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The relationship between AQP1 expression in the subventricular zone and severity of hydrocephalus in *Rattus Norvegicus* strain Sprague-Dawley rats

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ABSTRACT

Background: Hydrocephalus is a condition of dilation of the ventricles caused by disturbances production, distribution, or absorption of cerebrospinal fluid (CSF). One of the integral membrane proteins identified in facilitating water transport across the plasma membrane is aquaporin-1 (AQP1), which is frequently found in the plexus choroid. However, theoretically also transports water to the subventricular zone (SVZ) with an unknown mechanism. AQP1 expression will be raised in SVZ under hydrocephalus conditions. This study aims to evaluate the relationship between the severity of hydrocephalus and AQP1 levels in SVZ.

Methods: This research was conducted in an experimental design using Rattus Norvegicus rats of the Sprague-Dawley strain, which were injected with kaolin to create a hydrocephalus model. The study included 24 rats, divided into four groups of six each: the control group and the hydrocephalus induction group on day 7, day 14, and day 21. AQP1 expression was observed using immunohistochemical staining and counted semi-quantitatively.

Results: The average AQP1 expression increased with observation time in the rat model in each group. The ANOVA test showed a significant difference between the four study groups (p=0.001). The correlation showed a statistically significant difference (p=0.000). The results showed an increased expression of the SVZ with a higher severity of hydrocephalus.

Conclusion: The severity of hydrocephalus and AQP1 levels in SVZ are correlated, with the latter being higher, the more severe the degree of hydrocephalus.

Keywords: Aquaporin-1, Hydrocephalus, Sub-Ventricle Zone.
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INTRODUCTION

Dilation of the ventricles and subarachnoid space, known as hydrocephalus, is a disorder brought on by problems with cerebrospinal fluid (CSF) generation, distribution, or absorption.[1] Aqueductal stenosis, Chiari malformation, Dandy-Walker malformation, acquired (infection, tumor, hemorrhage), or idiopathic hydrocephalus are examples of congenital hydrocephalus.[1,2] The prevalence of congenital hydrocephalus in babies and young children ranges from 5 to 18 per 10,000 live births.[3] Globally, it is estimated that around 383,000 new cases of pediatric hydrocephalus are diagnosed each year.[4,5]

A class of integral membrane proteins known as aquaporins (AQP) facilitates water movement across the plasma cell membrane. The liquid-selective portion of AQP comprises six transmembrane helix proteins that come together to form a tetramer in the middle of the molecule.[6,7] The two subfamilies of aquaporins are aquaglyceroporins (glycerol aquaporin channels) and traditional aquaporins (water selective). There are 8 aquaporins expressed in the central nervous system (CNS), including AQP1, AQP3, AQP4, AQP5, AQP7, AQP8, AQP9, and AQP11.[8,9] AQP1 and AQP4 are the most prevalent and important AQPs in the CNS. The apical and basal membranes of the choroid plexus contain AQP1. By moving water to the perivascular area, AQP1 helps to lower the quantity of water in the ventricular system in both communicative and non-communicating hydrocephalus.[8]

An imbalance in CSF's creation, distribution, and absorption results in hydrocephalus. By moving water to the perivascular region, AQP1 works to lessen the amount of water in the ventricular system in hydrocephalus. In the case of hydrocephalus, an increase in AQP1 is highly likely. The severity of hydrocephalus and the expression of AQP1 levels were not well correlated in earlier work. Furthermore, there hasn't been any prior research examining AQP1 in the subventricular region. Based on the results presented above, the authors aims to evaluate the link between AQP1 levels and the severity of hydrocephalus.

METHODS

The research was conducted from January to December of 2021. The adaptation phase was carried out for 1 week, while the treatment and observation phases lasted 21 days from the intervention. The maintenance of experimental animals was carried out at the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. Tissue processing and immunohistochemical examination were conducted at the Department of Pathology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. The ethical standards of experiments were in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The research's operational framework is shown in Figure 1.

Experimental Units, Replication, and Randomization

This research was conducted using an experimental research design. The research subjects were Rattus Norvegicus rats of the Sprague-Dawley strain, injected with kaolin to create a hydrocephalus model. The rats were between 8 and 10 weeks old, weighed 150 and 200 grams, and were healthy.

The inclusion criteria were Rattus Norvegicus rats of the Sprague-Dawley strain that were healthy and had never been or were not currently receiving any intervention. The exclusion or dropout criteria were as follows: if the subject dies before the termination time according to the intervention group, hydrocephalus does not occur in the experimental unit, and organ or tissue damage occurs when sampling for immunohistochemical examination.

This study included 24 subjects, which were divided into four groups of six each: the control group (C), the hydrocephalus induction group on day 7 (D7), day 14 (D14) and day 21 (D21). The number of replications is calculated using the hypothesis test formula, and the result for each group is 6. The grouping of the samples into test groups was carried out by simple randomization using random numbers. A color code distinguishes each group; each sample gets a number code.

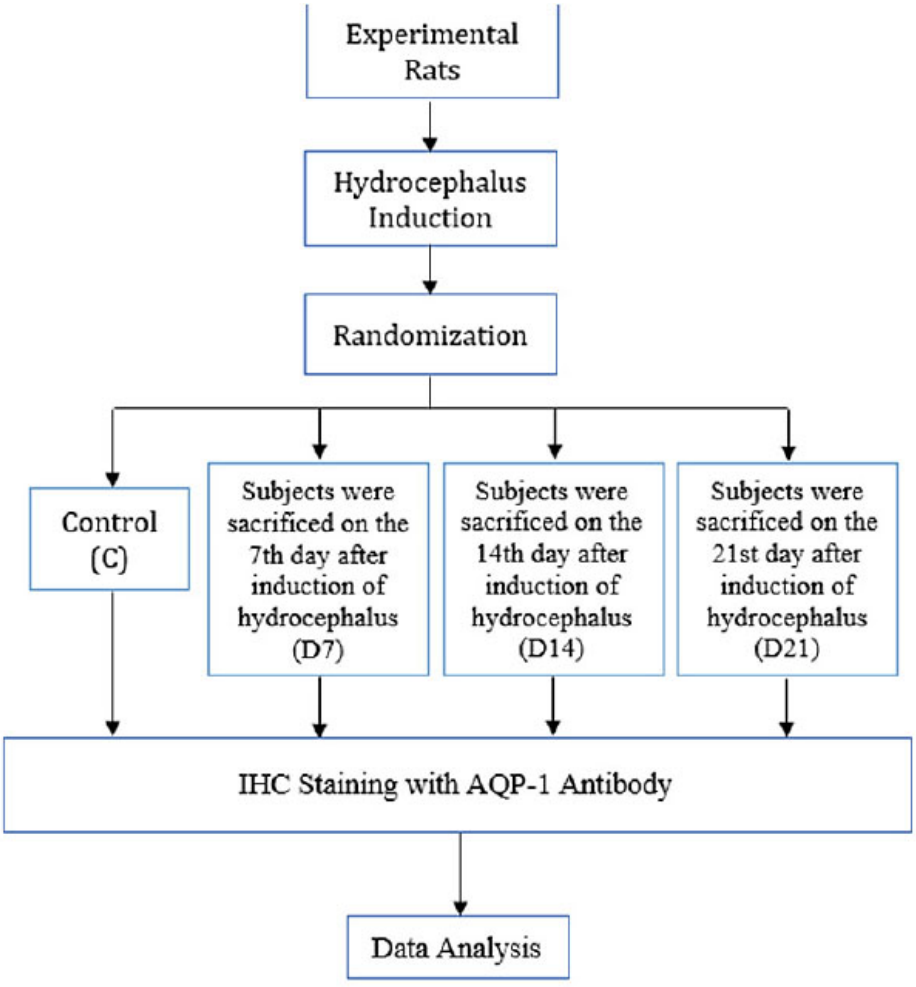

Figure 1. The operational framework of research implementation.

Research Variables

The independent variable was the length of time hydrocephalus was observed. The dependent variables were AQP1 levels and the severity of hydrocephalus. Control variables were the rat's species, maintenance management, examination methods and tools.

Research Materials and Instrumentations

The research material used was the AQP1 antibody. Materials for the preparation of experimental animals were a 5 ml syringe with 25G needle, 1 ml insulin syringe with 27G needle, betadine and sterile gauze, sterile open linen, ketamine and xylazine, disposable tubes (15 ml), 50 ml of alcohol (70% in concentration), sterile distilled water, and 10% povidone-iodine. The equipment used in this study was used for extracting the brain from the rats, preparing samples for immunohistochemical examination, and immunohistochemical staining.

Preparation of Kaolin-Induced Hydrocephalus Rat

Sprague-Dawley rats were treated according to guidelines for animal rearing. Sterile kaolin suspension is used to induce hydrocephalus. The procedure is performed under anesthesia. The suboccipital cleft is identified by palpating the space between the occipital and first cervical vertebral bones. After identification, 20-30 µL of sterile kaolin suspension (20% suspension in 0.9% saline) was injected percutaneously into the cisterna magna using a 27G needle.[10,11] The experimental animals were observed and monitored until they regained consciousness following the injection. The experimental animals were returned

to their respective cages, followed by standard maintenance and monitoring. After the induction with kaolin, experimental animals were observed for signs of hydrocephalus in the form of clinical appearance, such as enlarged head circumference, back neck bumping, gait disturbance, hindlimb paresis, and flat hindlimbs.

The extracted rat brains were prepared using standard paraffin block preparation procedures. The brain slices were put into gauze and dehydrated by soaking them in graded ethanol solutions for 60 minutes each at room temperature. The xylol-clearing process was then carried out three times for a total of 15 minutes at room temperature. Then, liquid paraffin was infiltrated thrice for 60 minutes each in a 60oC incubator. After being submerged in molten paraffin, the tissue is replaced at room temperature, solidifying into a paraffin block.

Statistical Analysis

Data analysis includes descriptive analysis and hypothesis testing. Ratio data are expressed in terms of mean, standard deviation (SD), frequency distribution, and percentage. Data analysis was performed with SPSS software version 24. The homogeneity test was assessed using the Levene test. Statistical test calculations were performed using ANOVA and Spearman correlation. The test results are significant if they have a p-value < 0.05. The post-hoc test is carried out to identify which groups are different if the results are significant.

RESULTS

The Sprague-Dawley rats used in this experimental study all met the inclusion criteria. The research sample was randomly divided into 4 groups (C, D7, D14, and D21), with the number of replications in each group being 6 rats. Examination of brain tissue with immunohistochemical staining and observations were then carried out under a light microscope with objective magnifications of 100x, 400x, and 1000x in 20 fields of view, where a positive AQP1 reaction was indicated by a brownish reaction, as seen in Figure 2.

The results of this study were analyzed descriptively to clarify the presentation of the research results. The lowest, highest, mean, and standard deviation values are shown in the data distribution table in Table 1. The distribution graph of AQP-1 expression demonstrated that AQP1 levels increased with time as hydrocephalus developed, as seen in Figure 3.

The data obtained had homogeneous variations based on homogeneity analysis using the Levene test (p>0.05). Following that, a parametric Oneway Anova test for comparative analysis was conducted. There was a significant difference in the results between the four study groups (p=0.001). A post-hoc test is therefore performed to identify the groups that differ. The post-hoc test showed a significant difference between the C group with the D14 (p=0.001) and D21 group (p=0.000). These significant differences were also seen between the D7 group with D14 (p=0.033) and D21 (p=0.004) group, as stated in Table 2. The relationship between the expression of AQP1 levels and the severity of hydrocephalus using the Spearman correlation test showed a statistically significant difference (p=0.000), as seen in Table 3.

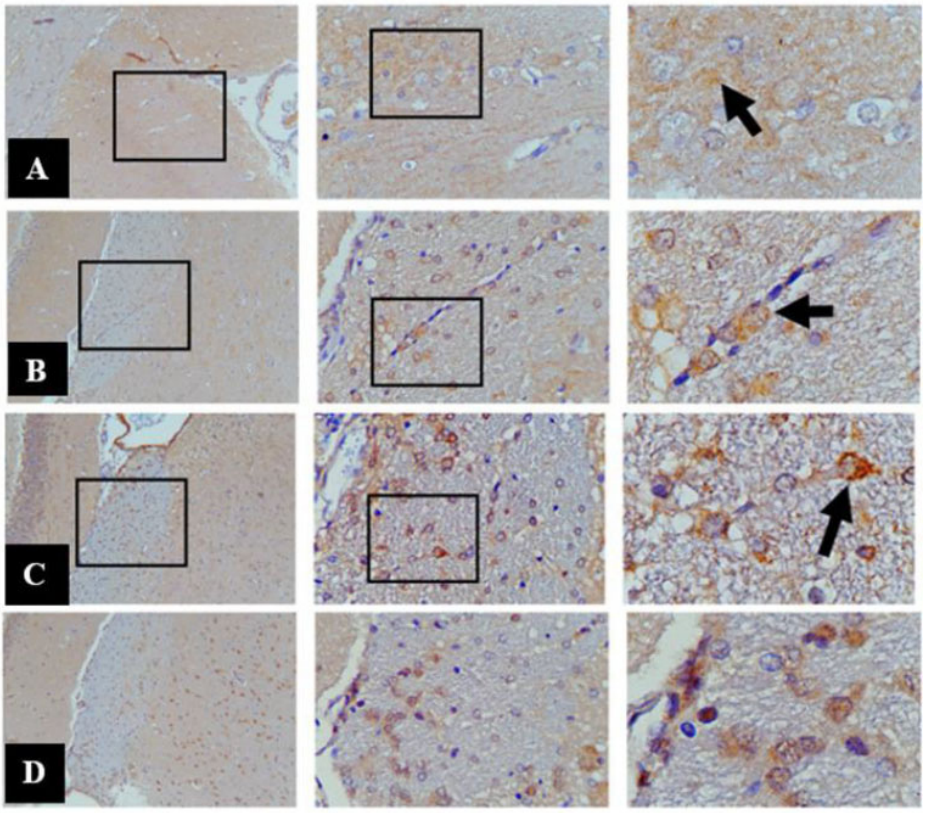

Figure 2. Results of AQP1 IHC staining from cross-sectional brains of rats. (A) Measured on day 7 (control group), (B) Measured on day 7 post-kaolin induction, (C) Measured on day 14 post-kaolin induction, (D) Measured on day 21 post-kaolin induction. Viewed under a microscope with magnification 100x left, 400x (center) and 1000x magnification (right). The black arrow indicates AQP1.

Table 1. Descriptive table of AQP1 expression in hydrocephalus rats

Group	Minimum	Maximum	Mean	Standard Deviation
Control	3	6	4.33	1.211
D7	3	9	6.00	2.191
D14	8	12	9.83	1.472
D21	3	16	13.17	1.941

DISCUSSION

Aquaporin-1 (AQP1), a protein that aids in cerebrospinal fluid (CSF) secretion, is expressed on the membrane that surrounds

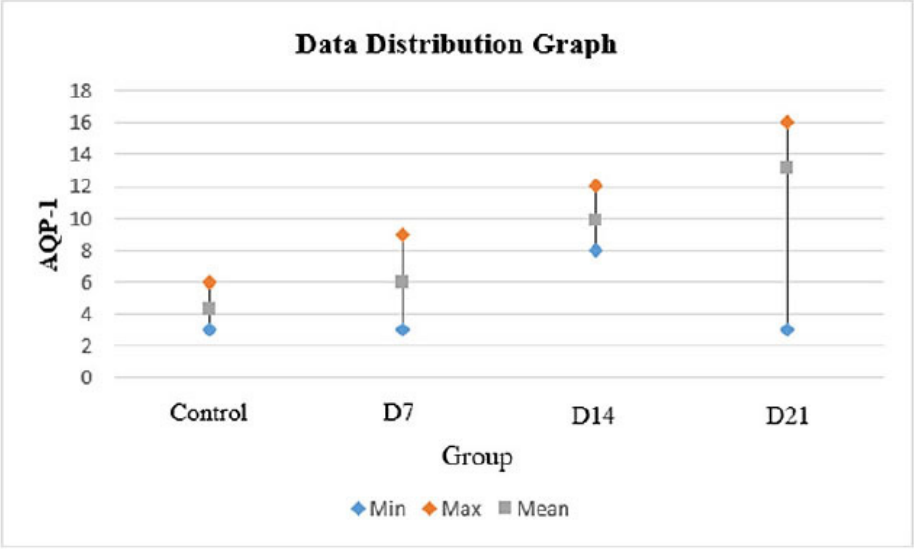

Figure 3. The distribution Graph of AQP-1 expression demonstrated that AQP1 levels increased with time as hydrocephalus developed.

Table 2. Post-hoc test of AQP1 comparison between study groups

AQP1 Comparison		P
Control group (C)	Intervention group on day 7 (D7)	0.514
	Intervention group on day 14 (D14)	0.001*
	Intervention group on day 21 (D21)	0.000*
Intervention group on day 7 (D7)	Control group (C)	0.514
	Intervention group on day 14 (D14)	0.033*
	Intervention group on day 21 (D21)	0.004*
Intervention group on day 14 (D14)	Control group (C)	0.001*
	Intervention group on day 7 (D7)	0.033*
	Intervention group on day 21 (D21)	0.751
Intervention group on day 21 (D21)	Control group (C)	0.000*
	Intervention group on day 7 (D7)	0.004*
	Intervention group on day 14 (D14)	0.751

*Statistically significant if p-value less than 0.05

Table 3. Spearman correlation of AQP1 with hydrocephalus severity

Variables	Spearman Correlation	p
AQP1 Expression	0.855	0.000*

*Statistically significant if p-value less than 0.05

the ventricles of choroid plexus epithelial cells.[12] The water-conducting channel protein AQP1 significantly contributes to the osmolarity of the permeability of cell membranes. According to earlier research, as a coping mechanism for obstructive hydrocephalus, choroid plexus AQP1 often declines.[13] However, how much AQP1 is present in the subventricular zone (SVZ) is unknown. According to Nguyen T et al., hydrostatic pressure interfered with AQP1-mediated transcellular or transendothelial flow.[14] In this work, it was postulated that AQP1 increases water distribution to the SVZ due to the differential in hydrostatic pressure between the ventricle and the periventricular zone.

Hydrocephalus is a neurological disorder marked by alterations in CSF flow that result in CSF accumulation in the cranial vault. Based on pathological research in the human brain and experimental and genetic animal models, the neuropathogenesis of hydrocephalus has been created.[15] Rats are the most extensively used experimental model animals because they are readily available, have simple breeding processes, are very straightforward to acquire, and can quickly be given hydrocephalus. Experimental animal models differ greatly between investigations.[16,17]

The intracisternal kaolin injection model is the most widely used technique for causing experimental hydrocephalus. A dose of around 0.01-0.2 mL of a 20–25% kaolin suspension (aluminum silicate) was injected into the cisterna magna when this model was first developed in the 1930s.[18] In the arachnoid and pia membranes, kaolin has a physical deposition effect and causes localized fibrosis.[19,20] At the base of the fourth ventricle, kaolin is deposited. It then spreads to the subarachnoid region, where it causes an inflammatory response and fibrous meningeal scarring. The forming scar tissue will result in ventricular hypertrophy and occlusion of the CSS channel close to the quadratus ventricular opening.[15] According to the available literature, this study employed a straightforward procedure that involved injecting kaolin through the suboccipital cleft into the cistern magna to cause hydrocephalus.

Our research revealed that, compared to controls without kaolin induction, the AQP1 protein was elevated in rat models receiving kaolin injections, suggesting a link between AQP1 and the development of hydrocephalus. Using Western Blot examination of choroid plexus tissue, Owler BK et al. discovered that AQP1 protein did not alter on days 3 and 5 following kaolin injection compared to saline-injected controls.[21] A rise in AQP1 expression was seen in a study by Long CY et al., and it peaked on day 3 of the choroid plexus epithelium.[22] McCoy E et al. discovered that AQP1 expression increased in astrocytes after brain damage. When there has been a subarachnoid hemorrhage (SAH), AQP1 is the main water channel controlling astrocyte water permeability.[23] These results all point to the possibility of higher AQP1 levels in a range of situations. In this work, we looked at AQP1 levels in the SVZ. We discovered a significant rise following the start of hydrocephalus and an increase related to the duration or severity of the hydrocephalus.

A visual representation of the elevation of AQP1 expression in this study shows how it gradually rose following kaolin injection. The D21 group had the highest

AQP1 level. Upregulation brought on by used neuroendocrine modulators may be the reason for this. The AQP1 channel's ability to function is regulated by atrial natriuretic peptide (ANP). ANP has neuroendocrine effects on the choroid plexus epithelium and inhibits CSF production.[24] The amount of AQP1 is decreased in the choroid plexus apical membrane and transferred from the apical membrane to the intracellular vesicles. The subventricular zone will have a higher concentration of AQP1 protein, as shown by our study. The downregulation of AQP1 expression on the apical membrane and this redistribution mechanism, along with the involvement of ANP, explain the pathological elevation of AQP1 in the rat's CSF brought on by hydrocephalus and the leaking of AQP1 into CSF.[25-27]

A correlation exists between the duration of AQP1 expression following kaolin injection and the degree of hydrocephalus. On the seventh day following kaolin injection, mild-moderate hydrocephalus will typically manifest. Macroscopically, ventricular enlargement (moderate-severe hydrocephalus) will be visible on the fourteenth and twenty-first days following kaolin injection.[19,28] González-Marrero I et al. found that hydrocephalus rats with systemic hypertension expressed more AQP1 and AQP4 in their CSF at 12 months compared to 6 months.[26] Injection of kaolin into a rat model of hydrocephalus for the previous studies failed to show any changes in AQP1 expression.[29,30] According to our research, the more AQP1 is elevated (in CSF), the more severe the hydrocephalus is.

The studies do not have any restrictions. First, our study's experimental design resulted in highly subjective research findings because of the potential for human error during the examination, intervention, and maintenance of experimental animals. Second, this study did not consider aspects like storage, material preparation, or the status of the experimental animals at the beginning of the treatment, which should be incorporated in further analysis. Instead, it simply looked at AQP1 expression at specific time points.

CONCLUSION

The study showed a significant difference in AQP1 expression levels in the subventricular zone between the hydrocephalus and normal rat groups. In addition, there was also an increase in AQP1 expression levels in the subventricular zone in the hydrocephalus rat group. The severity of hydrocephalus and AQP1 levels are correlated, with the latter being higher, the more severe the degree of hydrocephalus.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the manuscript.

ETHICAL CONSIDERATION

This study has received permission from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

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AUTHOR CONTRIBUTION

JNU contributed to the study's conceptualization, data collection, writing, and editing. MAF and WIH contributed to reviewing, editing, and finalizing the manuscript of the study. BU contributed to reviewing and analyzing the statistics of the study. AHB contributed to reviewing and finalizing the manuscript of the study. EAS contributed to reviewing and finalizing the manuscript of the study.

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