent dichlorofluorescein (DCF) The formation of the oxidized fluorescent derivative (DCF), measured by DCF fluorescence intensity, was recorded at 520 nm (480 nm excitation) 30 min after the addition of DCFH-DA to the medium. Results were expressed as % of control of AU (arbitrary units).

Detection of TBARS Level

Lipoperoxidation was assessed through the Thiobarbituric Acid Reactive Substance (TBARS) test (Ohkawa *et al*., 1979). One aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95ºC for 2h. Color reaction was measured at 532 nm and results were expressed as nmol of Malondialdehyde (MDA) per mg of protein.

Glutathione (GSH)

GSH levels were fluorometrically determined (Hissin and Hilf, 1976). An aliquot of homogenized sample was mixed (1:1) with perchloric acid ($HClO_4$) and centrifuged at 3000 g for 10 min. This mixture was centrifugated the protein pellet was discarded and free thiols (SH) groups were determined in the clear supernatant. An aliquot of supernatant was incubated with orto-phthaladehyde and fluorescence was measured at excitation of 350 nm and emission of 420 nm. Results were normalized by mg of protein and expressed as percent of control.

Glutathione Peroxidase (GPx) Activity

GPx activity was analyzed spectrophotometrically by the method of Paglia and Valentine (1967). GPx analysis was made by adding GSH, GR, NADPH and a peroxide to start the reaction, monitored at 340 nm as NADPH is converted to $NADP^+$.

Protein Determination

Protein content was measured colorimetrically by the method of (Bradford, 1976) using bovine serum albumin (1 mg mL^{-1}) as standard.

Statistical Analysis

Results are presented as mean ± Standard Error of the Mean (SEM). Results were analyzed by Two-way ANOVA with Bonferroni post-hoc test when appropriate.

Results

Behavioral Assessment

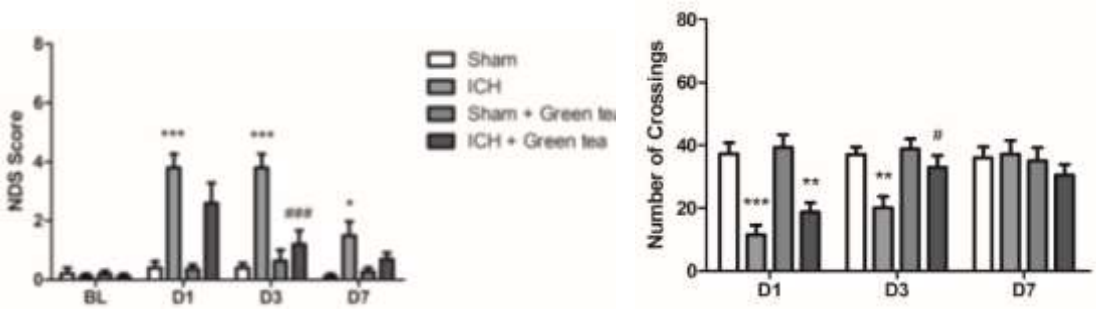
Green tea effects were assessed in NDS, OF and RR behavioral tasks. Figure 2A shows the effect of a 10-day GT administration (400 mg Kg^{-1}) on neuromotor deficit induced by ICH assessed in NDS. Two-way ANOVA showed that ICH induced neuromotor deficit on days 1 ($F(1,37) = 43.61$; $P = 0.0001$), 3 ($F(1,37) = 26.48$; $P<0.0001$) and 7 ($F(1,37) = 11.42$; $P = 0.001$) when compared to sham-operated rats. Besides, GT treatment attenuates this impairment on day 3 ($F(1,37) = 13.56$; $P = 0.0007$).

For locomotor activity assessed in OF the ICH group presented locomotor disability on days 1 ($F(1,37) = 43.95$; $P<0.0001$) and 3 ($F(1,37) = 11.40$; $P = 0.0017$) after surgery) (Fig. 2B). Besides, the treatment with GT did not change this effect on day 1 ($F(1,37) = 0.58$; $P>0.05$) though on day 3 GT reverted the locomotion deficit ($F(1,37) = 4.78$; $P<0.05$).

We also used the RR test to assess equilibrium and coordination. Figure 3 shows that motor deficit was induced by ICH. This was characterized by the lower latency to first fall (Fig. 3A) on days 1 ($F(1,37) = 48.61$; $P<0.0001$), 3 ($F(1,37) = 10.55$; $P = 0.0025$) and 7 ($F(1,37) = 22,7$; $P<0.0001$); and an increase on number of falls (Fig. 3B) on all days tested ($F(1,37) = 23.99$; $P<0.0001$ for day 01; $F(1,37) = 19.33$; $P = 0.001$ for day 3; $F(1,37) = 20,39$; $P<0.0001$ for day 7) in the ICH group. Green tea treatment was able to revert this motor impairment only on day 7 for both latency to first fall ($F(1,37) = 7.15$; $P = 0.011$) and number of falls ($F(1,37) = 6.65$; $P = 0.014$).

Biochemical Assays

To assess the effect of GT treatment on oxidative imbalance we considered ROS production (Fig. 4A), levels of lipid peroxidation (Fig. 4B), levels of GSH (Fig. 4C) and GPx activity (Fig. 4D). We observed that ICH induced the increase on ROS production ($F(1,37) = 8,91$; $P = 0.005$) and lipid peroxidation ($F(1,37) = 14.05$; $P = 0.0006$) when compared to sham-operated rats. There was difference between ICH + GT-treated group when compared to ICH group in ROS ($F(1,37) = 5.77$; $P = 0.02$), but not in TBARS ($F(1,37) = 2.44$ $P = 0.12$). However, TBARS levels were not different between sham and ICH + GT-treated group ($F(1,37) = 0.66$; $P = 0.42$). For antioxidant markers, we observed there were no differences between groups on GSH levels ($F(1,37) = 0.001$; $P>0.05$) and GPx activity ($F(1,37) = 0.26$; $P>0.05$).



(A) (B)

Fig. 2. Effects of green tea administration (400 mg Kg^{-1} p.o) on neuromotor deficit induced by ICH on Neurological Deficit Scale (A) and on locomotor activity (Open Field test) (B) evaluations. Data are presented as the mean ± S.E.M. Two-Way Anova *P<0.05; **P<0.01 and *** P<0.001 compared with vehicle-treated group, # P<0.05 and ## P<0.001 compared with ICH group (n = 10-11)

(A) (B)

Fig. 3. Effects of green tea administration (400 mg Kg^{-1} p.o) on neuromotor deficit induced by ICH on Rota Rod evaluation. (A) Latency to first fall in seconds on Rota Rod. (B) Number of falls on Rota Rod. Data are presented as the mean ± S.E.M. Two-Way Anova *P<0.05; **P<0.01 and *** P<0.001 compared with vehicle-treated group, # P<0.05 compared with ICH group (n = 10-11)

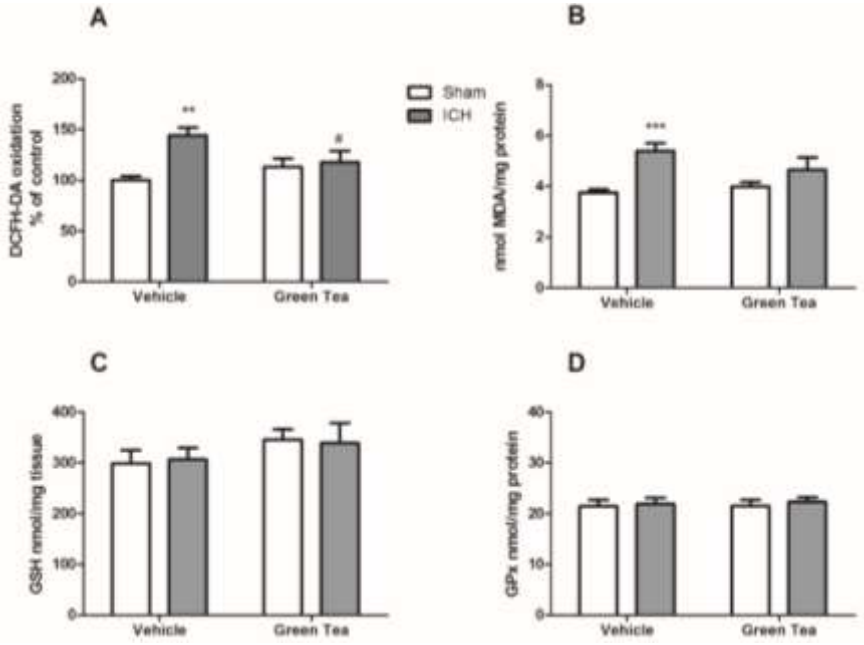


Fig. 4. Effects of green tea administration (400 mg Kg^{-1} p.o) on oxidative status after ICH. (A) Effect of green tea and ICH on ROS production, (B lipid peroxidation, (C) glutathione levels and (D) glutathione peroxidase activity. Data are presented as the mean ± S.E.M. Two-Way Anova **P<0.01 and *** P<0.001 compared with vehicle-treated group, #P<0.05 compared with ICH group (n = 10-11)

Discussion

It has been reported that long term green tea consumption is beneficial to human health because the leaves are a source of natural compounds with biological and pharmacological activities (Mandel *et al*., 2006). Indeed, habitual consumption is associated with a reduced risk of ischemic and hemorrhagic stroke (Tanabe *et al*., 2008). The beneficial effects of tea consumption are mainly due to bioactive components, catechins polyphenols and their derivatives, which have shown to act directly as radical scavengers and to exert indirect antioxidant effects through activation of transcription factors and antioxidant bioactive components, catechins polyphenols and their derivatives, which have shown to act directly as radical scavengers and to exert indirect antioxidant effects through activation of transcription factors and antioxidant enzymes, thus modulating the cellular redox state (see review: (Mandel *et al*., 2006)).

Despite the information regarding the effects of GT and GT polyphenols on several neurodegenerative disorders (reviewed in (Mak, 2012; Mandel *et al*., 2006), there is little information in literature about the use of GT to treat hemorrhagic stroke. In this study, using a collagenase model of ICH in rats, we show that GT treatment attenuates neuromotor deficit and striatum oxidative stress.

Green tea treatment starting four hours following ICH presented different levels of neuroprotection in these motor tests. In NDS, GT treatment shows positive effects only on day 3. On day 7, the ICH group showed spontaneous recovery, therefore it was not possible to assess the benefits of GT. Similarly to NDS, GT presented positive effects only on day 3 in the OF test. Our results are in accordance with the literature, since Lu *et al*. (2015) have previously demonstrated that ICH in basal ganglia induced motor deficit but spontaneous recovery occurs along the days. In the RR test, however, GT was effective only on day 7. This difference in motor recuperation and GT effect can be explained by the complexity to perform the motor test and RR is a more difficult task to perform than NDS and OF since it requires balance, grip strength and motor coordination. This set of data shows that GT attenuates neuromotor deficit induced by hemorrhagic stroke, as previously demonstrated with ischemic stroke. It has been previously shown that GT extract and EGCG administration (the major component of green tea) attenuate neuromotor deficit, oxidative stress and lesion volume in an experimental model of focal and global ischemia (Wu *et al*., 2012; Chang *et al*., 2014; Shah *et al*., 2010).

Chang *et al*. (2014) had previously studied the effect of Epicatechin (EC) on a model of hemorrhagic stroke and shown that EC induces the activation of Nrf2 and protects against oxidative stress, neuromotor deficit and lesion volume in mice (Chang *et al*., 2014). In our case, we studied the effects of green tea mixture, without isolating any compound, considering that antioxidant

characteristic of green tea is not related to a specific kind of polyphenol but to the combined activity of diverse antioxidants present in leaves, including phenolic acids and polyphenols (reviewed (Yang and Landau, 2000)). These data are important, considering that isolated catechins are not always available and GT is more accessible. Also, considering the multifactorial nature of ICH, treatments with specific targets could be ineffective. However, a single drug or cocktail of drugs with pluripharmacological properties could be more appropriate to be employed. ICH is the most devastating subtype of stroke with only ~20% of survivors regaining functional independence (Flower and Smith, 2011), emphasizing the importance to search for practices and effective strategies to treat it. At present there are no effective treatments and patient management is largely supportive.

It is well known that ROS is normal consequence of metabolic reactions, but high ROS levels can induce cellular death (Juranek and Bezek, 2005). Increasing evidence has shown that oxidative stress is implicated in the development of secondary neuronal injury, including cerebral edema, breakdown of the blood-brain barrier (Hu *et al*., 2016) and might also contribute to the outcome of ICH (Wang *et al*., 2006). Indeed, after autologous blood injection protein carbonyl formation, a measure of protein oxidation, have been found detected (Hall *et al*., 2000; Wagner *et al*., 2002).

In addition, brain edema and neuromotor were detected after intrastriatal infusion of lysed erythrocytes (Wu *et al*., 2002). Taken together this studies provide compelling evidences that ROS may contribute to ICH secondary brain injury.

In this study we showed that ICH induces an increase in ROS production and lipid peroxidation in the striatum. We have chosen the striatum because this structure is directly involved in motor control (Groenewegen, 2003) and because of the ICH model used here (see surgery methods). Importantly, green tea treatment avoids the increase of ROS and lipid peroxidation induced by ICH. In this sense, some studies have shown the efficacy of antioxidants as therapeutic agents for ICH. It has been previously shown that the administration of dimethylthiourea, deferoxamine, or edaravone reduces brain injury in experimental models of ICH (Chen *et al*., 2014; Aronowski and Hall, 2005). These results suggest that compounds that interrupt the free radical cascade might improve ICH outcome. Although there are a number of ongoing clinical trials (Xi *et al*., 2014), currently there is no FDA-approved treatment for ICH and the use of alternative means such as green tea, which could be part of people's diet, could be an interesting, inexpensive and easy way to avoid some ICH sequelae without side effects. In this study, we did not show any difference on GSH levels and GPx activity. Although these are not in agreement with the literature (Shang *et al*., 2013), they can be explained by differences in protocols of ICH induction and oxidative stress measurement and time.

The dose of 400 mg kg^{-1} of GT was chosen based on the study of Xu *et al*. (2006) in which they found memory improvement and reduced levels of pro-oxidants biomarkers after ischemic brain injury with a dose of 400 mg Kg^{-1} but not with a dose of 100 mg Kg^{-1}.

Neuroprotective interventions should be attempted before a stroke occurs (prevention) or very soon afterwards. The time to start treatment (four hours after ICH) was based on studies that revealed a therapeutic window (approximately 6 hours) between the injury onset and irreversible neuronal death related to stroke (Xu *et al*., 2006). ICH is a serious medical condition whose outcome can be impacted by early care (Hemphill *et al*., 2015). Green tea and green tea polyphenols are nontoxic, brain-permeable (Abd El Mohsen *et al*., 2002), natural compounds, reported to have multifunctional activities, being radical scavengers, iron chelators, anti-inflammatory and neuroprotectants as reviewed by (Mandel *et al*., 2004). However, the antioxidant/metal chelating activity attributed to catechin polyphenols are unlikely to be the only mechanism for their neuroprotective and neurorescue capacity. Thus, catechin polyphenols were found to invoke many mechanisms of action responsible for cellular survival (reviewed by (Weinreb *et al*., 2004).

Conclusion

Our study showed a neuroprotective effect of GT in an experimental model of ICH. GT attenuates neuromotor deficit and avoids the increase of ROS and lipid peroxidation. However, it is still necessary to investigate additional mechanisms that can be involved in the action of GT on this condition.

Acknowledgment

This research was funded with a student fellowship from CAPES-Brazil (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for M.S., C.A. and A.M. and was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; PROCAD 071/2013 granted to P.M.C.) and CNPq (Conselho Nacional de Pesquisa; Edital Universal 2013 granted to P.M.C.).

Author Contributions

Mauren Assis Souza: Designed and conducted the research and had primary responsibility for the final content.

Caroline Dalla Colletta Altermann, Alexandre dos Santos Martins and Priscila Marques Sosa: Parricipated in all experiments, coordinated the data-analysis and contributed to the writing fo the manuscript.

Cristiano Chiapinotto Spiazzi: Conducted the research and contributed to the writing of the manuscript.

Pamela Billig Mello-Carpes: Designed the research and had primaty responsibility for the final content.

Francieli Weber Santos: Provieded essential reagents, or provided essential materials and contributed to the writing of the manuscript.

Ethics

The authors strictly observed the ethical guidelines provided in the use of the animals for this research work and ehical approval for the study was obtained from the Ethical committee for Animals Use of Federal University of Pampa. No other ethical issues are anticipated.

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