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ORIGINAL ARTICLE

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Evaluation of the effects of quercetin on brain lesions secondary to experimental hydrocephalus in rats

G. B. Sampaio[1] · **S. C. Da Silva**[2] · **T. H. Romeiro**[2] · **P. D. S. Beggiora**[2] · **H. R. Machado**[2] · **L. D. S. Lopes**[2]

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Abstract

Introduction Hydrocephaly is a disease that affects not only the dynamics of the cerebrospinal fluid, but also other structures of the central nervous system. Although shunt is effective in reducing ventriculomegaly, many neurological damages are not reversed with surgery. Several studies demonstrate that oxidative stress is involved in the genesis of hydrocephalus lesions.

Objective Evaluate the neuroprotective response of quercetin in hydrocephalus.

Materials and methods Male newborns rats were used, which received the 15% kaolin injection in the cisterna magna for induction of hydrocephalus. They were divided into control group (C), untreated hydrocephalic (HN), shunted hydrocephalic (HD), hydrocephalic treated with distilled water (HA), hydrocephalic treated with distilled water and shunt (HDA), hydrocephalic treated with quercetin peritoneal (HQp), hydrocephalic treated with quercetin peritoneal and shunt (HDQp), hydrocephalic treated with quercetin by gavage (HQg), and hydrocephalus treated with quercetin by gavage and shunt (HDQg).

Results Quercetin significantly improved the immunohistochemical markers, mainly caspase and GFAP. There were no significant changes in clinical/behavioral assessment. The use of isolated quercetin does not alter the volume and ventricular size, and the realization of ventriculo-subcutaneous shunt in newborn rats with hydrocephalus presents a high morbi-mortality.

Conclusion The use of quercetin shows laboratory improvement of the effects of glial lesion and corpus callosum fibers and is therefore not justified by the use of the routine substance as neuroprotective.

Keywords Hydrocephalus · Neuroprotective drugs · Oxidative stress · Shunt

Introduction

Hydrocephaly may be defined as an increase in the amount of cerebrospinal fluid (CSF) in the ventricular or subarachnoid spaces, generating intracranial hypertension. The imbalance between the production and absorption of CSF leads to ventricular dilatation and consequent distortions in the cerebral parenchyma, compressing the hemispheres against the inner surface of the skull [2]. The first affected structure is the ventricular parenchyma, which undergoes compression and stretching; being destroyed in isolated or even totally ruptured [3]. Another injured structure is the white matter, which in later cases of ventricular dilation, may also lose myelin.

A significant and important advance in the history of the treatment of hydrocephalus was the introduction of unidirectional shunt systems, with the objective of retrieving excess fluid from the cerebral ventricles to other body cavities, canceling the pathophysiological basis of intracranial hypertension. This advance promoted a marked reduction in mortality and morbidity in children with hydrocephalus [9].

Oxidative stress (EO) occurs in hydrocephalus, due to the aggression caused to the cerebral parenchyma by the forces of compression, stretching and consequently ischemia of the brain tissue. This action leads to tissue damage, with the production of free radicals, predominantly through lipid peroxidation, which has a toxic effect on the walls of cerebral arterial vessels. EO is a biological condition in which the imbalance between the production of reactive oxygen species (ROS) and their detoxification occurs through biological systems that remove and/or repair them. However, all organisms have an intracellular environment of reductive nature, so that there is a balance between the

✉ G. B. Sampaio
gugsampaio@gmail.com

1 Hospital da Criança e Maternidade, Av. Jamil Feres Kfouri, 60 - Jardim Panorama, São José do Rio Preto, SP 15091-240, Brazil

2 Developmental neuropathology laboratory - Faculdade de Medicina da Universidade de São Paulo, Campus Ribeirão Preto - SP, Av bandeirantes, 3900, Ribeirão Preto, São Paulo 14040-900, Brazil

oxidized and reduced molecules, thus keeping the enzymes at the expense of metabolic energy.

Quercetin is a natural flavonoid that has pharmacological properties, such as anti-inflammatory, anticarcinogenic, antiviral, antihistamines, and antioxidants. It has protective cardiovascular activity, reducing the risk of death due to coronary diseases and reducing the incidence of myocardial infarction. [13].

Among its main actions, it can remove free radicals, exerting a cytoprotective role in situations of risk of cellular damage. Quercetin has been shown to inhibit the in vitro oxidation of low density lipoprotein (LDL) by macrophages and to reduce the cytotoxicity of oxidized LDL [7].

It is the most abundant flavonoid present in the human diet, representing about 95% of the total flavonoids ingested [13]. Their estimated daily intake ranges from 50 mg to 500 mg daily [4]. It is present in vegetables and fruits. Being found in high concentrations in apples, onions, tea, red wine, and broccoli. It is extracted very easily, because it is in great quantity.

The protective effect of quercetin was superior to that of vitamin C, by preventing the effect of reducing glutathione, and protecting the brain from oxidative stress induced by neurotoxicity due to its structural properties and physiological benefits [7]. Quercetin inhibits the enzymes cyclooxygenase and lipoxygenase, which reduces the production of the main inflammatory mediators: prostaglandins and leukotrienes. It inhibits histamine production, stabilizing basophils and mast cells. Quercetin inhibits the process of free radical formation in three different steps, initiation (by interaction with superoxide ions), formation of hydroxyl radicals (by chelating iron ions), and lipid peroxidation (by reacting with lipid peroxy radicals).

Many studies report that flavonoids in their free or glycosylated form are absorbed in the gastrointestinal tract and metabolized to glucuronidate or conjugated sulfate. These metabolites circulate in the blood being excreted in bile and urine. Quercetin is completely converted into methylated conjugates in plasma, after administration, in both rats and humans. Quercetin is absorbed in the intestinal microflora and excreted in bile and urine as glucuronidate and conjugated sulfate within 48 h. Subsequently, it is degraded by intestinal bacteria in phenolic acid, 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid within ring B.

As cerebral permeability is controlled by psycho-chemical characteristics such as hydrophobicity or lipophilicity, quercetin may enter brain regions benefiting from antioxidant and biological functions, protecting against hydrogen peroxide-induced cytotoxicity. [4].

Neuroprotection is an intervention, not necessarily pharmacological, whose objective is to avoid damage to the neural cells or when already injured, to avoid the progression of the lesion. Quercetin acts as a neuroprotectant directly in the intracellular mechanisms of the ischemic cascade, aiming at the rescue of the area of hypoperfusion, still viable, surrounding the infarct. Therefore, the study of the neuroprotective effects of quercetin in hydrocephalus is extremely important and may represent a new treatment for the disease.

Material and methods

All procedures involving the animals are in accordance with the guidelines established by the Brazilian College of Animal Experimentation (COBEA) and approved by the local committee held by the Ethics Committee on Animal Experimentation (CETEA)—University of São Paulo at Ribeirão Preto Medical School (FMRP-USP), Protocol No. 32/2016. All efforts were made to minimize the suffering and the number of animals used in this research.

At 7 days of age, the animals were randomly selected and submitted to hydrocephalus induction by intracisternal injection of kaolin or maintained without intervention. The space between the posterior margin of the foramen magnum in the occipital bone and the cranial border of the dorsal arch of the first cervical vertebra was identified. With a short bevel Mise 0.3 dental needle, a suboccipital puncture and slow percutaneous injection of 0.04 ml of a 15% kaolin (Merck®) suspension in sterile autoclaved distilled water was performed.

At 7 days after induction of hydrocephalus with kaolin, the animals were anesthetized with 10% Isoflurane (BioChimo®) with continuous oxygen therapy, followed by administration of Cephalotin 50 mg/kg for preoperative infectious prophylaxis, degermation of the head, neck, and trunk with deodorant iodine and painted with alcoholic iodine. A cut was performed in the cranial region near the right coronal suture and a trepanation forward of the right coronal suture using a drill and a rotary punch; the dorsal region of the trunk of the animal was dissected using a dissector. Next, a ventricular punction was performed using a ventricular catheter. Using a prolene wire 5.0, the proximal catheter was attached to the bone. After reviewing the surgical technique, the procedure was performed the closure of the skin in a single plane with prolene 5.0 (Fig. 1). The technique was developed in the research laboratory, detailing the procedures recently published [16] (Figs. 2 and 3).

Experimental groups

Control (C): Animals without hydrocephaly, without surgical intervention or drug treatment;

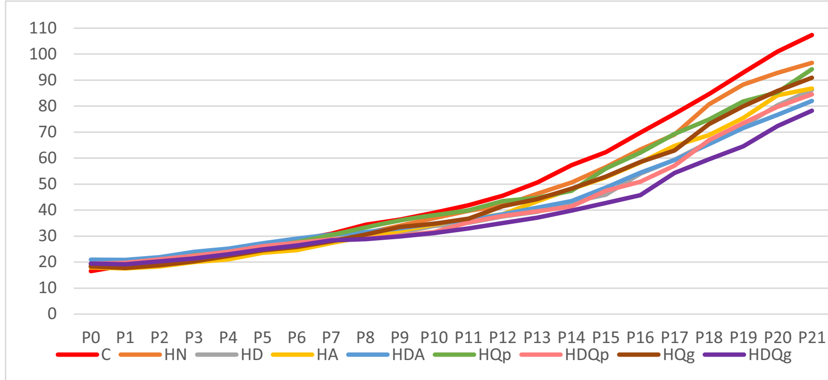


Fig. 1 Analysis of the average daily weight of the experiment between the groups studied

Hydrocephalic not treated with medication: *Shunted hydrocephalic (HD)*, animals with hydrocephalus treated with DVSC. *Non-shunted hydrocephalus (HN)*, animals with hydrocephalus without surgical intervention or drug treatment.

Hydrocephalic treated with the vehicle, distilled water: *Shunted hydrocephalic (HDA)*, hydrocephalic animals receiving peritoneal distilled water and DVSC. *Non-shunted hydrocephalus (HA)*, animals that received intraperitoneal distilled water.

Treated with quercetin by gavage: *Shunted hydrocephalic (HDQg)*, animals that received quercetin by gavage and DVSC. *Non-shunted hydrocephalus (HQg)*, animals that received quercetin by gavage.

Treated with peritoneal quercetin: *Hydrocephalic shunted (HDQp)*, animals treated with intraperitoneal quercetin and DVSC. *Non shunted hydrocephalus (HQp)*, animals treated with intraperitoneal quercetin.

Results

Weight

The animals were weighed every day and the daily and the final weight gain percentage was used for weight evaluation, as well as the average daily weight (Fig. 1), and there was no significant difference in the analysis of the weight in the comparison between the groups.

Solochrome cyanine corpus callosum

In the analysis of solochrome cyanine, control animals exhibited a greater thickness of the corpus callosum than the other groups, presenting statistical difference only in comparison with the HA group (C × HA $p = 0.0007$ IC 0.04975–

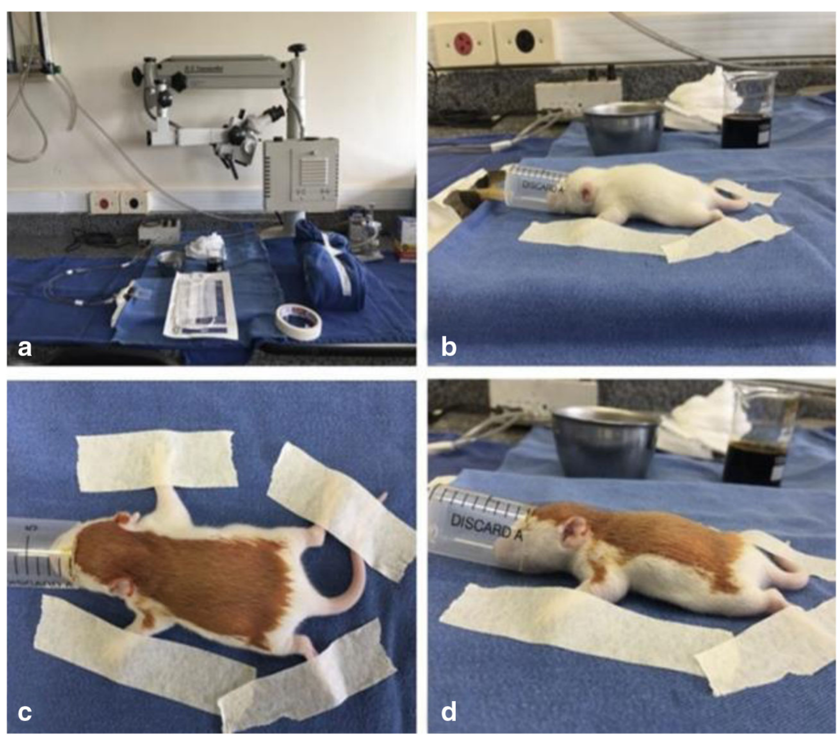


Fig. 2 Surgical procedure. **a** Materials used during surgery. **b** Animal with 14 days of life in ventral decubitus under inhalation anesthesia. **c** Asepsis with topical iodine. **d** Disinfection with alcoholic iodine. Source: Laboratory of Brain Protection in Childhood [8]

0.2991). Animals that received quercetin as treatment and DVSC had a higher mean corpus callosum thickness than the other groups and were statistically significant in comparison with the HA group (HDQp × HA $p = 0.0264$ CI − 0.2313 a − 0, 00783 and HDQg × HA $p = 0.0092$ IC − 2.403 to − 0.0195).

Compared only with the animals shunted (HD, HDA, HDQp and HDQg) and non-shunted animals with the exception of the control group (HN, HA, HQp and HQg), it was observed that the control group presented a higher increase of the corpus callosum compared to the group of non-shunted animals, presenting a significant change (C × Non-shunted $p = 0.0062$ CI 0.2348–0.1708). There was no significant difference in the comparison between the groups C × shunted and shunted × not-shunted (Figs. 4 and 5).

Caspase cerebral cortex

The cell density labeled with the caspase-3 antibody in the cerebral cortex of the animals studied was evaluated. The group that presented the highest cellular marker of antibodies was the HN, whereas the control group had the lowest cell density between the groups, presenting a significant difference when compared (C × HN; $p < 0.001$ CI − 28.45 to − 7.55). There was also a significant difference in the comparison of caspase-3-labeled cell density in the cerebral cortex between the HN and HDA groups (HN × HAD; $p = 0.0105$ IC 1.714 to 22.09), between the HN and HDQp groups (HN × HDQ p; $p = 0.0013$ CI 3.714–24.09); HN and HQg (HN × HQg; $p = 0.0116$ IC 1.614–21.99) and HN and HDQg (HN × HDQg; $p = 0.0155$ IC 1.314–21.69) (Figs. 6 and 7).

GFAP Cospus callosum

Fibrillar acidic acid antibody (GFAP) antibody showed a higher density of labeled cells in the corpus callosum of HDQp and HN animals, presenting a significant change when compared to group C (C × HDQ; $p = 0.0007$ CI − 2.118 to − 0.371; C × HN; $p = 0.0176$ IC − 1896 to − 0.1039) and in the comparison between the HDQp and HQg groups (HDQp × HQg $p = 0.0343$ IC − 1.452 to 0.3406). There was no significant difference between the other groups (Fig. 8).

Discussion

Hydrocephaly causes anatomical changes culminating in a significant increase in ventricular volume due to the accumulation of cerebrospinal fluid. This increase causes distortion of adjacent structures, reducing the cortical mantle and compressing the white matter. Therefore, distortion of adjacent structures, such as compression of the cerebral veins, cause cell metabolic changes that determine the damage of the cerebral parenchyma. From the second day on, the animals receiving the kaolin injection had the main clinical signs associated with hydrocephaly such as: lethargy, pronounced curvature of the spine, claw toes, bulging of the bones of the skull, eyes directed downward, and gait with broad base (Parinaud sign), rarefaction of the hairs mainly of the cranial region, difficulty of weight gain. In the study conducted by Lopes et al. [10], from the second day after the induction of hydrocephalus with kaolin, the same signals observed in this study were already visible. These clinical changes observed in animals are similar to those observed in humans. In

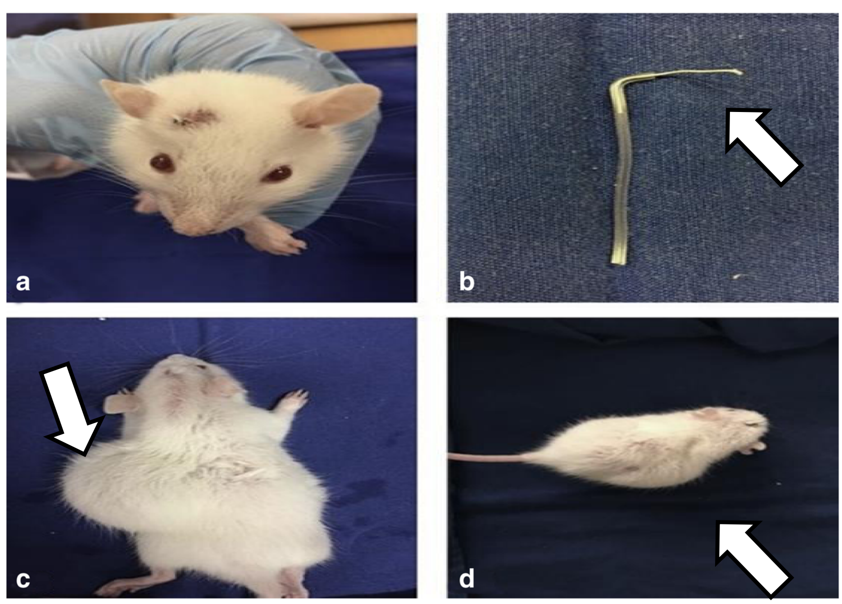


Fig. 3 **a** Surgical scar on the 21st day of study. **b** Catheter used to perform the DVSC with the metallic guide (white arrow) to maintain the angulation of 90°. The guide was removed before catheter implantation. **c** The white arrow shows the accumulation of cerebrospinal fluid in the subcutaneous space of the animal at P21, in the upper view. **d** The white arrow shows the accumulation of liquor, in the same animal, in the subcutaneous space region in a lateral view. Source: Laboratory of Brain Protection in Childhood (2017)

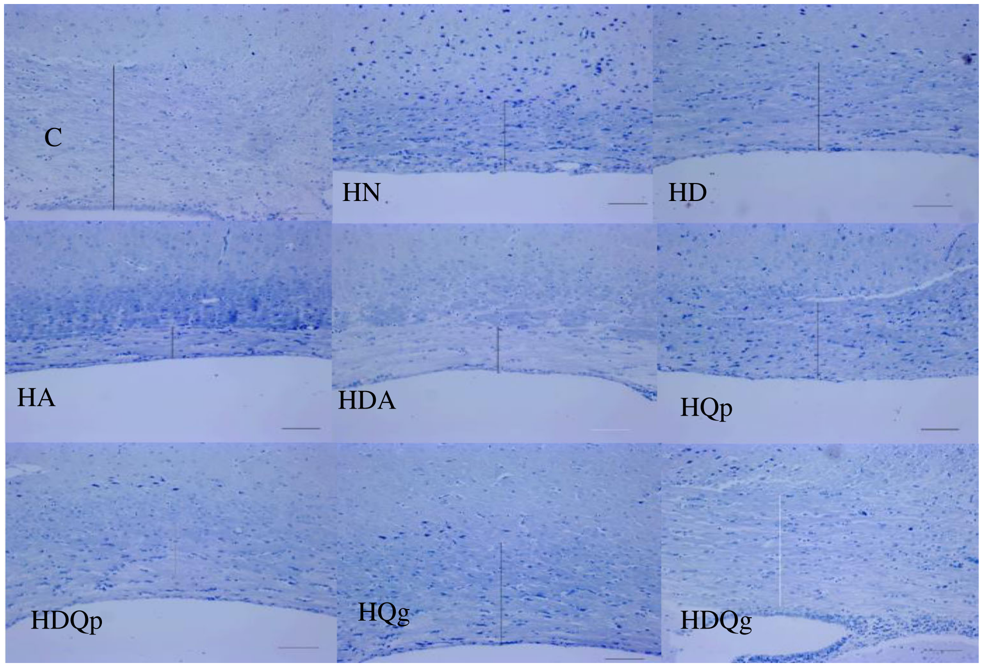


Fig. 4 Histological sections of the corpus callosum, visualized in × 10 objective, stained in solochromo-cyanine. The transverse lines represent 10 μm. C Control. HD Hydrocephalic shunted. HA Hydrocephalic treated with distilled water. HDA Hydrocephalic treated with distilled water and DVSC. HQp Hydrocephalic treated with peritoneal quercetin. HQg Hydrocephalic treated with quercetin by gavage. HDQp Hydrocephalic treated with peritoneal quercetin and DVSC. HDQg Hydrocephalic treated with quercetin by gavage and DVSC. There was no significant difference

patients, hydrocephaly manifests itself according to the age, cause, and speed of evolution. In newborns, we found an increase in the cephalic perimeter and remission of sutures forming the anterior fontanelle, making its late closure, in addition to other clinical signs, such as the "sunset gaze," also known as Parinaud's compression of the pineal recess [14], bulging of the anterior fontanelle, engorgement of the cranial veins, and difficulty feeding. Another frequent clinical sign is papillary edema, which is widely used when it is doubtful if the presented symptoms are due to intracranial hypertension, its presence, depending on the context, practically confirms the diagnosis of intracranial hypertension.

Animals that were not treated with subcutaneous ventricle shunt were less attentive, slower, and showed less social interaction, some carelessness with hygiene, frequent self-isolation, signs of aggression, loss of self-care capacity, lack of interest in the environment, and inadequate behavior to danger. While the animals that were derived were more active and alert, they further exploited the environment and exhibited more often natural behaviors such as play and self-cleaning body.

Once again, the physiological adaptation mechanism to the surgical procedure is discussed, since it was expected that the animals submitted to the combined treatment performed better than the animals with isolated quercetin. The surgical procedure for the installation of DVSC despite treating the pathophysiology of intracranial hypertension in this age group is extremely aggressive, and it takes time for the body of the animals to recover and at the same time recover the damages caused by hydrocephaly. For children under 1500 g, the procedure for the

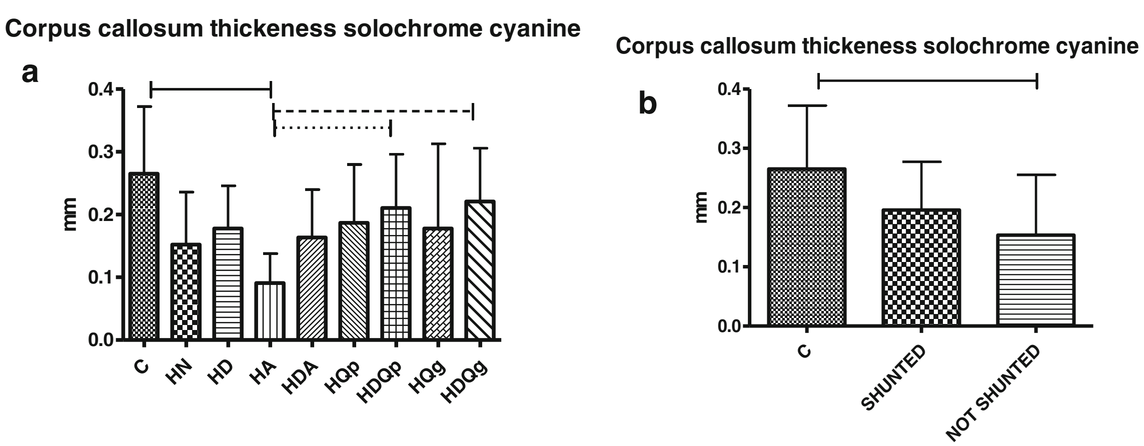


Fig. 5 **a** Evaluation of the mean corpus callosum thickness in millimeters between the groups studied. Dashed, dotted, or rectilinear lines showed significant changes between the groups and in **b** the evaluation of mean corpus callosum thickness between groups of animals with DVSC and without DVSC, there was a significant difference in the comparison between group C and shunted

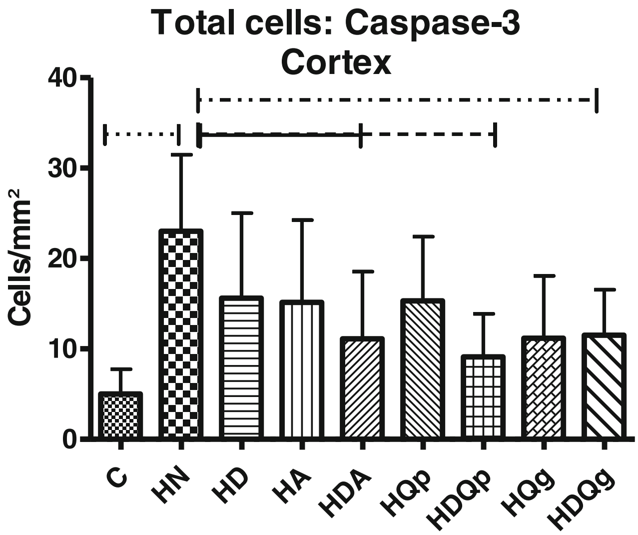


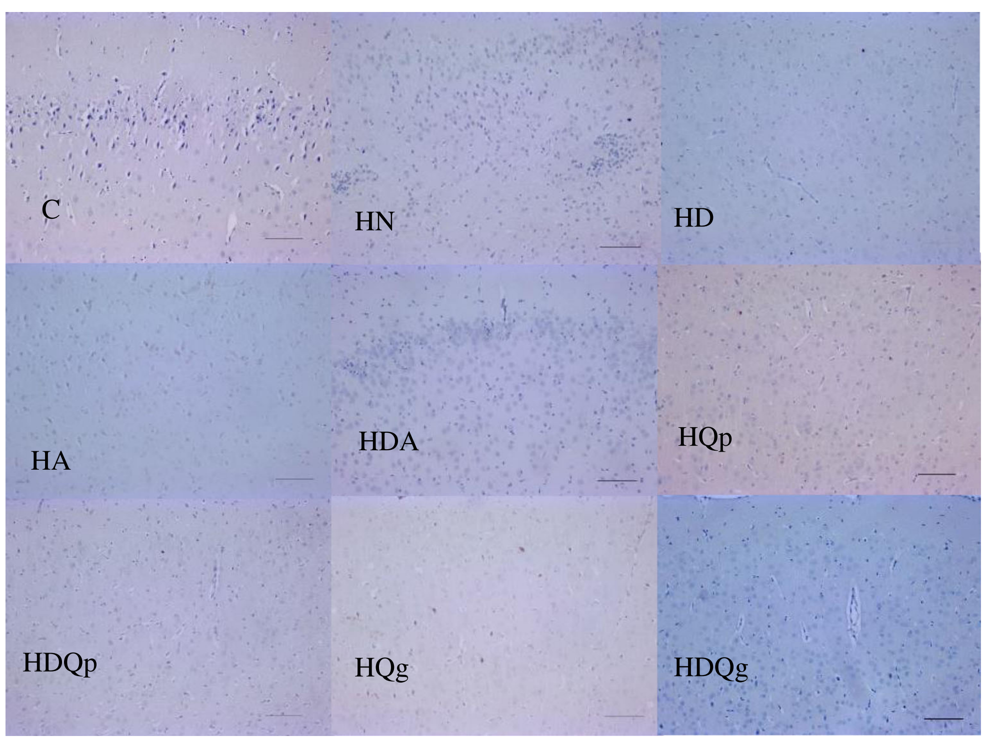
Fig. 6 Graphic representing the cellular density of caspase-3 in square millimeters between the studied groups. The upper crossbars connecting two groups represent the significant difference between the groups

installation of DVP is contraindicated by most neurosurgeons due to the complications occasioned in this period; therefore, hydrocephalus alterations are less deleterious than the surgical procedure for its treatment [15]. The same happens in our animals, the fact of operating them at 14 days of life makes the surgical procedure very aggressive and its recovery slower, we believe that observation for only 14 days after the procedure is not enough to obtain the adequate response expected.

Histological analysis by solochrome-cyanine in the hydrocephalic animals would show weaker blue tint than the control animals, indicating that hydrocephalus reduces the amount of myelin present in the brain tissue. The involvement of myelination in hydrocephalic rodents was also observed by other researchers using the same methodology [11], but it was also seen in rats.

Histological analysis by solochome-cyanine in the hydrocephalic animals would show weaker blue tint than the control animals, indicating that hydrocephalus reduces the amount of myelin present in the brain tissue. However, it was also observed in adult hydrocephalic rats [1]. Unfortunately through the methodology used to generate hydrocephalus, we did not control the degree and the evolution of the same, by this fact during the evaluation of histological analysis of SC, the animals of the HN group presented a lower corpus callosum thickness than the HA group, both are untreated animals, the only difference is that the HA group was subjected to daily stress through peritoneal application of distilled water, the vehicle used for the dilution of quercetin. The control group had a significantly lower CC thickness than the group that used only distilled water. Animals in the combined treatment groups, quercetin and DVSC, had a significantly lower CC thickness compared to the group of animals receiving only peritoneal distilled water. Referring to think that hydrocephaly plus daily stress caused by the administration of medication are deleterious to the body and the combined treatment was able to reverse the effects caused by both. When all animals of the shunted groups were placed compared to all the animals of

Fig. 7 Microscopic sections marked with caspase-3, 40x, antibody evaluating the cerebral cortex of the studied groups. The straight lines represent 3 μm. C Control. HD Hydrocephalic shunted. HA Hydrocephalic treated with distilled water. HDA Hydrocephalic treated with distilled water and DVSC. HQp Hydrocephalic treated with peritoneal quercetin. HQg Hydrocephalic treated with quercetin by gavage. HDQp Hydrocephalic treated with peritoneal quercetin and DVSC. HDQg Hydrocephalic treated with quercetin by gavage and DVSC. There was a significant difference in the comparison between the groups C × HN, HN × HAD, HN × HDQp, HN × HQg, HN × HDQg



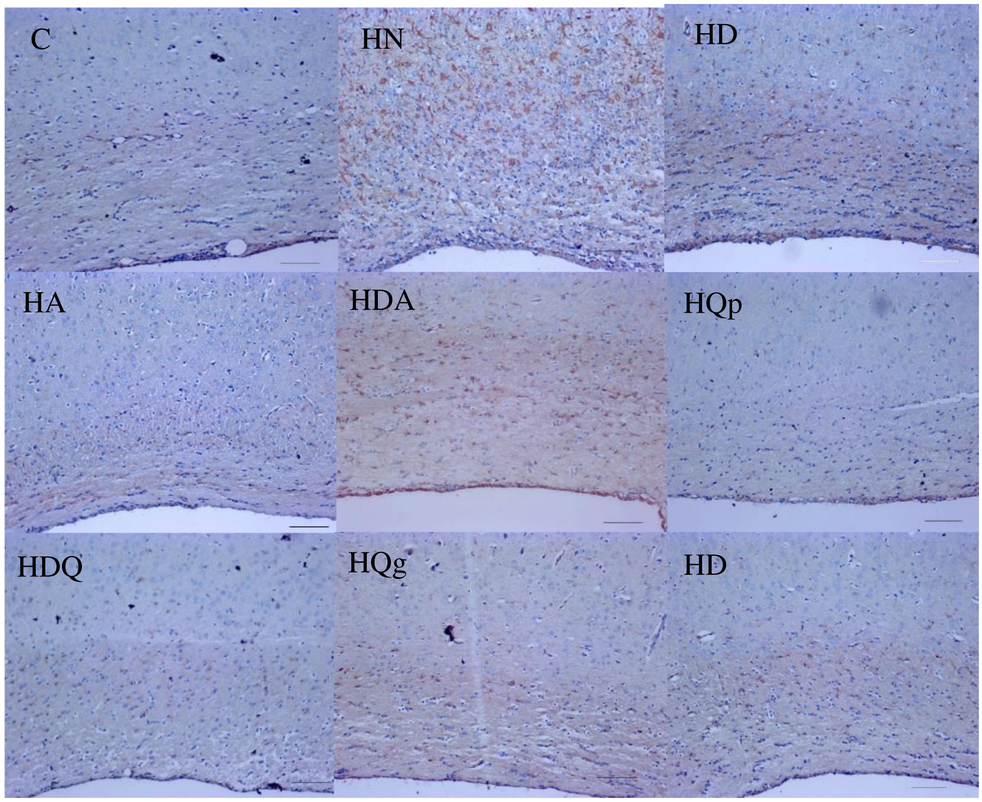


Fig. 8 Microscopic cuts stained with GFAP, 10x, in the germinative matrix of the animals of the studied groups. The straight lines represent 5 μm. C Control. HD Hydrocephalic derivative. HA Hydrocephalic treated with distilled water. HDA Hydrocephalic treated with distilled water and DVSC. HQp Hydrocephalic treated with peritoneal quercetin. HQg Hydrocephalic treated with quercetin by gavage. HDQp Hydrocephalic treated with peritoneal quercetin and DVSC. HDQg Hydrocephalic treated with quercetin by gavage and DVSC. There was a significant difference in the comparison between the groups C × HDQ p and HDQ p × HQg

the non-shunted groups, except for the control group, it was observed that there was a significant difference in the corpus callosum thickness of the non-derived animals compared to the control group, showing that shunts lead to a decrease in ventricular volume and consequently decompression of the corpus callosum, but this improvement is not significant enough to return to previous ventricular anatomy.

In the analysis of CC thickness, the hydrocephalic animals had a lower average thickness than the control animals, due to the compression/stretching suffered as a result of ventricular dilation. When analyzing the groups of animals shunted and not shunted, we can show a significant difference in the comparison between the control and non-shunted groups. In contrast, there was no significant difference in the comparison between the shunted and non-shunted groups. Once again, we discussed the time required for the anatomical response of the animal organ after the shunt to be performed, and if the benefits of the procedure actually exceed the risks since the brain is still developing and the closure of the skull cap has not yet occurred completely.

The best time to perform hydrocephalus treatment in premature infants is still much controversal, the treatment is ideal when symptoms of intracranial hypertension are evident, since treatment without clinical / radiological evidence of intracranial hypertension may mean adding all the inherent risks of the surgical procedure The astrocytes are the most abundant glial cells in the CNS and play essential functions for their homeostasis, such as maintenance of the ionic levels of the extracellular environment, uptake, and release of several neurotransmitters, participation in the formation of the blood-brain barrier and in the functioning of the synapses [6]. One of the most notable features of astrocytes is their response to various neurological insults. Reactive astrogliosis is characterized by high proliferation of astrocytes and extensive hypertrophy of the cell body and cytoplasmic processes, both at the site of injury and in neighboring areas. In the immunohistochemical analysis of GFAP, we observed that HN animals showed intense astrocytic reaction, with intensely branched astrocytes with hyperplastic and hypertrophic cell bodies, which synthesize numerous amounts of the astrocyte-specific protein, GFAP. These characteristics indicate lesions in the nervous tissue, observed in various CNS aggression modalities, such as in viral infections, demyelinating diseases, inflammations, acute traumatic brain injuries, and in neurodegenerative diseases [12]. In the immunohistochemical analysis of the GFAP of the corpus callosum, this study showed that there was a significant change between the HDQp × C and HN × C groups, showing that untreated animals, HN, present more reactive astrocytes than the control group, hydrocephaly causes significant glial injury, a fact that has already been demonstrated in other laboratory studies [17]. While the HDQp group presented greater glial lesion when compared to the control and HQg groups, it was probably in this case that the ventricular lead catheter itself caused glial lesion, perhaps because of the suboptimal positioning compared to the other derived groups. In the analysis of the GFAP of the cortex and germinative matrix, we did not find significant changes between the groups, which shows that the glial lesion in the initial periods is predominantly located in the ventricles and adjacent tissues. Reactive astrocytes play an important role in the repair phase of the CNS, actively monitoring and controlling the molecular and ionic contents of the extracellular space [5].

Conclusion

After the administration of quercetin in the hydrocephalic animals, we can conclude the following: The treatment does not produce clinical beneficial effects in behavioral tests as well as in weight. Quercetin does not alter the rate of cell division in the germ matrix. Quercetin does not alter the cellular apoptosis rate of the corpus callosum, but when associated with ventricular shunting, it improves the cellular apoptosis rate of the cerebral cortex. Quercetin does not improve cellular damage to astrocytes in the corpus callosum and cerebral cortex. The administration of quercetin does not modify ventricular size. Ventricular shunting decreases ventricular size. The use of quercetin shows laboratory improvement of the effects of glial lesion and corpus callosum fibers and is therefore not justified by the use of the routine substance as neuroprotective.

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