Aquaporin-4 expression related to hydrocephalus severity in hydrocephalus mice model

Fachri Balafif1, Muhammad Arifin Parenrengi1*, Wihasto Suryaningtyas3, Dyah Fauziah2, I Ketut Sudiana3, Budi Utomo4

ABSTRACT

Background: Aquaporin-4 (AQP4), a water channel protein, is important in regulating brain water distribution. We hypothesized that increased expression of AQP4 in the kaolin-induced hydrocephalic mice brain is associated with the severity of hydrocephalus. This study aims to evaluate the AQP4 expression related to hydrocephalus severity in hydrocephalus mice model.

Methods: Hydrocephalus was induced in 8-10 weeks Sprague-Dawley mice by kaolin injection into cisterna magna. The mice were randomly divided into normal control and hydrocephalus groups and were sacrificed on days 7, 14, and 21 after kaolin induction. The brains were analyzed for AQP4 expression by histological and immunohistochemistry analysis. Data were analyzed using SPSS version 25.0 for Windows.

Results: Histopathological analysis showed an increase in AQP4 expression in periventricular zone astrocytes with the duration of hydrocephalus (p < 0.001). A significant difference in AQP4 expression in this study was found in the hydrocephalus induction group on day 21.

Conclusion: The results showed that the expression of AQP4 increased with the severity of hydrocephalus. Expression of AQP4 in the kaolin-induced hydrocephalic mice brain was significantly altered depending on the length of time after kaolin induction. Changes in AQP4 expression in periventricular zone astrocytes may be a compensatory mechanism resulting in drainage of CSF accumulation.

Keywords: AQP4, Aquaporin-4, CSF, Hydrocephalus, Kaolin.

INTRODUCTION

Hydrocephalus is an active distention of the brain’s ventricular system resulting from an inadequate flow of cerebrospinal fluid (CSF).1 Water homeostasis and neuronal activity are two things that are difficult to separate, are crucial in the physiology of the nervous system and are important in the pathophysiology of hydrocephalus. Brain fluid secretion and the transport of cerebral fluid between different compartments and cellular structures have been known for centuries.24 Shifting of intracerebral water across the plasma cell membranes in response to osmotic gradients is greatly facilitated by aquaporins, a family of water channels.5 Aquaporin-4 (AQP4) is the primary aquaporin in the central nervous system and shows a protective effect in cases of hydrocephalus, where AQP4 can play a relevant role in astrocytes and ependymal cells.67 Since AQP4 is mainly found at the blood-tissue and tissue-CSF borders, it may be capable of fast water transport dependent on osmotic gradients between those compartments.8 AQP4-null mice showed significantly increased CSF content and accelerated ventricular enlargement progression after kaolin injection to reproduce obstructive hydrocephalus, increasing brain parenchymal water content by 2-3%.9 After five days, AQP4-deficient mice mortality was 34% compared with 16% of wild-type mice.10 At the same time, in mice brains with severe hydrocephalus, there was an enhanced AQP4 immune response.11 These results suggest that the upregulation of AQP4 expression may be interpreted as a compensatory mechanism to allow for trans ependymal and parenchymal CSF absorption. In this study, we hypothesized that an increase in AQP4 expression following induction of hydrocephalus in mice corresponds to the severity of hydrocephalus.

METHODS

Animal
Sprague-Dawley rats were carried out according to animal-rearing guidelines, and experiments were approved by the Animal Care and Use Committee of Faculty at the Veterinary Medicine of Universitas Airlangga. Male Sprague-Dawley mice aged 8-10 weeks, weighing 150-200 grams (n = 24), were obtained.
from the Experimental Laboratory of Universitas Gajah Mada and randomly divided into normal control and hydrocephalus groups. They were kept in standard cages and provided with a 12-hour cycle of dark and light. Water and food were given in sufficient quantities. The rats in the hydrocephalus group were sacrificed on the 7th, 14th, and 21st days after kaolin induction.

Hydrocephalus induction and kaolin administration
Hydrocephalus was induced in anesthetized Sprague–Dawley mice using sterile kaolin suspension. The mice's necks were shaved to width, making it possible to identify the suboccipital region where the kaolin suspension would be injected. The Rats were positioned prone on a 15 cm pad, with the head and neck protruding so that the space between the occipital bone and the first cervical vertebra was large enough to be identified by palpation. Once properly identified, a sterile needle was used to percutaneously inject 20–30 μL of clean kaolin suspension (20% suspension in 0.9% saline) into the cisterna magna. The rats were closely observed after the injection procedure until they were no longer dependent on the anesthetic drugs. After seven days of induction with kaolin, the mice were observed for signs of hydrocephalus from its clinical appearance, such as a raised head circumference, back neck bumping, flat hind limbs, rear limb paresis, and gait change.

Brain sampling
Standard paraffin block preparation techniques were used to prepare the extracted brains. The brain slices were placed into gauze and soaked in 70%, 80%, 90%, 100%, 100% and 100% ethanol solution for 60 minutes each to dehydrate. The following step involved clearing with xylol thrice for 15 minutes at room temperature. Following the clearing procedure, the infiltration process with liquid paraffin was performed three times for 60 minutes each at a temperature of 60°C in an incubator. The tissue is then submerged in molten paraffin and cooled at room temperature to create a paraffin block.

Histological and immunohistochemistry studies
The brain tissue-containing paraffin blocks were later de-paraffinized. Rehydration was then accomplished using ethanol at a lower concentration, followed by three times Phosphate Buffer Saline (PBS) rinses for 5 minutes each. The tissue preparation was then cooled for 20 minutes at room temperature after being incubated in DAKO® Antigen Retrieval Buffer for 20 minutes at 94°C in the microwave. The preparations were washed thrice with PBS solution for 5 minutes each and set in Peroxidase Block (Novocastra®) for 20 minutes. This procedure was repeated twice, followed by overnight incubation for 12-18 hours with AQP4 antibody (1:500) at 4°C. The next step was rinsing three times with PBS solution for 5 minutes each and incubating with post-primary and post-protein solution for 45 minutes. The procedure was followed by secondary antibody incubation (Novolink® Horse Radish Peroxidase (HRP)) for 60 minutes and was washed three times with PBS solution for 5 minutes each, followed by hematoxylin (Novocastra®) counterstaining. Next, dehydration was accomplished using ethanol and clarification with xylol, followed by mounting.

AQP4 expression calculation method
Each tissue sample was sliced into 4-μm sections, then immunohistochemistry (IHC) detection of AQP4 expression in Sprague-Dawley rats brain cells modeled hydrocephalus. By examining the brown color of the astrocytes in the periventricular zone, AQP4 expression was analyzed and calculated. Calculations were carried out on each slide of 20 fields/field of view with 1000x microscope magnification under a Nikon E100 microscope and camera Sony ZV-e10. Quantitative data are expressed as mean ± standard deviation (SD). The AQP4 data was analyzed using a One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test to assess the statistical significance among different groups. Results were considered statistically different at p < 0.05. Data were analyzed using SPSS version 25.0 for Windows.

RESULTS
In this study, we analyze the distribution of AQP4 expression to the severity of hydrocephalus. After receiving a kaolin injection, Sprague-Dawley mice were kept under observation for seven days to look for signs of hydrocephalus from the clinical appearance, which included an enlarged head circumference, back neck bumping, altered gait, hind limb paresis, and flattening of the hind limbs. The research sample was then randomly divided into four groups (N, T-7, T-14, and T-21), with the number of replications in each group being 6 rats. AQP4 IHC staining results on

Figure 1. The results of AQP4 IHC staining on a cross-section of the mice brain (A) normal control, (B) 7 days after kaolin injection (T-7), (C) 14 days after kaolin injection (T-14), (D) 21 days after kaolin injection (T-21). Viewed under a 1000x magnification microscope. The brown color indicates AQP4. (E) coronal section of brain gross anatomy 21st days after induction of kaolin.
AQP4 is presented as an effective water channel on the membranes of living cells and microorganisms. AQP4 shows a protective effect in cases of hydrocephalus, where the role of AQP4 is involved in the cerebral vasculature. Various types of mice experimental studies as animal models have been widely reported because they are available in relatively large quantities, easily accessible, and easily induced into hydrocephalus. The standard method many researchers use for inducing experimental hydrocephalus is the intracisternal kaolin injection. Kaolin is an inert silica derivative and causes both non-communicating and communicating hydrocephalus according to the injection site by combining the physical deposition effects and the response of local fibrotic on the arachnoid and pia mater membranes. The severity of hydrocephalus correlated with the length of days after the kaolin injection. On the seventh day following the kaolin injection, mild-moderate hydrocephalus will appear macroscopically. Moderate-severe hydrocephalus will generally occur on the 14th and 21st days after the kaolin injection.

One of the most abundant aquaporins involved in the CSF homeostasis system is AQP4, which is found mainly in the ependymal cells that make up the lateral part of the ventricles and the astrocyte end-feet. The involvement of AQP4 may explain a change in extracellular volume that occurs in neuronal activation and provides a pathway for transport through the plasma membrane. The main role of AQP4 appears to be in regulating the distribution of brain water. AQP4 promotes water accumulation in cytotoxic edema and removes excess water from the brain.

In hydrocephalus, interstitial edema affects the brain parenchyma surrounding the ventricles. AQP4 undergoes enhanced regulation of excess water content in interstitial spaces in compensatory response to facilitate CSF elimination. This up-regulation is associated with the presence of neuroendocrine modulators. The process explains the relationship between AQP4 and hydrocephalus.

### Table 1. AQP4 descriptive table

<table>
<thead>
<tr>
<th>Groups</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
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<td>3</td>
<td>9</td>
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<tr>
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<td>9</td>
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<tr>
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### Table 2. Tukey's post hoc test analysis results

<table>
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<td>T-7</td>
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<td>Normal control</td>
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<td>T-14</td>
<td>0.023</td>
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<tr>
<td>T-21</td>
<td>&lt;0.001</td>
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<tr>
<td>T-14</td>
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<tr>
<td>Normal control</td>
<td>0.001</td>
</tr>
<tr>
<td>T-7</td>
<td>0.023</td>
</tr>
<tr>
<td>T-21</td>
<td>0.044</td>
</tr>
<tr>
<td>T-21</td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T-7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T-14</td>
<td>0.044</td>
</tr>
</tbody>
</table>

### DISCUSSION

This experimental study was conducted to determine the AQP4 expression in the periventricular zone for each hydrocephalus severity. AQP4 is presented as an effective water channel on the membranes of living cells and microorganisms. AQP4 shows a protective effect in cases of hydrocephalus, where the role of AQP4 is involved in the cerebral vasculature. Various types of mice experimental studies as animal models have been widely reported because they are available in relatively large quantities, easily accessible, and easily induced into hydrocephalus. The standard method many researchers use for inducing experimental hydrocephalus is the intracisternal kaolin injection. Kaolin is an inert silica derivative and causes both non-communicating and communicating hydrocephalus according to the injection site by combining the physical deposition effects and the response of local fibrotic on the arachnoid and pia mater membranes. The severity of hydrocephalus correlated with the length of days after the kaolin injection. On the seventh day following the kaolin injection, mild-moderate hydrocephalus will appear macroscopically. Moderate-severe hydrocephalus will generally occur on the 14th and 21st days after the kaolin injection.

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Our study showed significant differences in AQP4 expression in mice models without hydrocephalus compared to the 14th and 21st days after hydrocephalus induction. The difference in AQP4 expression in this study was significant in the hydrocephalus group between the T-7 and T-14, T-7 and T-21, and T-14 and T-21 groups. Jeon T et al. reported that 14 days after kaolin injection, AQP4 expression significantly increased compared to controls. Skjolding AD et al. reported a decrease in AQP4 expression in mice models after kaolin injection in the cerebral cortex and periventricular region. After 2 weeks of hydrocephalus induction, there was a noticeable rise in periventricular AQP4. Mao X et al. reported 21 days after kaolin injection; there was an increase in perivascular AQP4 immunoreactivity in the brain of kaolin-induced mice models. It is explained that the effect is a compensatory mechanism against hydrocephalus. Shen XQ et al. reported increased AQP4 expression in congenital hydrocephalus mice models and its association with developing alternative CSF circulation pathways. However, the choroid plexus’ overexpression of aquaporin may increase CSF production. On the other hand, hydrocephalus can result from the down-regulation of AQP4 expression in ependymal cells, glia limitans, and astrocyte end feet.

In this study, the up-regulation of AQP4 expression is depicted in Figure 2, where AQP4 expression increased gradually at the 7th, 14th, and 21st days after kaolin injection. The highest AQP4 expression was found in the T-21 group. Our study data showed that the number of days following the kaolin injection correlates with the severity of the hydrocephalus. The AQP4 up-regulation expression results in this experimental study were supported by other research findings using a mice model of kaolin injection or congenital hydrocephalus, representing a physiological defense against hydrocephalus. Other studies have also shown that AQP4-null rats exhibit a more severe form of hydrocephalus. Therefore, it can be concluded that the AQP4 expression in the periventricular zone is related to the severity of hydrocephalus.

CONCLUSION

This study provides significant evidence that AQP4 expression in the kaolin-induced hydrocephalic mice brain increases with the severity of hydrocephalus. The expression of AQP4 significantly changed depending on the length of time after kaolin induction. The highest AQP4 expression was found on the 21st day after the kaolin induction group. The differentiation of AQP4 expression in periventricular zone astrocytes could play an important neuro-defensive role as a compensatory mechanism resulting in CSF drainage.

ETHICAL CONSIDERATIONS

All procedures performed and materials included in the study are in accordance with the ethical standards of the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (Ethical number 2.KE.061.06.2020).

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

FUNDING

None.

AUTHOR CONTRIBUTIONS

All authors equally contribute to the study from the conceptual framework, data acquisition, data analysis, until reporting the study results through publication.

REFERENCES