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ORIGINAL PAPER

# *Camellia sinensis* neuroprotective role in experimentally induced hydrocephalus in Wistar rats

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**Abstract**
*Purpose* This study tested possible neuroprotective effects of *Camellia sinensis*-extracted polyphenols in experimental hydrocephalus in young rats.
*Methods* Seven-day-old Wistar rats were used in this study. Pups were subjected to hydrocephalus induction by 20 % kaolin intracisternal injection. The polyphenol was administered intraperitoneally for 9 or 20 days from the induction of hydrocephalus. Clinical observations and behavioral tests were performed once a day. The animals, deeply anesthetized, were sacrificed by cardiac perfusion with saline 10 or 21 days after induction of hydrocephalus and their brains were removed. Preparations were made for histological analysis by hematoxylin and eosin, solochrome-cyanine, and immunohistochemistry for GFAP.
*Results* Histopathological analysis showed that animals treated with the polyphenol for 9 consecutive days displayed reduction on astrocyte activity on the corpus callosum and external capsule, shown by GFAP immunostaining. They also displayed thicker and myelinated corpus callosum, exhibiting a more intense solochrome-cyanine blue staining.
*Conclusion* Although these results demonstrate a possible neuroprotective effect at the initial onset of the disease, additional studies should be performed to obtain an effective and safe therapy for deeper studies in clinical trials.

**Keywords** Hydrocephalus · *Camellia sinensis* · Polyphenol · Immunohistochemistry

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## Introduction

Hydrocephalus cannot be considered a simple clinical identity, but a disease with complex physiopathology, that not only strikes cerebrospinal fluid (CSF) dynamics, but affects also other structures of the central nervous system. The imbalance between CSF production and absorption puts forward ventricular dilation and consequent distortions on the brain parenchyma, by compressing the hemispheres against the skull inner surface. The whole skull enlarges when cranial sutures are still apparent, in response to cerebral ventricles, thus increasing brain trauma by adding stretching force to the compression force. The first structures affected by intracranial pressure and ventricle dilation are the ependyma and the white matter near the brain ventricles [4, 5, 8, 14]. Furthermore, the change in dynamics, along with the accumulation of CSF in the dilated ventricle system, cause stagnation and difficulty in metabolites elimination [2]. This congestion of metabolites favors lipid peroxidation and free radicals formation, concurring to the appearance of tissue wounds. Additionally, the amount of proteolytic enzymes in the CSF causes digestion of the axonal myelin [11].

Catechin polyphenols have been used in different nervous system disorders including neurodegenerative diseases, cerebral ischemia, models of oxidative stress by hydrogen peroxide and hydrocephalus [7, 12]. They are found in abundance in the leaves of *Camellia sinensis* and correspond to approximately 26.7 % of the compounds present in green tea. This chemical group was chosen due to their potent antioxidant role as a free-radical scavenger, given by the number of hydroxyl radicals linked to their rings A and B [1, 3, 15].

Hydrocephalus treatment comprehends the derivation of the CFS circulation through implanting derivation systems

(shunts) or by third ventriculostomy. Nevertheless, it is still difficult to know exactly when this surgery should be performed, for there is a possibility that the ventricle stabilizes spontaneously. On the other hand, some patients need to undergo immediate intervention, in spite of having severe clinical troubles, such as infections, heart diseases, immune system shortfalls, etc. During the waiting time, there may appear irreversible lesions in the brain, caused by ischemia, lipid peroxidation and increasing free radicals formation. Therefore, the search for drugs or ways to protect the nervous tissue must be a part of hydrocephalus study. The purpose of this work is to demonstrate the neuroprotective benefits to different regions and brain structures, through behavior and histopathological studies on antioxidant polyphenols present in *C. sinensis* (green tea).

## Methods

All animals were treated in accordance with the Brazilian College of Animal Experimentation guidelines; protocols were approved by the local animal ethics committee. Fifty-six male Wistar rats were used in the composition of experimental groups. Animals were divided into six groups, separated according to the hydrocephalus evolution and polyphenol use (Table 1).

### Hydrocephalus induction and polyphenol administration

Seven-day-old pups were submitted to hydrocephalus induction by 20 % kaolin injection method [9]. After palpation and identification of the space existent in between the dorsal edge of the foramen magnum and the first cervical vertebra, a percutaneous suboccipital puncture was performed. Slowly, 0.04 ml of 20 % kaolin (aluminum silicate; Sigma, St. Louis MO) suspension in distilled water was injected.

From the second day after the hydrocephalus induction, the animals from groups HT10 and HT21 received a once-a-day dose of *C. sinensis* extract, rich in catechin polyphenols. The freeze-dried extract was diluted in saline in a proportion of 100 mg/ml and administered intraperitoneally, at a rate of 50 mg/kg.

### Behavior studies

From the fifth day after the hydrocephalus induction, an Open Field test was performed. The animals were put in an acrylic arena and watched for 2 min in order to evaluate hygiene cares, environment exploration and stance, according to the following scale: 4=alert, with normal exploration and stance; 3=discretely lethargic, with reduced activity but normal stance when stimulated; 2=kyphotic, walking, but its stance has a widened, unstable or ataxic base; 1=barely walking, but still eating; 0=near death or euthanized. The animals were submitted to a memory test in a T-Maze on days 9, 14, and 20 after the kaolin injection. This test evaluated their memorization capacity in choosing between the two branches on the labyrinth. They were timed since the lobby of the bigger branch until they reached the end of the chosen branch.

### Sample collecting

Each animal was deeply anesthetized with an intraperitoneal injection composed of 10 % ketamine and 10 % xylazine (at the rates of 0.1 and 0.05 mg for each 100 g of body weight). After a transcardiac perfusion, the cerebrum was removed in block, through a vertex craniectomy and fixed in 3 % paraformaldehyde diluted in phosphate-buffered saline 0.1 M (7.3–7.4 pH). Each cerebrum was then sectioned in the coronal plane, dividing it into anterior portion (frontal) and posterior portion (parietal), taking the optic chiasm as reference. Each portion was dehydrated in crescent alcohol solutions, diafanized in xylol, and blocked in paraffin.

### Histopathological studies

#### *Histology*

Anterior portions were cut coronarily into sections 5-μm thick, which were put upon histological slides and colored with hematoxylin and eosin (H&E) stain, in order to observe the general cytoarchitecture, structure distribution and cellular density. In the sections stained with solochrome cyanin, the myelination degree of the periventricular white matter and the thickness measure of the corpus callosum were observed.

**Table 1** Different experimental groups composition

| Group | Designation | Hydrocephalus | Use of polyphenol | Sacrifice (days old) | Sacrifice (induction days) |
|---|---|---|---|---|---|
| 1 | H10 | Yes | No | 17 | 10 |
| 2 | H21 | Yes | No | 28 | 21 |
| 3 | HT10 | Yes | Yes | 17 | 10 |
| 4 | HT21 | Yes | Yes | 28 | 21 |
| 5 | C10 | No | No | 17 | – |
| 6 | C21 | No | No | 28 | – |

*Immunohistochemistry*

The immunohistochemical method was used in order to detect reactive astrocytes. Sections were incubated with polyclonal anti-GFAP rabbit primary antibody (DAKO Z0334; Glostrup, Denmark), diluted 1/6,000 in overnight BSA at 4 °C. Afterwards, incubation with biotin conjugated secondary antibody (goat anti-rabbit dilution at 1/300; Santa Cruz Biotechnology SC-2040) occurred, as well as with tertiary antibody (steptravidin HRP at 1/400 dilution; Thermo Scientific JG 122591). Finally, sections were detected with diaminobenzidine (DAB, Sigma D5905) for 1 min. Negative controls were processed without the primary antibody.

*Photo documentation and morphometric analysis*

Photo documentation of the slides was performed with an AxiosKop2 (Carl Zeiss) optical microscope and an AxioCam Hrc (Carl Zeiss) digital camera, connected to a Pentium II computer, equipped with Axio Vision 3.1 software. Forty times objective magnification was used. Photographs of the corpus callosum region and measurement of its thickness were taken of the solochrome cyanin stained slides. As for the GFAP-immunostained slides, pictures were taken of the corpus callosum region and the external capsule. ImageJ (NIH) software was used to note the corpus callosum thickness in the solochrome cyanin stained slides and to count the cells in the GFAP-immunostained photomicrographs.

*Statistical analysis*

Data was analyzed using one-way ANOVA variance test, followed by Tukey–Kramer post-hoc test. Kruskal–Wallis variance analysis was performed, with post-hoc Dunn test to analyze the Open Field and the T-Maze behavior tests. Significant statistical difference was considered when $p<0.05$. Software used was Bioestat version 5.3 (Mamirauá Institute; Manaus, Brazil).

## Results

Clinic and behavior observations

Clinic and behavior aspects were observed daily after hydrocephalus induction. The affected animals showed macrocrania, arched skull roof, low eye position (similar to those of children with hydrocephalus), lethargy and broad-based stance with tiptoed stepping. On the back, they had an acute kyphotic curve, similar to a hunchback. The Open Field and T-Maze tests results were not sensitive enough to detect significantly different changes between the experimental groups in motor and exploratory activities, and in learning and memory development.

Histochemical analysis

The analysis of the general cytoarchitecture of the hydrocephalic rats cerebral stained with H&E showed evident signs of destruction, especially in the animals with severe ventricular dilation, regardless of the time of the disease progress. Morphological changes in the corpus callosum and in the external capsule were similar, characterized by strong edema and shredded fiber. Ependyma destruction could also be seen in the corpus callosum. As for the cortex, only a light edema of the cerebral parenchyma was observed. In the germinal matrix, located at the external angle of the lateral ventricles (an intense cell proliferation area), the quantity of cells undergoing the process of mitosis was taken into special account. Cellularity was more intense in this region when the animal was younger and had lesser ventricular dilation (Fig. 1).

The analysis of the stained corpus callosum with solochrome cyanin focuses on indirectly measuring the myelination degree on that structure. In visual confrontation of the corpus callosum of the control rats (C10 and C21) it was noted that the blue tonality was more intense in the older animals, indicating progressive myelination of this structure (Fig. 2a, b). Hydrocephalic animals (H10 and H21) had remarkably lighter blue stained corpus callosum than their controls (Fig. 2a–d). The animals from groups HT10 and HT21 showed lighter blue color than their controls, but stronger blue when compared to hydrocephalic animals without treatment (Fig. 2c–f). The thickness of the corpus callosum in the solochrome cyanin stained cerebrum was also measured. Averages are shown in Fig. 3.

Immunohistochemical analysis

Immunostained reactive astrocytes were observed and counted through the histochemical method for GFAP. Their density in the corpus callosum region and in the external capsule region was calculated by the same method. It was noted that the animals from H10 group presented intensely marked astrocytes in both regions, with its extensions rougher; whereas the animals from the group HT10 presented more discrete immunostaining, with thinner astrocytes extensions. The animals from control group (C10) did not exhibit noticeable reactive astrocytes in the studied regions (Fig. 4). Density averages of the corpus callosum and external capsule reactive astrocytes are shown in Fig. 5.

## Discussion

The analysis of the cerebral cytoarchitecture stained with H&E showed morphological changes mainly in the corpus

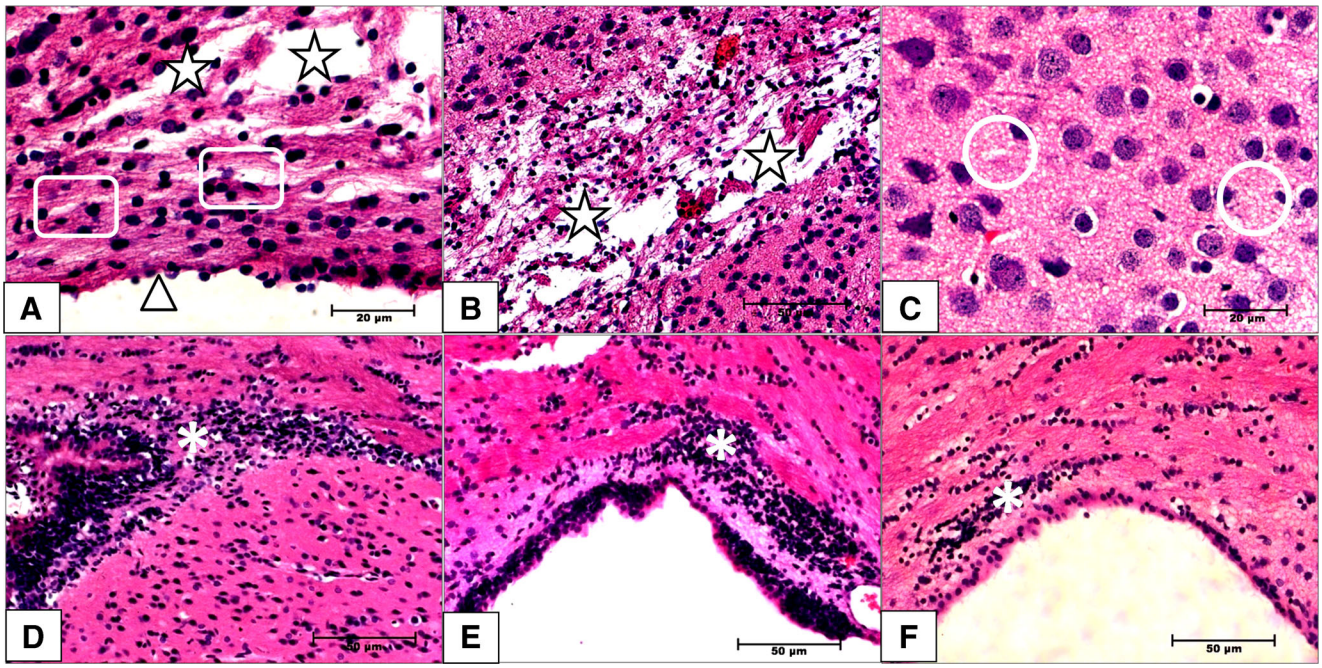


**Fig. 1** Photomicrographs of different cerebrum regions in 17- and 28-day-old rats (10 and 21 days after kaolin injection, respectively): **a** H21 (corpus callosum). **b** H21 (external capsule). **c** H21 (cerebral cortex). **d** C10 (germinal matrix). **e** H10 (germinal matrix with discrete dilation). **f** H10 (germinal matrix with severe dilation). Note the denudation of the ependyma (*arrowhead*), shredding (*stars*), severe edema (*squares*), and light edema (*circles*). Staining: hematoxylin and eosin. Objective magnification: external capsule and germinal matrix ×20; corpus callosum and cerebral cortex ×40. *Asterisks* cells undergoing mitosis in the germinal matrix

callosum, external capsule, dorsal cerebral cortex and germinal matrix regions. Probably due to their proximity to the ventricular cavities; where the lengthening and compression forces resultant of the ventricular dilation compromise the adjacent structures more intensely. We noted that all morphological changes were present in several degrees and directly related to the ventricular size, regardless of time, and the disease progress.

The corpus callosum myelination in rats begins around the 13th day postnatal, increasing considerably at the end of the first month. It goes on until they reach 10 months of age [6]. Postnatal and progressive maturation of the corpus callosum can be explained by the expressive quantity of neuroglia cells found in this structure in young rats. Studies have shown an increment in the number of these cells in 14-days old animals, demonstrating its function in the axonal myelination process. As time goes by and the quantity of myelinated fibers increase, neuroglial cells tend to decrease in number, indicating the end of this process [10].

Our results show a delay in the myelination in animals with hydrocephalus perceivable by the lighter intensity of the blue color when compared to its respective controls. This delay was even more severe when the ventricular dilation was bigger. On the other hand, there was distinct visual difference in the intensity of the blue color between hydrocephalic animals and hydrocephalic animals treated with the polyphenol. In both ages, 17 and 28 days old (10 and 21 days after the hydrocephalus induction, respectively), the corpus callosum was more strongly stained in blue in the group of hydrocephalic rats treated with polyphenol, suggesting a better myelination pattern in that region, due to the possible neuroprotective effect of the polyphenol that was administered to these animals.

The ventricular dilation causes axonal degeneration and demyelination of the periventricular tissue in hydrocephalus. The mechanism of injury can occur both by stretching and compression forces in the brain parenchyma, and by the edema formation and accumulation of proteolytic enzymes. Later, astrogliosis occurs, replacing the injured axons and myelin [11].

Through measuring the corpus callosum thickness, we attempted to directly obtain a parameter regarding the ventricular dilation degree, and to indirectly obtain its association with tissue lesion levels. We observed that rats with hydrocephalus that were treated with polyphenol exhibit a thicker and more preserved corpus callosum when compared to untreated ones. Nevertheless, this difference was significant only in animals 17-days old (HT10 and H10), not being seen in older groups. This can be explained perhaps by the polyphenol protective tendency, especially in the acute period of the disease, when the ventricular dilation starts. It is known that the polyphenol possible neuroprotective effects are not able to directly prevent or reduce ventricular dilation progression, but it can do so in its biomolecular mechanism related to the oxidative cascade, which, by its turn, damages the cerebral parenchyma through free radicals production. This occurs especially in the acute phase of the ventricular dilation [13].

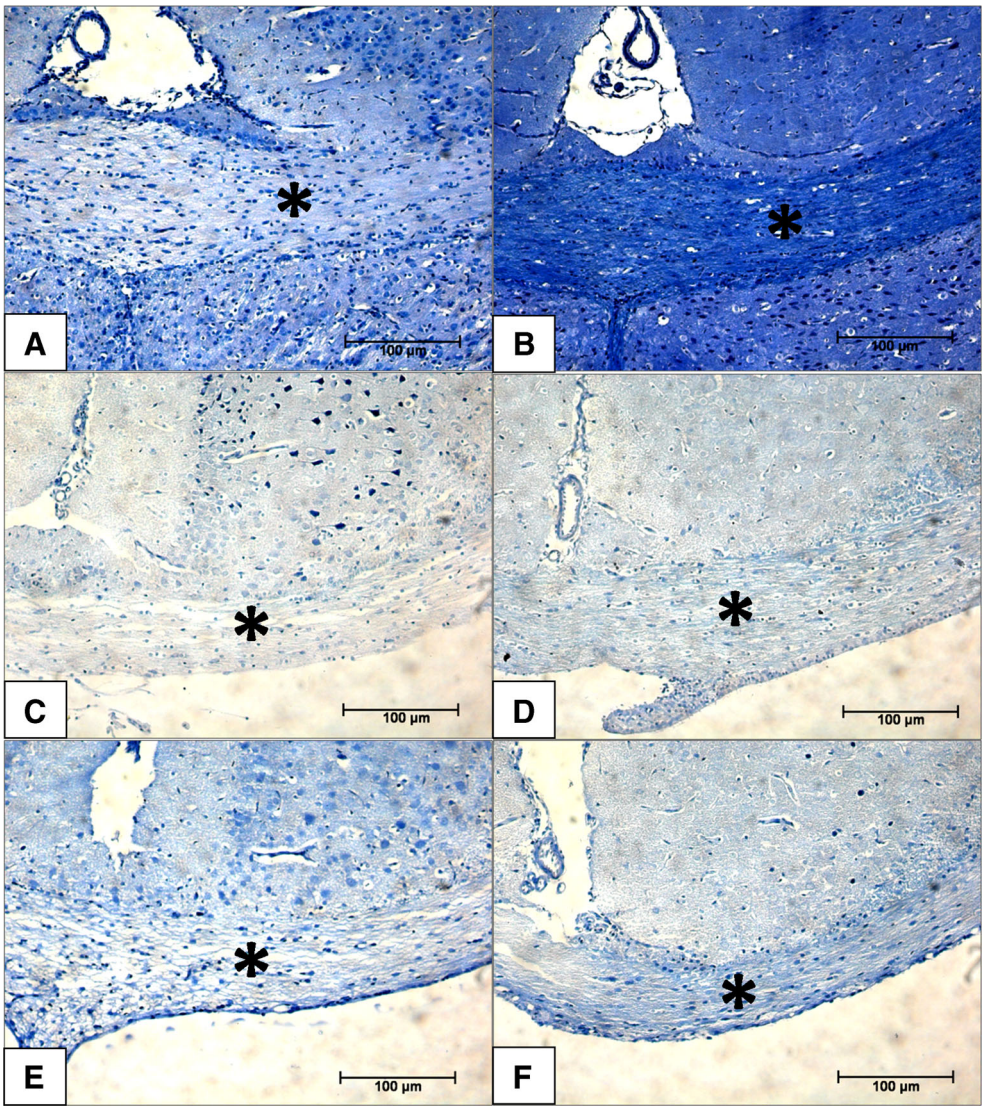


**Fig. 2** Photomicrograph of 17 and 28 days old rats' corpus callosum (10 and 21 days after the kaolin injection, respectively): **a** C10. **b** C21. **c** H10. **d** H21. **e** HT 10. **f** HT21. Staining: solochrome cyanin. Objective magnification: ×10. Light exposure: 90.0 ms. *Asterisks* corpus callosum

Correlating thickness with intensity blue hue of the corpus callosum, we believe the neuroprotective action of catechins has enabled a better pattern of myelination and therefore acted indirectly in preserving the thickness of the corpus callosum in the early days of hydrocephalus progression.

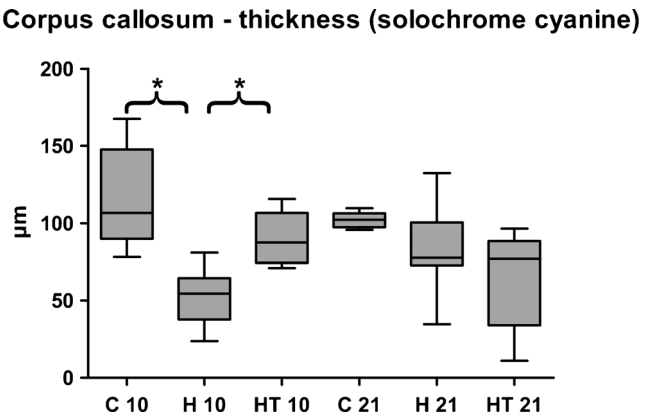


**Fig. 3** Representative graph for the thickness of the corpus callosum in the different experimental groups. *$p<0.05$

Atrocytes that were strongly immunostained by GFAP suggest intense cell activation to repair and heal the cerebral parenchyma submitted to different types of aggression. Lesser astrocyte density found in the corpus callosum and in the external capsule of the HT10 group demonstrates a smaller aggression degree in those brain structures in the animals treated with the polyphenol when compared to the H10 group. This suggests that the *C. sinensis*-extracted polyphenol might have had a neuroprotective role through its known antioxidant properties, thus protecting the tissue against damages caused by the releasing of reactive oxygen species. Other authors [7] came to satisfactory results in the biochemical field by administrating EGCG catechin in induced experimental hydrocephalus in 3-week-old rats. The authors concluded that the EGCG intraperitoneal injection (50 mg/kg) once a day for 15 days has significantly reduced

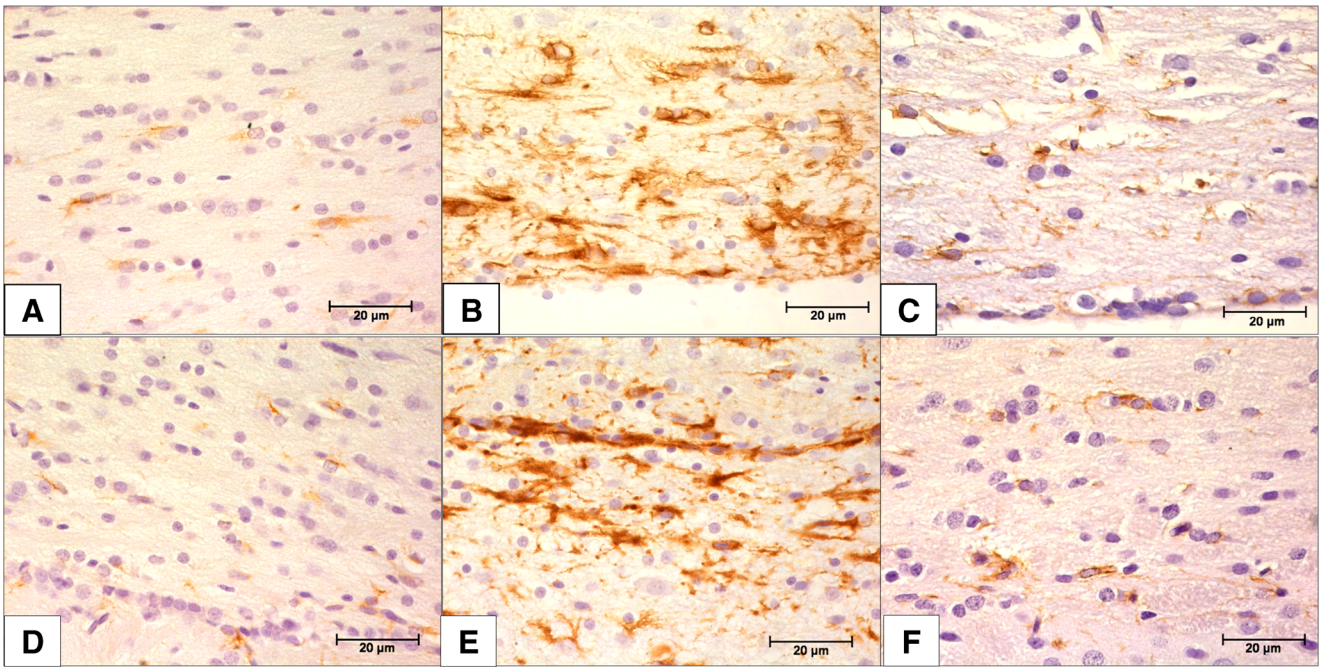


**Fig. 4** Photomicrograph of the corpus callosum (*upper pictures*) and external capsule (lower pictures) with immunostaining for 17-days old rats GFAP (21 days after kaolin injection): **a** and **d** C10. **b** and **e** H10. **c** and **f** HT10. Objective magnification: ×40 (immersion)

malondialdehyde levels in the periventricular white matter of treated rats when compared to hydrocephalic rats that had not received any treatment.

The beneficial role of the polyphenol only in the young animals (which were sacrificed 10 days after the disease's evolution) can be explained by the subsequent lesions found in the rats with more time in the hydrocephalus progression. In these animals, the ongoing increase of pressure gradient between the ventricles and the cerebral parenchyma would get worse the ventricular dilation, intensifying tissue aggression. We can also infer that the polyphenol role was effective only in the first days of the disease progress. Catechins do not act in structural bases to stop or slower ventricular dilation, but they work specifically in biochemical and molecular mechanisms, in an attempt to ameliorate the oxidative stress poisonous effects. Therefore, the polyphenol anti-oxidative effect would not be efficient to attenuate the harmful effects of progressive ventriculomegaly.

## Conclusions

The polyphenol that was administered for nine consecutive days after hydrocephalus induction reduced the astrocyte activity, pointed by the GFAP immunostaining on the corpus callosum and on the external capsule. Similarly, the thickness and myelination patterns in these animals were better preserved, showing a possible neuroprotective role in the initial phase of the disease. Nevertheless, additional studies must be performed in order to obtain efficient and safe therapies for deeper clinical studies.

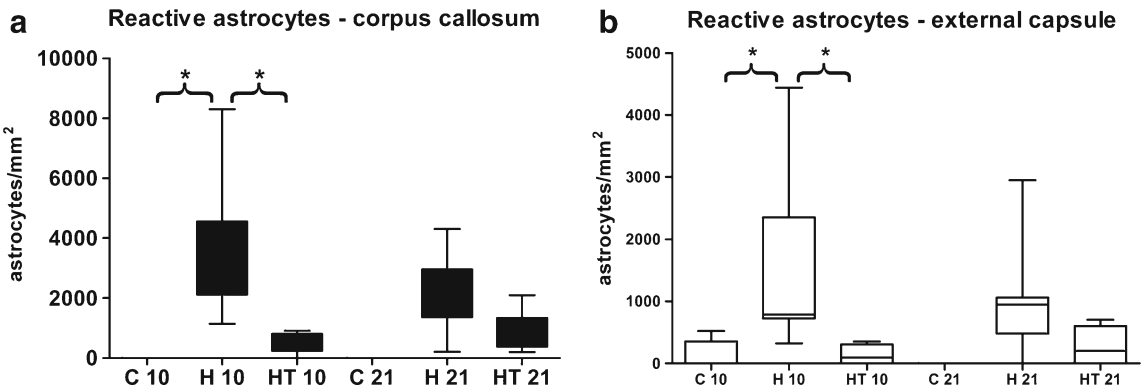


**Fig. 5** Representative graphs for the astrocytes density immunostained by GFAP antibody in the corpus callosum region (**a**) and in the external capsule region (**b**) in the different experimental groups. *$p < 0.05$

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**Conflict of interest** The authors declare that they have no conflict of interest.

## References

1. Anghileri LJ, Thouvenot P (2000) Natural polyphenols–iron interaction: its biological importance. Biol Trace Elem Res 73:251–258
2. Castro-Gago M, Rodriguez IN, Rodriguez-Núñez A, Guitián JP, Rocamonde SL, Rodriguez-Segade S (1989) Therapeutic criteria in hydrocephalic children. Childs Nerv Syst 5:361–363
3. Cook NC, Samman S (1996) Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. J Nutr Biochem 7:66–76
4. Del Bigio MR, Bruni JE, Fewer HD (1985) Human neonatal hydrocephalus. An electron microscopic study of the periventricular tissue. J Neurosurg 63:56–63
5. Del Bigio MR, Wang X, Wilson MJ (2002) Sodium channel-blocking agents are not of benefit to rats with kaolin-induced hydrocephalus. Neurosurgery 51:460–466
6. Elberger AJ (1982) The functional role of the corpus callosum in the developing visual system: a review. Prog Neurobiol 18:15–79
7. Etus V, Altug T, Belce A, Ceylan S (2003) Green tea polyphenol (−)-epigallocatechin gallate prevents oxidative damage on periventricular white matter of infantile rats with hydrocephalus. Tohoku J Exp Med 200:203–209
8. Kiefer M, Eymann R, von Tiling S, Müller A, Steudel WI, Booz KH (1998) The ependyma in chronic hydrocephalus. Childs Nerv Syst 14:263–270
9. LdaS L, Slobodian I, Del Bigio MR (2009) Characterization of juvenile and young adult mice following induction of hydrocephalus with kaolin. Exp Neurol 219:187–196
10. Lopes LS, Machado HR, Lachat J-J (2003) Study of callosum in experimental hydrocephalic Wistar rats. Acta Cir Bras 18:10–13
11. Mabe H, Suzuki K, Nagai H (1990) Cerebral blood flow after ventriculoperitoneal shunt in children with hydrocephalus. Childs Nerv Syst 6:388–391
12. Mandel S, Avramovich-Tirosh Y, Reznichenko L, Zheng H, Weinreb O, Amit T, Youdim MB (2005) Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway. Neurosignals 14:46–60
13. Socci DJ, Bjugstad KB, Jones HC, Pattisapu JV, Arendash GW (1999) Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. Exp Neurol 155:109–117
14. Tirapelli DP, Lopes Lda S, Lachat JJ, Colli BO, Tirapelli LF (2007) Ultrastructural study of the lateral ventricle choroid plexus in experimental hydrocephalus in Wistar rats. Arq Neuropsiquiatr 65:974–977
15. Rice-evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956