Behavioral and Biochemical Features of the Course and Surgical Treatment of Experimental Obstructive Hydrocephalus in Young Rats

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Keywords

Cytokines · Experimental hydrocephalus · Interleukins · Ventricular-subcutaneous shunt

Abstract

Introduction: Hydrocephalus is a multifactorial disease, affecting the dynamics of cerebrospinal fluid (CSF) and leading to severe neurological impairment in children; in spite of the recent advances in hydrocephalus research, it has many physiopathological aspects that still remain poorly understood, especially after treatment. **Objectives:** To analyze the clinical, radiological, histopathological, and biochemical aspects of kaolin-induced hydrocephalus in an experimental model, both in the acute phase and after shunt treatment, by means of behavioral tests, magnetic resonance imaging (MRI) scans, histopathological studies, and level of inflammatory interleukins in the CSF. **Methods:** Seven-day-old Wistar rats were used and subdivided into three subgroups: treated hydrocephalic ($n = 24$), untreated hydrocephalic ($n = 17$), and controls ($n = 5$). The hydrocephalic groups underwent cisternal injection of 15% kaolin for induction of hydrocephalus at 7 days of age. The treated group was submitted

to a ventricular-subcutaneous shunt (VSCS) 1 week after induction. All animals were euthanized at 21 days of age. They underwent motor function and memory testing as well as brain MRI scans. Histopathological analysis for glial fibrillary acidic protein and Ki-67 was done, and CSF was collected for measurement of IL-1 β , IL-6, and TNF- α . **Results:** The average time to reach the water maze platform was highest in the untreated hydrocephalic group. The magnetization transfer rates were 37.21 and 33.76 before and after shunting, respectively. The mean astrocyte counts were 2.45, 1.36, and 90.5 for shunted, untreated, and control animals, respectively. The mean CSF IL-1 β concentrations were 62.3 and 249.6 pg/mL, the average IL-6 levels were 104.2 and 364.7 pg/mL, and the average TNF- α values were 4.9 and 170.5 pg/mL for the treated hydrocephalic group and the untreated group, respectively. **Conclusions:** Pups treated with a CSF shunt showed better performance on memory tests. VSCS did not revert demyelination caused by hydrocephalus. Likewise, reactive astrocytosis and cell proliferation over the germinal matrix were not reversed after shunting. Hydrocephalic animals had raised levels of inflammatory interleukins, which returned to normal after treatment.

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Introduction

Hydrocephalus is one of the commonest pathological conditions affecting the central nervous system (CNS) during the neonatal period and infancy. It has an overall estimated incidence of 1:2,000 live births and is associated with about one-third of all CNS congenital malformations [1, 2]. Hydrocephalus is defined by an increase in the volume of cerebral ventricles secondary to abnormal secretion, circulation, or resorption of cerebrospinal fluid (CSF), leading to raised intracranial pressure and appearance of clinical symptoms and signs [3].

The mechanisms of brain damage in this disease remain widely unclear, in spite of significant advances provided recently by clinical studies and experimental methods [4]. Furthermore, among the currently available therapeutic modalities, shunts are still the option used more often [5]. Nevertheless, it is not possible to ratify that shunts are able to reverse the cellular and biochemical injury caused by hydrocephalus, even with early treatment. Therefore, the present study aimed at analyzing, in the context of experimental hydrocephalus induced in young rats, the effects of an adapted (ventricular-subcutaneous) shunt, specifically on their motor and memory skills, on the microstructural and biochemical alterations seen by means of histological examinations, and on the presence of CSF inflammatory biomarkers.

Methods

Forty-six Wistar rats were used at 7 days of age; hydrocephalus was induced in all of them, except for controls, by intracisternal injections of kaolin as described previously by Lopes et al. [6]. In this technique, each animal is positioned with a flexed neck to expose the dorsal cervical region. After identification of the space between the inferior rim of the foramen magnum and the cranial border of the first cervical vertebra, a percutaneous suboccipital puncture is performed for slow injection of 0.04 mL of 15% kaolin suspended in distilled water, previously sterilized at 60 °C.

Afterwards, animals were divided into three experimental groups: (1) hydrocephalic nontreated (HNT) group ($n = 17$): pups were sacrificed 14 days after kaolin injection (at 21 days of age); (2) hydrocephalic treated with ventricular-subcutaneous shunt (HT-VSCS) group ($n = 24$): pups shunted with the ventricular-subcutaneous shunt (VSCS) 7 days after induction of hydrocephalus and sacrificed 7 days after shunting (also at 21 days of age); (3) control group ($n = 5$): pups sacrificed when 21 days old without having undergone any procedure.

Implantation of the VSCS was performed according to the following steps (Fig. 1) [7]: after intraperitoneal anesthesia with a combination of ketamine 10% and xylazine 10% (0.1 and 0.05 mg/100 g of body weight, respectively) and prophylactic antibiot-

ics (cefalotin 50 mg/kg, also administered intraperitoneally), a short skin incision was performed over the calvaria 3 mm from the midline to the right and 2 mm anterior to the coronal suture, enclosing all layers of the scalp, for exposure of the skull. With the aid of a delicate twist drill (1 mm wide), a burr hole was fashioned and the dura mater was pricked. The right lateral ventricle was then directly punctured with a polypropylene catheter (internal diameter of 0.7 and 8 mm long), bent at a right angle, and connected to a distal catheter (8–10 cm long) which, after tunneling through the cervical subcutaneous tissue, was placed in a pocket created bluntly close to the rat's sacrum. The skin was then closed with 2 or 3 Mononylon 5.0 stitches. A drop of cyanoacrylate glue was laid over the incision.

Motor and Memory Assessment

All animals underwent motor and memory assessments by means of a modified Morris water maze test. It consists of a round pool (diameter 100 cm, height 50 cm) and a transparent platform (diameter 8 cm, height 34 cm) positioned 2 cm underwater. Initially, animals went through an adaptation and training period in which they were let free to swim around the pool, without the platform. Thereafter, the platform was placed into the water tank in one specific quadrant. The pool was stored in a completely dark room, except for a tiny light bulb fixed next to the north side of the tank, so that the pup could identify the reference point to be memorized. The test was then performed on the 10th and 11th postinduction days, when animals were put into the pool in one of the four cardinal points on each of the four consecutive sessions, repeated twice daily. The time needed to reach the platform from the moment they were put in the water was recorded in seconds. A resting break of 15 s was given between sessions.

Analysis of White Matter Myelination by Magnetic Resonance Imaging

For this matter we used the magnetization transfer rate (MTR) technique, whose goal is to analyze interactions between non-aqueous hydrogen protons with those moving in water, which are usually not analyzed on conventional magnetic resonance imaging (MRI). Scans were acquired in a 3-Tesla large bore gantry scanner (Philips Achieva™, Rotterdam, The Netherlands), using a specific coil for small rodents, and performed at 14 days of age. Animals of the shunted group were also scanned 7 days after shunting to assess ventricular size and thus adequate functioning of the catheter. Slice thickness was 1 mm, resulting in a total of 28 slices. As mentioned above, the protocol included a 3D T2-weighted image and two identical 3D MTR sequences (Fig. 2). T2-weighted scans were chosen to increase the contrast between CSF and brain matter as well as to provide a precise spatial resolution. Magnetization transfer was used with defined standard pulses on the scanner consisting of 121 perpendicular radiofrequency pulses and four rectangular elements with a duration of 275 ms. Then MTR values on each pixel were calculated using the following equation: $MTR (\%) = (PI_{woMT} - PI_{wMT}) \times 100 / PI_{woMT}$, where PI_{wMT} and PI_{woMT} represent pixel intensity before and after scanning with the MTR protocol, respectively. The MTR was obtained on the coronal plane using previously defined regions of interest, one ventral and one dorsal to the left lateral ventricle, along with the whole brain. Voxel size was $0.3 \times 0.32 \times 0.3$ mm.

Fig. 1. Sequential iconographic representation of the VSCS technique in a 14-day-old Wistar rat. **a** Prepping and fur clipping. **b** Skin incision with a small number 15 scalpel. **c** Tunneling of the distal catheter. **d** Burr hole with a 1-mm twist drill. **e** Ventricular

puncture. **f** A drop of cyanoacrylate glue was put in to secure the catheter and avoid its migration. **g** Skin closure. **h** Another drop of glue was placed over the incision. VSCS, ventricular-subcutaneous shunt.

Sampling

At the end of all experiments, animals were sacrificed after deep anesthesia with intraperitoneal ketamine and xylazine (0.2 and 0.1 mg/100 g of body weight, respectively). Following transcardiac perfusion with phosphate-buffered saline (PBS), brains were removed en bloc through a vertex craniectomy and fixed in 3% paraformaldehyde diluted with a 0.1 M phosphate buffer solution (pH 7.3–7.4). Thereafter, all samples were sectioned on the coronal plane of the optic chiasm, dividing them into anterior (frontal) and posterior (parietal) sections. Subsequently, samples were dehydrated with increasing concentrations of ethanol solutions, diaphanized in xylol, and paraffin soaked.

Immunohistochemistry

Glial fibrillary acidic protein (GFAP) assays were performed to assess the distribution and morphological aspects of astroglia, as well as Ki-67 for evaluation of the germinal matrix on the external angle of the lateral ventricle.

Samples were initially prepared by extraction of paraffin in a 60°C incubator for 30 min and sequential washouts with xylol and ethanol. After antigenic recovery, when needed, blockage of en-

dogenous peroxidase was carried out by the acidification and warming technique, with methanol-diluted 3% hydrogen peroxide. Thereafter, blockage was also performed with adequate serum in 10% PBS, for 30 min, in a wet chamber. Soon afterwards, these slices were incubated overnight at 4°C with the respective primary anti-GFAP antibody 1:300 (DAKO Z0334™, Denmark), anti-Ki-67 1:500 (Santa Cruz Biotechnology SC-23900™), diluted in bovine serum albumin. Then, the primary antibody was withdrawn, and the appropriate secondary antibody was added (biotin-labeled goat anti-rabbit antibody [Santa Cruz Biotechnology SC-2040™] or anti-mouse antibody [Santa Cruz Biotechnology SC-2039™]), diluted 1:300 in bovine serum albumin. At this point, the assays were incubated with tertiary antibody streptavidin conjugated with horseradish peroxidase (BioLegend™ cat. 405210), diluted 1:400 in PBS. They were revealed with 3,3-diaminobenzidine (Sigma Aldrich™, St. Louis, MO, USA). Finally, slides were countercolored with hematoxylin, washed in running water, dehydrated by serial crescent ethanol solutions and xylol, and Permount™ coverslips were put on.

Photographic documentation was made with an Axioskop 2 plus light microscope (Carl Zeiss™) and an AxioCam HRc digital

Fig. 2. Number and average number of reactive astrocytes of the experimental groups over the corpus callosum (**a**) and germinal matrix (**b**). HTVSCS, hydrocephalic treated with ventricular-subcutaneous shunt; HNT, hydrocephalic nontreated; C, controls.

camera (Carl ZeissTM), connected to a Pentium II computer equipped with the Axio Vison 3.1 software. The corpus callosum regions, as well as the germinal matrix, of GFAP- and Ki-67-immunomarked slides were also documented, along with cell counting. Cell counting was made on ImageJ software (National Institutes of Health, Bethesda, MD, USA).

CSF Interleukin Levels (IL-1 β , IL-6, and TNF- α)

After the vertex craniectomy, prior to removal of the brains, CSF was collected by a direct transcortical ventricular puncture, using a 23-gauge needle and a 1-mL sterile polycarbonate syringe (Becton DickinsonTM, Franklin Lakes, NJ, USA). About 0.25 mL were withdrawn, which was enough for further analyses. Demonstration of the interleukin levels was performed by commercially available sandwich ELISA kits (all reagents from AbcamTM, Cambridge, UK), according to the manufacturer's instructions, using a rat anti-IL-1 β antibody, a mouse anti-IL-6 antibody, and a rat anti-TNF- α antibody.

In 96-well plates (NUNC 442404, Sigma AldrichTM), 50 μ L of specific antibody for the respective cytokine (IL-1 β , IL-6, and TNF- α) were added, diluted in PBS, and incubated overnight at 4°C. Plates were then washed three times with a solution containing PBS and 0.05% of the nonionic detergent polyethylene glycol sorbitan monolaurate (Tween 20, Sigma AldrichTM). After that, 100 μ L of buffer solution (PBS + 1% of bovine serum albumin) (Thermo Fisher ScientificTM, Waltham, MA, USA) were added so that plates could be incubated for 2 h at ambient temperature. Another triple wash with PBS and Tween 20 was performed, followed by another incubation at 4°C overnight. Subsequently, 50 μ L of detection antibodies were added and incubated at ambient temperature, plates were triple washed with PBS and Tween 20 again, and mixed with 50 μ L of avidin-peroxidase. Another triple wash with PBS and Tween 20 was performed. Lastly, the reaction between the content of the plates and 50 μ L of chromogenic substrate tetramethylbenzidine protected from light was observed for approximately 10 min, until the appearance of a blue color. The reac-

tion was then interrupted with 50 μ L of sulfuric acid for reading of its optic density in a spectrophotometer with a wave length of 450 nm. Results were expressed in pg/mL for each interleukin.

Statistical Analysis

Statistical tests for comparisons among groups were chosen as follows: For continuous variables, a linear mixed-effects model for the evaluation of performance in the Morris water maze test and a Student *t* test for quantification of MTRs were used. For categorical variables, such as GFAP-labeled reactive astrocytes and Ki-67 mitotic cell counts, analysis was performed via double Poisson regression. Regarding the concentration of CSF inflammatory interleukins, after verification that they were not normally distributed, a Kruskal-Wallis test with Dunn's post hoc test and a focused log-binomial Bayesian model were utilized. The statistical softwares SAS 9.2, R 2.15.1 (SAS/STATTM, Version 9.2, 2008, Cary, NC, USA) and GraphPad Prism[®] 6.0 (GraphPad SoftwareTM, La Jolla, CA, USA) were used. Results were deemed significant when *p* < 0.01.

Results

Morris Water Maze

The average time intervals to reach the platform, in the morning (M1, M2) and afternoon (A1, A2) sessions of the 10th and 11th experimental days, respectively, as well as the standard deviations (SDs), are summarized in Table 1.

The treated (HTVSCS) group did not show better performances (shorter time intervals) when subsequent sessions were compared, nor did the HNT group. Control animals performed gradually better when M2-A1 and

Table 1. Average time intervals (in s) to reach the water maze platform for the experimental groups

Group	Session	Average time	SD
HTVSCS	M1	41.6	8.9
	A1	38.8	13.2
	M2	38.6	13.0
	A2	36.1	12.1
HNT	M1	47.5	9.8
	A1	53.2	8.4
	M2	51.6	6.0
	A2	43.1	15.5
Controls	M1	44.3	12.1
	A1	51.6	5.1
	M2	34.3	12.4
	A2	34.7	12.6

A1, afternoon period of the first day; A2, afternoon period of the second day; HNT, hydrocephalic nontreated; HTVSCS, hydrocephalic treated with ventricular-subcutaneous shunt; M1, morning period of the first day; M2, morning period of the second day; SD, standard deviation.

Table 2. MTRs of the whole brain (WB) and dorsal (DR) and ventral (VR) regions of the left lateral ventricle

Time	Variable	Average MTR	SD
Before shunting	WB	37.21	1.82
	DR	41.07	5.9
	VR	37.06	1.48
After shunting	WB	33.76	10.06
	DR	26.26	14.77
	VR	33.36	6.92

MTR, magnetization transfer rate; SD, standard deviation.

Table 3. Average and SD of the number of mitotic cells found in the germinal matrix samples of all experimental groups

Group	Average	SD
HTVSCS	2.45	3.70
HNT	1.36	2.24
Controls	90.50	22.23

HNT, hydrocephalic nontreated; HTVSCS, hydrocephalic treated with ventricular-subcutaneous shunt; SD, standard deviation.

A1-A2 were compared ($p < 0.01$). Comparison among the performance of the three different groups, however, demonstrated that HTVSCS pups reached the platform more quickly than HNT ones in sessions A1 and M2 ($p < 0.01$ for both). Besides that, in session M2, the HNT group's performance was significantly worse than that of controls ($p < 0.01$); HTVSCS animals, on the other hand, performed similarly.

Magnetization Transfer

All shunted animals included in the study had a clear reduction in ventricular size, as seen in postshunt MRI scans. The preshunt mean ventricular index was 0.67, whereas it became 0.5 after shunting. Average MTRs are described in Table 2. Statistically significant differences were not found in hydrocephalic pups before and after shunting. Comparison among pre- and postshunt MTR rates with controls also failed to show any significant difference, although there seemed to be a tendency towards improved myelination in the whole-brain analyses of the HTVSCS group as compared to the control group ($p = 0.02$).

Immunohistochemistry Analyses

Reactive astrogliosis was sought in two distinct regions: corpus callosum and germinal matrix. Average reactive astrocyte count and SDs for all experimental groups are displayed in Figure 2.

These data attest that there were significant differences among all three groups, except for the number of reactive astrocytes in the corpus callosum of the HNT and HTVSCS groups, which tended to be higher in the latter ($p = 0.02$). Therefore, there was no reduction in the number of reactive astrocytes after treatment; conversely, treated animals showed higher counts than untreated ones. Comparatively, one could also observe greater reactive astrogliosis in shunted and unshunted pups than in controls.

Regarding Ki-67 mitotic cell immunolabeling, the average number of marked cells, along with SDs, are summarized in Table 3. Statistically significant differences between the HTVSCS/HNT groups and controls were detected, but not between themselves; ergo, in this study, hydrocephalus reduced the presence of mitotic cells over the germinal matrix, even though treatment with VSCS did not bring any benefits in this regard ($p = 0.35$). It is interesting to point out that, despite the absence of a statistical difference, the number of mitotic cells was greater in the treated group as compared to the untreated one (2.45 vs. 1.36, SD 3.7 vs. 2.24, respectively).

Table 4. Mean CSF concentrations of inflammatory interleukins of treated and untreated hydrocephalic animals

Group	Mean IL-1, pg/mL	Mean IL-6, pg/mL	Mean TNF- α , pg/mL
HTVSCS	62.3	104.2	4.9
HNT	249.6	364.7	170.5

CSF, cerebrospinal fluid; HNT, hydrocephalic nontreated; HTVSCS, hydrocephalic treated with ventricular-subcutaneous shunt.

CSF Cytokines

The mean CSF concentrations of the inflammatory interleukins dosed herein are summarized in Table 4. None of the control animals had raised levels of any of the cytokines studied, i.e., CSF concentrations were always zero in this group.

A direct statistical comparison among groups confirmed these data to be significantly different, the HNT group showing the highest levels, significantly higher than those in the HTVSCS and control groups ($p < 0.01$), which, in turn, did not differ between themselves. Nevertheless, all experimental groups had at least one animal in which CSF interleukin concentrations, whether IL-1 β , IL-6, or TNF- α , was null. Therefore, another statistical evaluation using a focused log-binomial Bayesian model was performed, which failed to confirm a strong statistical correlation between raised levels in hydrocephalic pups and their reduction in treated ones, for the number of animals used herein.

Discussion

The pathophysiology of hydrocephalus, especially in children, remains unknown in various aspects, in spite of important advances achieved in the last decades by centers studying experimental and clinical hydrocephalus. Obviously, this knowledge is essential for the understanding of clinical manifestations of hydrocephalus and might also have therapeutic implications. The development of new treatment modalities should target early physiopathological mechanisms, both on the cellular and molecular levels.

Therefore, the present study aimed at analyzing and correlating clinical, radiological, cellular, and biochemical alterations of hydrocephalus induced by intracisternal kaolin in 7-day-old rats. Injury to the brain parenchyma

was studied and compared to healthy controls; nonetheless, the second and principal objective of this study was to analyze the effect of hydrocephalus treatment by means of a VSCS, with emphasis on short-term recovery of the damage resulting from ventricular dilation and raised intracranial pressure.

The goal of the water maze test is to assess swimming and thus motor skills of rats, along with their ability of spatial location, which also provides information about associative memory [8]. It is a fairly easy test whose main virtue is the possibility of fast training [8]. The disadvantages of the method include the fact that it does not allow motivational variations and is completely manual, which makes evaluations longer. Besides, immersion in water may cause endocrinological and organic distress [9].

In the present work the HTVSCS and HNT groups, when analyzed individually, did not show progressive improvement after training and subsequent sessions in the water maze, contrary to the control group, which showed significantly shorter intervals for reaching the platform as the experiments went on. When comparing all groups, it was noticed that HTVSCS animals had a clearly superior performance in the afternoon session of day 10 and in the morning session of day 11, along with a tendency to perform better in the afternoon session of day 11. In addition, in the afternoon session of day 11, shunted animals performed rather similarly to controls. Thus, treated animals were able to memorize their way to the platform similarly to healthy controls and more easily than untreated counterparts, indicating reestablishment of spatial memory after shunting.

Del Bigio et al. [10], studying slightly older rats, published analogous results and even pointed out that rats shunted 7 days after hydrocephalus induction had a slightly superior performance than those shunted after 4 weeks. Williams et al. [11] found that it takes more time for hydrocephalic rats to reach the platform 28 days after induction, and that there is a linear correlation between severity of hydrocephalus (according to the degree of ventricular dilation) and poorer results in the water maze test.

Magnetization transfer is a widely used MRI technique for quantification of myelin and the concentration of metabolic products of tissue degradation; many authors have studied MTR in distinct CNS conditions, such as multiple sclerosis, optic neuritis, Wallerian degeneration, and intracranial hemorrhage [12–15]. It is based on the magnetic exchange between protons linked to large, slow-moving molecules (in this case, myelin) and those free in water [13, 14, 16]. MTR is utilized to analyze the exten-

sion of parenchymal destruction of the brain, since lower MTR levels are attributed to reduction in the integrity of macromolecules, reflecting damage to myelin and to the axonal membrane or presence of inflammatory edema [13, 17].

In addition to the whole brain, the dorsal and ventral areas of the lateral ventricle were chosen due to their susceptibility to stretching forces secondary to ventricular dilation, and because, at this age, fibers found at such regions have already become myelinated, according to Jacobson [18]. Nevertheless, no statistically significant differences were found between pre- and postshunt rates nor in the comparison between shunted animals and controls. We postulate that the progression of hydrocephalus in this time schedule was insufficient to produce any lesion detectable by MTR in the periventricular white matter. Some other limitations of MTR application in this context include heterogeneous myelination of the rat's whole brain at this age [18], since MTR techniques compare adjacent cerebral areas, and use of a nonspecific scanner which reduces the sensitivity of MTR [19, 20].

Discrepant results have been published in the medical literature in this regard, however. Hähnel et al. [21], analyzing MTR rates in five distinct brain areas of patients with chronic hydrocephalus, observed significantly lower rates in hydrocephalic patients, for all regions except the thalamus, as compared to healthy individuals. They concluded that MTR provides additional information in relation to traditional MRI scans and that there is indeed myelin injury in the white matter, which is in accordance with the findings from Harrison et al. [22]. In the study of Rocha Catalão et al. [4], a functioning shunt returned MTR rates to normal (control) levels in young Wistar rats treated 7 days after hydrocephalus induction with kaolin.

Astrocytes are glial cells widely distributed over the CNS, whose functions include neuronal nutrition and regulation [23]. In situations of CNS distress or injury, they are activated and start to overexpress GFAP, a process called reactive astrogliosis that limits tissular damage and restores homeostasis. The number of reactive astrocytes in all experimental groups was counted in the corpus callosum and germinal matrix, with interesting results: the HTVSCS and HNT groups had increased reactive astrocytes counts in relation to controls, meaning that hydrocephalus activates glial reparatory processes. Surprisingly, HTVSCS rats also had greater counts than HNT counterparts, suggesting that reactive astrogliosis continues even after shunting, and possibly that shunt implantation and presence of a foreign material in the brain has potentiated defensive cellular mechanisms.

These inferences need to be further confirmed by histological evaluations of the shunt implant sites.

Increased density of reactive astrocytes in hydrocephalus, mostly over periventricular white matter, is a well-established phenomenon [6, 24–26]. Several papers have analyzed the effect of shunting in this context. Rocha Catalão et al. [4] found that the number of reactive astrocytes was elevated in the corpus callosum, external capsule, and germinal matrix of hydrocephalic pups, and reduced after treatment. Miller and McAllister [27] studied GFAP profiles in the H-Tx model of hydrocephalic rats before and after VSCS, and postshunt reductions in GFAP expression of 18, 23, and 18.3% were seen in the parietal and occipital cortex and periaqueductal white matter, respectively.

Furthermore, Ki-67 immunolabeling was studied in order to evaluate cell proliferation over the germinal matrix (located in the external angle of the lateral ventricles). This is a protein expressed in all phases of the mitotic cycle, whereas it is absent in cells which are not replicating (G0); for this reason, it is considered an excellent marker to specify the growth fraction of a given tissue [28, 29]. We observed that controls had intense cellularity in this region, with a great number of labeled cells, whereas hydrocephalic pups, treated or not, showed less evident labeling; objective counting confirmed fewer Ki-67-marked cells in these latter groups, which did not differ between each other. This is a strong indicator that hydrocephalus reduces cell proliferation in the germinal matrix, and shunting does not seem to reverse this phenomenon. To the best of our knowledge, there are no other studies which addressed the effects of shunting in these circumstances, although there are some papers describing a reduction of Ki-67-labeled cells in purely untreated hydrocephalic rats [6, 24, 26].

Lastly, the final analysis of this study concerned the role of inflammatory mechanisms in the pathophysiology of hydrocephalus. Interleukins comprise a group of cytokines that mediate and trigger inflammatory responses among various types of cells [30]. The immature CNS is particularly vulnerable to inflammation-related injury [31]. Proinflammatory cytokines are produced by T lymphocytes, and when released, they promote migration of immune cells into the brain as well as microglial and astrocyte activation [31]. T lymphocytes have been found to migrate across the choroidal epithelium forming the blood-CSF barrier and to inspect the CNS environment for cognate antigens along perivascular spaces [19, 20].

Although the level of specific interleukins such as insulin-like growth factor 1, hepatocyte growth factor, vas-

cular endothelial growth factor, interferon-gamma, and IL-18 is known to be raised in patients with neonatal hydrocephalus, their role has not been completely clarified [2, 31, 32]. Few papers have addressed this issue, and the majority of these studied inflammation in patients with normal-pressure hydrocephalus, which is a condition specific of the elderly and might not represent other types of hydrocephalus [33–36].

Moreover, there is scarce information regarding the consequences of shunt therapy. Despite the increase in cerebral perfusion and the reduction in mechanisms of direct injury that shunting brings, it does hinder inflammatory mechanisms specifically, and therefore microglial damage can continue even after the intervention [31]. Since they constitute biomarkers whose levels can be measured, interleukins could also help in the diagnosis and evaluation of the efficacy of treatment [37].

We selected three interleukins, based on previous studies and the individual features of each cytokine: IL-1 β , IL-6, and TNF- α . IL-1 β increases the proliferation, differentiation, and production of antibodies by B lymphocytes and also has proinflammatory activity within the CNS, such as induction of neurodegeneration and fever [33]. IL-6 is produced by T lymphocytes in response to antigenic stimuli and acts on late stages of B cell differentiation [38]; its blockage leads to a reduction of neuronal regeneration mechanisms [39]. TNF- α is the key initiator of immune-mediated inflammation in the brain and has intense expression in neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and vascular dementia [35, 36]. These three markers have all been implicated in inflammatory cerebral injury [19, 33, 36, 38].

The results of this study suggest that there is an increase in the abovementioned interleukins in kaolin-induced hydrocephalus and that treatment with VSCS reduced their levels, indicating that inflammation might be part of the physiopathology of hydrocephalus. In the medical literature, there are very few studies exploring the relation between interleukins and experimental hydrocephalus; nevertheless, there is only one study analyzing the effects of treatment with shunts. Wostrack et al. [40] measured CSF concentrations of IL-6 in 84 patients with posthemorrhagic hydrocephalus. They found that IL-6 concentrations were clearly elevated and that they correlated with shunt dependency, along with progression of the disease. They also stated that high IL-6 levels might predict the need for shunting.

Merhar [30], in a meta-analysis of inflammatory biomarkers in neonatal hydrocephalus, mentions two other

studies: the one by Sävman et al. [35], which found high levels of IL-1 β , IL-6, IL-8, and TNF- α in preterm children with germinal matrix hemorrhage, and the one by Schmitz et al. [38], who reported raised concentrations of this interleukin and interferon-gamma in the same setting.

Generally speaking, there is a gradually bigger body of evidence confirming the occurrence of inflammation in hydrocephalus, consequently elevating the levels in CSF interleukins. Studies with larger samples, alongside correlation with clinical hydrocephalus, are certainly necessary. The possibility of measuring the concentrations of these markers in CSF and blood is also a potential target for future research.

Conclusion

The VSCS for treatment of kaolin-induced hydrocephalus, for the time intervals used herein, was able to improve memory performance in rats. Contrarily, periventricular white matter myelination, as evaluated by magnetization transfer techniques, is damaged by hydrocephalus and is not restored after shunting. Likewise, shunt treatment was not sufficient to enable reduction of reactive astrogliosis processes within periventricular regions and cell proliferation over the germinal matrix. Finally, kaolin-induced hydrocephalus in rats increases the CSF levels of inflammatory interleukins IL-1 β , IL-6, and TNF- α , and VSCS reduced these levels, indicating the presence of inflammation processes in the physiopathology of this condition.

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Statement of Ethics

All experimental and surgical procedures involving animals were strictly in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL) and the Brazilian College of Animal Experimentation (COBAL), and approved by the Animal Experimentation Ethics Committee at the authors' institution.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception and design: M. Volpon Santos, L. da Silva Lopes, and R. Santos de Oliveira. Acquisition of data: M. Volpon Santos and L. da Silva Lopes. Analysis and interpretation of data: M. Volpon Santos and R. Santos de Oliveira. Drafting of the article: all authors. Critical revision of the article: L. da Silva Lopes, R. Santos de Oliveira, and H.R. Machado. Review of the final version of the manuscript and approval for submission: all authors.

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