Pharmacogn J. 2022; 14(6) Suppl: 1054-1060
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Aquaporine 4 Expression on End Feet Astrocyte Before and After Cerebrospinal Fluid Drainage of Hydrocephalus Mice Model

Mustaqim Apriyansa Rahmadhan[1], Muhammad Arifin Parenrengi[1,*], Wihasto Suryaningtyas[1], Dyah Fauziah[2], I Ketut Sudiana[2], Budi Utomo[3]

ABSTRACT

Background: *Aquaporin* (AQP) is a family of integral membrane proteins that function as water channels. AQP facilitates the transport of water across the plasma cell membrane. AQP lining the periventricular wall in the presence of edema may impair the function of the AQP to prevent or facilitate proper movement of water. **Result**: We analyze the effect of hydrocephalus and CSF drainage on the expression levels of *aquaporin* 4 (AQP4) end feet astrocytes in a hydrocephalus mice model. The test was carried out using a mice model of hydrocephalus induced with kaolin, then CSF drainage was performed on the 7th and 14th day, and compared the levels of AQP4 expression in each group. Data showed an increase in AQP4 excretion levels in astrocyte end feet along with the duration of hydrocephalus ($p = 0.001$) in comparison between hydrocephalus mice on the 7th, 14th, and 21st days. AQP4 before and after CSF drainage, comparison of the hydrocephalus group on day 21 with the group of mice undergoing CSF drainage ($p<0.05$). The results showed that the CSF drainage treatment was proven to reduce the level of AQP4. **Conclusion**: This is the first study to describe immunohistochemical distribution of AQP4 after drainage hydrocephalus model in mice end feet astrocyte. The AQP4 expression and distribution in after drainage hydrocephalus model was comparable 14th and 21st day of hydrocephalus but 7 days after drainage. Larger studies are needed to substantiate the influence of breed and ageing on AQP4 expression after drainage of hydrocephalus model.

Key words: Hydrocephalus, Drainage, CSF, Aquaporin 4, AQP4.

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History

- Submission Date: 09-11-2022;
- Review completed: 10-12-2022;
- Accepted Date: 17-12-2022.

DOI : 10.5530/pj.2022.14.209

Article Available online

http://www.phcogj.com/v14/i6

BACKGROUND

Cerebrospinal fluid (CSF) plays an important role in maintaining fluid balance in the brain.[1] The classic hypothesis regarding CSF hemodynamics is that it is simply and schematically a slowly flowing river that forms in the ventricles of the brain, which flows irregularly along the CSF system into the cortical subarachnoid space, where it is absorbed into the sinus venosus.[2]

One of the fundamental assumptions of the classical theory is that the brain parenchyma is impermeable to CSF, and could not absorb CSF that accumulates in the ventricles.[3] The molecular basis of this permeability involves a specific ion channel that facilitates the movement of water with ions, namely the aquaporin channel, which facilitates the free movement of water without changing the surrounding ionic environment.[3] One of the most abundant aquaporins involved in the CSF homeostatic system is aquaporin 4 (AQP 4), which is found in ependymal cells that make up the lateral part of the ventricles and in the legs of astrocytes.[3,4] AQP4 permeates throughout the brain and retina, most prominently in astroglia at the brain-liquid interface. The end-feet membrane adjacent to the ventricles, capillaries, and subarachnoid space contain 10–15 times the amount of AQP4 protein compared to the non-end-feet membrane, with the microvilli not expressing AQP4 at all.[5,6]

Hydrocephalus is described by the Rekate model as a disease state that evolves after obstruction of the CSF circulation. Obstruction points may be found in various places and regions. Given the role of aquaporins in both production (*via* AQP1) and absorption of CSF (*via* AQP4), it could be a potential therapeutic target. AQP1-null mice had reduced osmotic water permeability compared to the wild-type, reduced CSF production by 20%-25%, and intracranial pressure by 56%.[7,8]

Because of its involvement in the cerebral vasculature, AQP4 exhibits a protective effect in hydrocephalus cases. AQP4-null mice exhibit significant ventriculomegaly after kaolin injection to reproduce obstructive hydrocephalus, increasing intracranial pressure by 2%-3%. After 5 days, the mortality of AQP4-deficient mice was 34% compared with 16% of the wild type. After kaolin-induced hydrocephalus in wild-type mice, AQP4 expression increased 3–4 weeks after injection, with highest levels in the lower vasculature, parietal cerebrum and hippocampus, ependymal lining, and glia limitans.[8]

METHODS

Research material

The research materials used were the monoclonal antibody Aquaporin-4 Immunoblot, and kaolin. Consumables materials for the preparation of experimental animals are 5 ml syringe with 25G needle, 1 ml syringe with 27G needle, betadine and sterile gauze, sterile perforated linen, ketamine and xylazine, 15 ml disposable tube, alcohol 70 %, 10% povidone iodine, surgical blade.

Kaolin-induced hydrocephalus mouse preparation

Sprague-Dawley mice were treated according to guidelines for animal rearing. To induce

Cite this article: Rahmadhan MA, Parenrengi MA, Suryaningtyas W, Fauziah D, Sudiana IK, Utomo B. Aquaporine 4 Expression on End Feet Astrocyte Before and After Cerebrospinal Fluid Drainage of Hydrocephalus Mice Model. Pharmacogn J. 2022;14(6)Suppl: 1054-1060.

hydrocephalus, sterile kaolin suspension is used. The procedure is performed under anesthesia. After the animals were anesthetized, the necks of the mice were shaved to a width sufficient to allow identification of the suboccipital area where the kaolin injection would be administered. Mice were placed in a prone position on a 15 cm, head and neck were placed protruding on the edge of the pad so that the rat's scapula was parallel to the edge of the pad, then the head is flexed with sufficient curvature (approx. 90°). An aseptic procedure using 10% povidone iodine solution was performed in the nape of the neck and craniovertebral junction prior to injection.

The neck is flexed so that the gap between the occipital bone and the first cervical vertebra is wide enough to be identified by palpation. The suboccipital cleft is identified by palpating the space between the occipital and first cervical vertebral bones. The pitfall of this technique is the process of inaccurate neck flexion which will open gaps in cervical vertebrae 2 and 3 or below. Once properly identified, 20-30 µL of sterile kaolin suspension (20% suspension in 0.9% saline) was injected per cutaneously into the magna cistern using a 27G needle.

The experimental animals were observed and kept under close observation after the injection procedure until they were independent of the anesthetic drugs. After seven days of induction with kaolin, the experimental animals were examined for signs of hydrocephalus from its clinical appearance such as enlarged head circumference, back neck bumping, gait change, hind limb paresis, flat hind limbs.

CSF drainage procedure

CSF drainage on the 7th day and 14th day after kaolin induction was carried out by tapping of CSF through estimated projection of ventricle. The target the ventricular drain age in our experimental animals is 1 cm to the right lateral side, perpendicular to the skull bone, with a depth of about 1.5 cm. It is hoped that from this projection, drainage will enter the lateral ventricle.

Brain sampling

The extracted brains were prepared using standard paraffin block preparation procedures. The brain slices were put into gauze, dehydrated by soaking in graded ethanol solutions of 70%, 80%, 90%, 100%, 100% and 100% for 60 minutes each at room temperature. The next process was clearing using xylol for 15 minutes at room temperature three times. After the clearing process, the infiltration process was carried out with liquid paraffin 3 times for 60 minutes each in an incubator at 60 °C. The tissue is then immersed in molten paraffin and cooled to room temperature so that it becomes a paraffin block.

Preparation and IHC staining preparations

Paraffin blocks containing brain tissue were then de-paraffinized. Next, rehydration was carried out with decreasing concentration of ethanol, followed by rinsing with Phosphate Buffer Saline (PBS) for 3x5 minutes. The tissue preparation was then incubated in DAKO® Antigen Retrieval Buffer in the microwave at 94°C for 20 minutes and cooling it for 20 minutes at room temperature.

The preparations were then washed with PBS solution for 3x5 minutes, and incubated in Peroxidase Block (Novocastra®) for 20 minutes. Then, the preparations were washed again with PBS for 3×5 minutes and incubated in the Protein Block for another 20 minutes. After that, it was washed again with PBS for 3×5 minutes and incubated overnight (12-18 hours) with AQP4 antibody (1:500) overnight at 4°C. The next step was rinsing with PBS for 3×5 minutes and incubating with post primary and post protein solution for 45 minutes and followed by secondary antibody incubation (Novolink® Horse Radish Peroxidase (HRP)) for 60 minutes at room temperature. After incubation, the preparations were washed with PBS for 3×5 minutes and counterstained with hematoxylin (Novocastra). Next, dehydration was carried out using increasing concentrations of ethanol. The next process is clarification with xylol, then mounting.

AQP4 expression calculation method

This study used Sprague Dawley mice brain tissue model of hydrocephalus. There are 30 slides (number of slides), consisting of 6x5 Examination groups. Each tissue sample was made into slices with a thickness of 4 um, then IHC detection of AQP4 expression in the brain cells of Sprague Dawley mice modeled hydrocephalus was detected. For calculation purposes, the coded slides are closed with a code number and randomly assigned a new number so that the examiner does not know the slide being examined is a sample of what group (Blind). Examination and calculation of AQP4 expression was observed by observing the brown color in the cytoplasm of the brain cells of Sprague Dawley mice with hydrocephalus model. Calculations were based on Soini *et al*, (1998) and Pizem and Cor (2003) and the calculations were carried out on each slide of 20 fields/field of view with 1000x microscope magnification (ocular – objective). The results of each calculation are written on a worksheet and the average value per field of view is taken. Hematoxylen-Eosin staining was performed which was used as a structural comparison. Statistical analysis when all results have been returned to the real code. In order to ensure representation and reduce error in the results, it is necessary to observe in approximately 20 fields of view with 1000x magnification, each containing approximately 1500 cells.[9,10]

Data analysis

The data collected is coded, tabulated and entried into the computer. Data analysis includes descriptive analysis and hypothesis testing. Ratio-scaled data is expressed in terms of mean, standard deviation, frequency distribution and percent in descriptive and frequency analysis. Data analysis was performed with SPSS statistical software.

The normality test of the data used the Shapiro-Wilk statistical test. Statistical analysis was done to compare the expression levels of aquaporin-4 hydrocephalus mice on days 7, 14, 21 and mice with hydrocephalus on days 14, 21 which underwent CSS drainage for 7 days. The One Way ANOVA test was used followed by the TUKEY test. The test result is said to be significant if it has a p value of < 0.05.

RESULTS

Sprague-Dawley mice that had been injected with kaolin were observed for 7 days to be examined for signs of hydrocephalus from the clinical appearance which included enlargement of head circumference, back neck bumping, gait change, hind limb paresis, hind limb flattening. The research sample was then randomly divided into 5 groups with the number of replications in each group being 6 mice (K-7, K-14, K-21, P-14, and P-21). AQP4 IHC staining results on K-7 group (Figure 2), K-14 group (Figure 3), K-21 group (Figure 4), P-14 (Figure 5), P-21 (Figure 6) are shown in the figure below.

Distribution of AQP4 expression results

The data from the research results were analyzed descriptively to get an overview of the distribution and summary of the data in order to clarify the presentation of the research results. The descriptive table in this study, consisting of five groups, aims to provide the distribution of data from the variables studied which are seen from the lowest (min), highest (max), mean, median, and standard deviation values (Table 1). Graph of AQP4 expression assessment results is shown on Figure 4.

The ANOVA test table is an analytical test used to test the differences for >2 groups. Data in each group is declared to have a significant value if the p-value was <0.05. In this comparison test. it was found that there

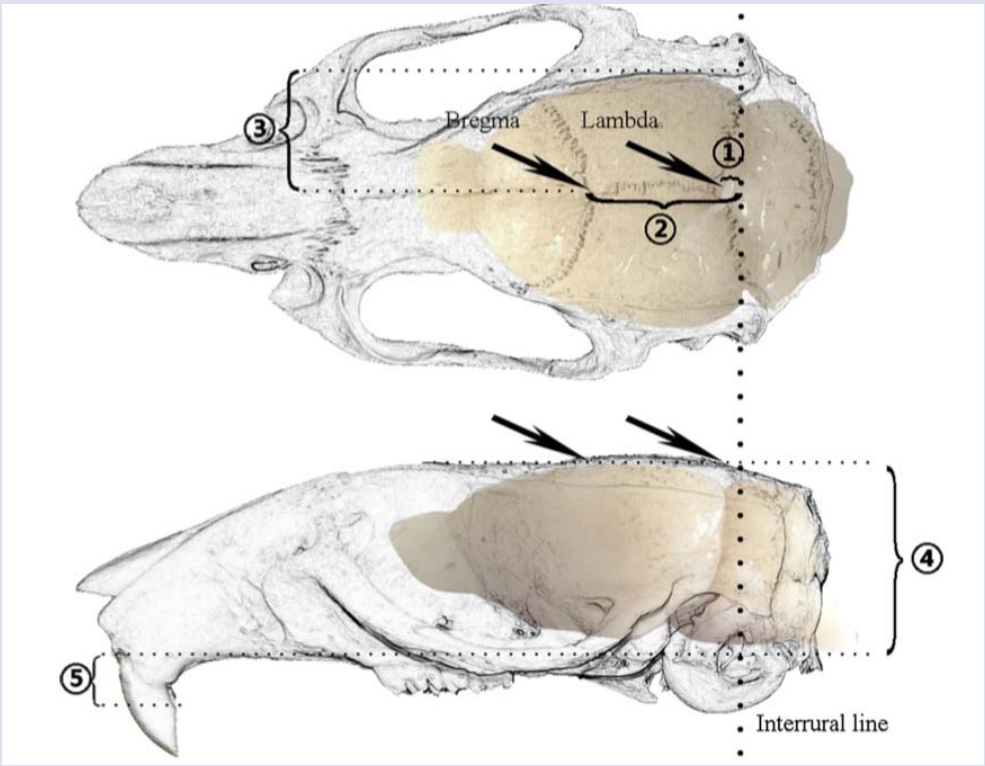

Figure 1: Figure of skull and craniometric of **Sprague** sp. 1). Lambda. 2) distance between bregma and lambda. 3) horizontal distance from the tip of roof orbita to sagitalis suture. 4).Vertical height lambda to interneural line. 5). Vertical distance from incisor bar to interneural line.[24]

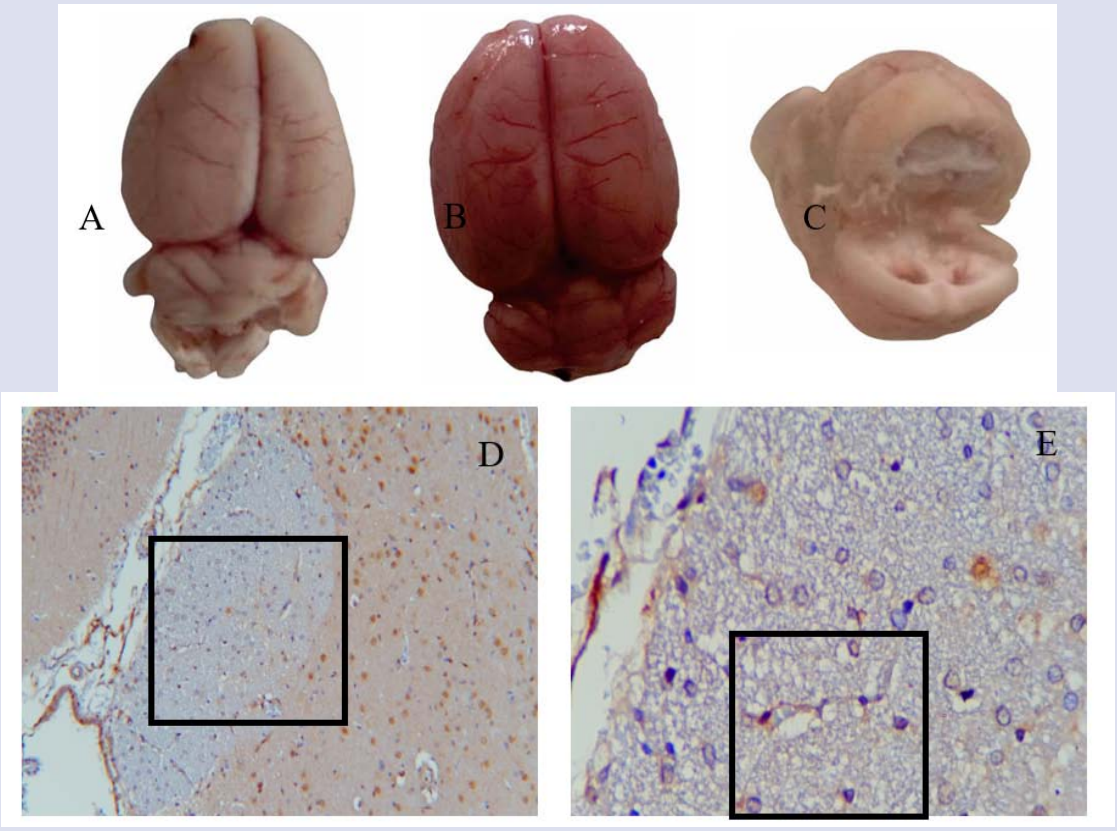

Figure 2: Picture of brain gross anatomy (A) brain after induction of kaolin 7th day(K-7), (B) after 21st day (K-21); (C) sagittal section of brain after induction of kaolin 14th day (K-14); (D) differentiation of 7th day and (E)21st after kaolin injection. AQP4 IHC staining on a cross-section of the mouse brain that were measured on day 7 after being induced of kaolin (K-7). Viewed under a microscope with 100x (left), 400x (middle) The black arrow indicates AQP4.

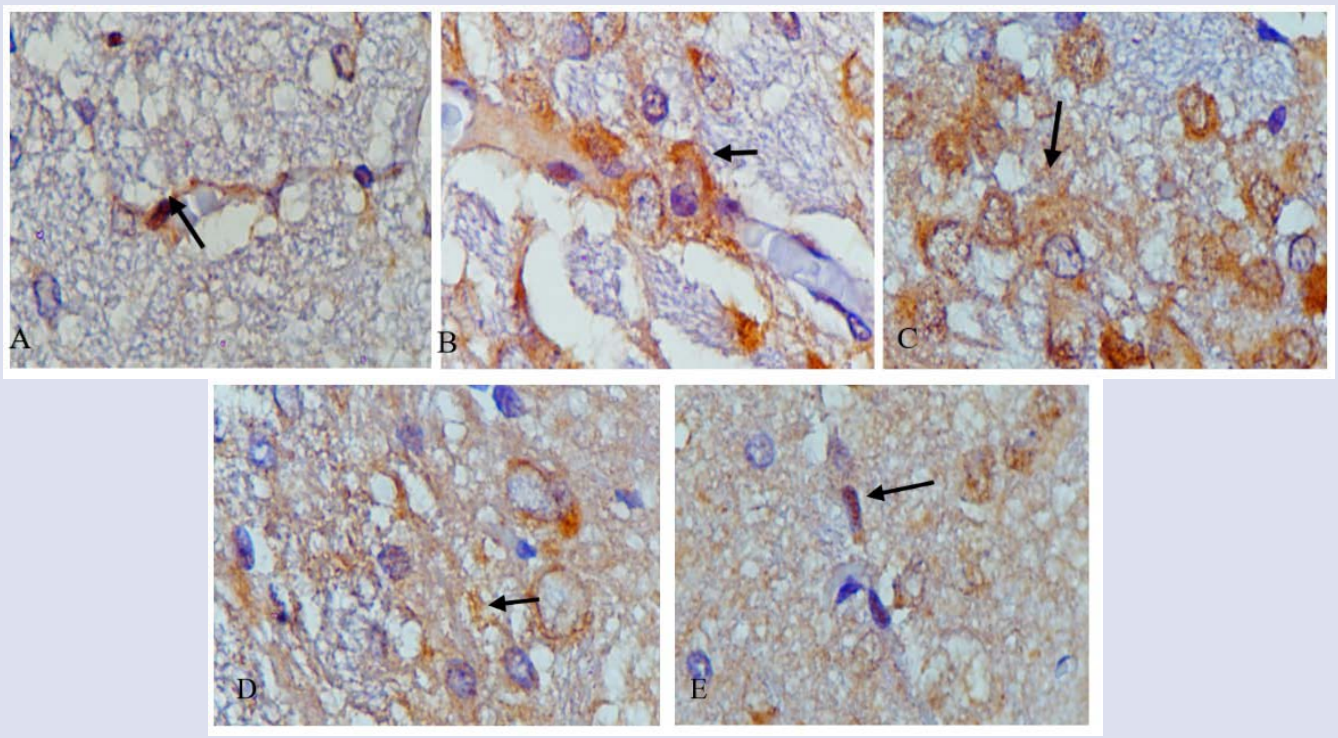

Figure 3: The results of AQP4 IHC staining on a cross-section of the mouse brain that were measured on day 7th (A), 14th(B), 21st(C) after being induced by kaolin (K-7, K-14, and K-21), and on day 7th, 14th after being induced by kaolin followed by CSF drainage (7 days) (P-14 (D) and P-21 (E)) different brightness of brown colors. Viewed under a microscope with 1000x magnification. The black arrow indicates AQP4.

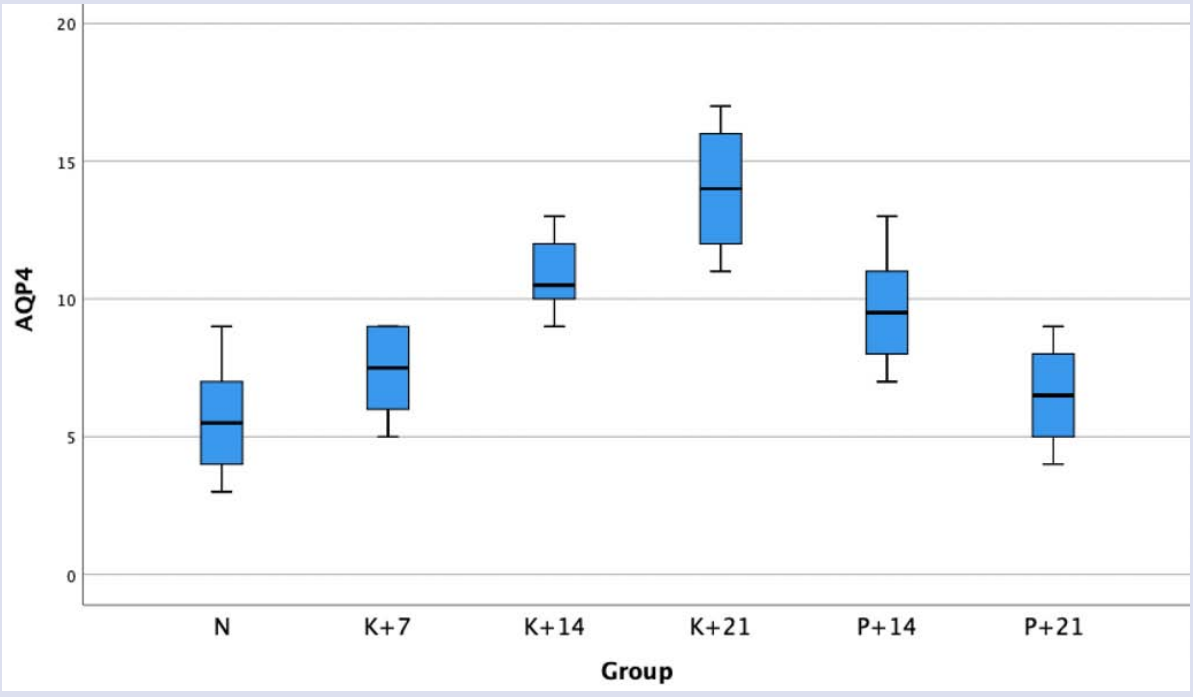

Figure 4: Graph of AQP4 expression assessment results by group.

Table 1: AQP4 descriptive table.

Groups	n	Min	Max	Mean	Median	SD
Negative control (N)	6	3.00	9.00	5.67	5.50	2.160
D-7 Positive control (K 7)	6	5.00	9.00	7.33	7.50	1.633
D-14 Positive control (K 14)	6	9.00	13.00	10.83	10.50	1.472
D-21 Positive control (K 21)	6	11.00	17.00	14.00	14.00	2.280
Control with D-14 CSF drainage (P 14)	6	7.00	13.00	9.67	9.50	2.160
Control with D-21 CSF drainage (P 21)	6	4.00	9.00	6.50	6.50	1.871

Table 2: Tukey's analysis results in the negative control group.

AQP4 Comparison		p-value
Negative control group (N)	D-7 Positive control (K 7)	0.680
	D-14 Positive control (K 14)	0.001*
	D-21 Positive control (K 21)	0.001*
	Control with D-14 CSF drainage (P 14)	0.015*
	Control with D-21 CSF drainage (P 21)	0.975

was a significant difference in the results between the five study groups because a significance value of p<0.05 was obtained (p=0.001). Tukey analysis test was then carried out to test differences between groups which were declared to have a significant value in the post hoc test if p-value was <0.05.

DISCUSSION

The purpose of this experimental laboratory study was to determine the effect of CSF drainage in cases of hydrocephalus in reducing the amount of AQP4 expression in astrocyte end feet to reduce the effect of hydrocephalus. This experimental research requires experimental animals in the form of mice which have been widely reported in other studies because they are available in relatively large quantities. easily accessible. breeding does not require tools and materials that are too complex. and easily induced to hydrocephalus both genetically and inductive using external material.[11-13]

The standard technique used by many researchers in hydrocephalus research is to use the induction method with kaolin. The standard technique was chosen in this study because the procedure is simple and does not require other special equipment to induce hydrocephalus.[14,15] The injected kaolin will spread to the subarachnoid cavity as a place for cerebrospinal fluid to drain. As a result, there will be a dam at the outlet of the quadratus ventricle. Mild-moderate hydrocephalus will generally occur on the 7th day after the kaolin injection. Ventricular enlargement (moderate-severe) will be seen macroscopically on the 14th and 21st day after kaolin injection.[14]

Relationship between aquaporin 4 and hydrocephalus

AQP4 is an aquaporin which is known to have high levels in the membrane. Previous research confirmed AQP4 as a cerebral aquaporin that has the widest and highest expression in the rat central nervous system. Although AQP4 is detected at very low levels in neurons. its expression is mainly localized to ependymal cells and astrocytes. with significant differences in expression levels between the various astrocyte types.[15]

AQP4 is located in large quantities in the brain parenchyma. especially in the astrocyte end feet. which line the blood-brain barrier and glia boundaries and are related to the physiology of water in the brain.[13] AQP4 functions in the physiology of water movement in the brain.[16] AQP4 facilitates water accumulation in cytotoxic edema and excess water clearance in the brain in vasogenic and interstitial edema.[17] In acute hydrocephalus. the brain parenchyma surrounding the ventricles is subject to interstitial edema. AQP4 undergoes up-regulation due to excess water content in the interstitial space as a compensatory response to facilitate elimination of CSS.[13,16,17] This up-regulation is related to the presence of neuroendocrine modulators.[18]

Our study showed a significant difference in AQP4 expression in K 7 compared to K 14 and K 21. Skjolding *et al.* reported a decrease in AQP4 in the periventricular region and cerebral cortex at 2 days after kaolin injection. However, there was a marked increase in periventricular AQP4 after 2 weeks of hydrocephalus.[19] Jeon *et al.* reported a significant increase in AQP4 expression compared to controls after 14 days after kaolin injection.[13] Mao *et al.* also reported that in a kaolin-induced mouse model. there was increased perivascular AQP4 immunoreactivity in the cerebrum after 3 weeks of kaolin injection (day 21). This effect is described as a compensatory mechanism for hydrocephalus.[20] Shen *et al.* reported increased expression of AQP4 in a mouse model of congenital hydrocephalus and its association with the development of an alternative pathway of CSF circulation.[21] The literature evidence confirms the results of the study that there is a relationship between AQP4 and hydrocephalus.

These changes demonstrate that AQP4 responds as a compensatory process to prevent further hydrocephalus. However, over-expression of aquaporins in the choroid plexus can lead to increased CSF production. In contrast. down-regulation of AQP4 expression in ependymal cells. glia limitans. and astrocyte end feet can reduce CSF exchange/evacuation leading to hydrocephalus.[15]

Previous studies have demonstrated that AQP4 expression is an adaptive response that varies depending on the severity of hydrocephalus.[22] The up-regulation of AQP4 expression in this study is illustrated in Figure 4 where AQP4 expression increased gradually at 7- (K 7). 14- (K 14). and 21- (K 21) days after kaolin injection. The highest levels of AQP4 were found in the K 21 group. The difference in AQP4 expression in this study was found to be significant between the K 7 and K 14 groups. K7 with K 21. and K 14 with K 21. AQP4 expression in the K 14 group was also significantly different from the negative control group. The severity of hydrocephalus correlated with the duration of AQP4 examination post-kaolin injection. The up-regulation of AQP4 expression observed in this study corroborates the findings of other studies using a mouse model of congenital hydrocephalus[21] or kaolin injection *via* the cisterna magna.[20] and represents a protective physiological mechanism. against hydrocephalus.[13,15,22] Other studies have also shown a more severe degree of hydrocephalus in AQP4-null mice.[23] Therefore. it can be concluded that the expression of AQP4 levels is indirectly related to the severity of hydrocephalus with a positive linear correlation.

Changes in aquaporin expression after drainage

The results of AQP4 expression in the astrocyte end feet of the P 14 and P 21 groups were significantly different with the group of mice that were not induced with kaolin. This difference was also shown in the P 14 group with K 21 and P 21. and the P 21 group with K 14 and K 21. According to the graph in Figure 4. the drainage which was delayed 7 days compared to the P14 group showed lower AQP4 expression. Changes in AQP4 expression after the drainage procedure were clearly illustrated in the graph compared to K 14 and K 21 groups. AQP4 levels after the drainage procedure in groups P 14 and P 21 were relatively lower than K 14 and K 21 groups. respectively. In general, the AQP4 levels of post-draining hydrocephalus mice were relatively lower compared to hydrocephalus mice without drainage. However, the analysis showed that the difference was not statistically significant.

Invasive actions with drainage or installation of shunts can disrupt the up-regulation of the AQP4 channel as a compensatory mechanism for the body to increase CSS reabsorption from the ventricles.[12] However. further explanation regarding the relationship between the

two was not found. Theoretically. the condition of hydrocephalus in mice that are drained will experience a slow resolution. This process is certainly related to CSF homeostasis which will have an impact on AQP4 expression. A possible theory can be proposed based on literature evidence. The drainage carried out in this study decreases the mechanism for the formation of natural AQP4 from the mouse body. This leads to down-regulation of AQP4 in astrocyte end feet after drainage procedure. In accordance with this theory. this study showed a decrease in AQP4 levels in hydrocephalus mice after drainage procedure. This provides a new concept that the drainage method yields different aquaporin results in addition to the conventional concept that invasive or operative measures can lower intracranial pressure.

Larger-scale studies involving experimental animals are required to reduce bias and confounding variables like the volume of CSF secreted and the high mortality rate of the animals.

CONCLUSION

We found a significant differentiation of AQP4 protein to intracellular vesicles following kaolin-induced hydrocephalus in mice that appears to involve AQP4 endocytosis. The differentiation of AQP4 End Feet Astrocyte Expression may be a compensatory mechanism resulting in drainage CSF accumulation. AQP4 deficiency results in reduced baseline ventricular size and less severe ventriculomegaly than in early induced hydrocephalus mice. We conclude that AQP4 is a potential indicator target for the treatment of hydrocephalus and mechanical drainage could make significant differentiation of reduction csf accumulation.

CONFLICTS OF INTEREST

None to declare.

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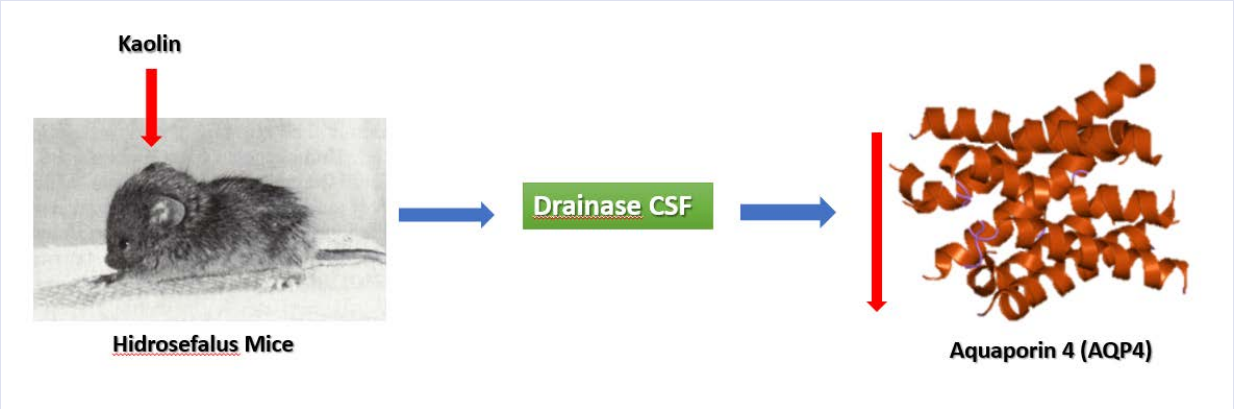
GRAPHICAL ABSTRACT



Cite this article: Rahmadhan MA, Parenrengi MA, Suryaningtyas W, Fauziah D, Sudiana IK, Utomo B. Aquaporine 4 Expression on End Feet Astrocyte Before and After Cerebrospinal Fluid Drainage of Hydrocephalus Mice Model. Pharmacogn J. 2022;14(6) Suppl: 1054-1060.