BRAIN AQUAPORIN-4 EXPRESSION IN THE RAT SEPTIC MODEL (IMMUNOHISTOCHEMICAL STUDY)

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Abstract. Brain aquaporin-4 expression in the rat septic model (immunohistochemical study). Shulyatnikova T.V., Tumanskiy V.O. The study aimed to determine aquaporin-4 expression in different brain regions was performed in Wistar rats subjected to cecal ligation and puncture (CLP) septic model. The immunohistochemical study of aquaporin-4 was carried out in the sensorimotor cortex, white matter, hippocampus, thalamus and caudate nucleus/putamen regions between 20 and 38 h after CLP. From the 12th h after CLP all animals showed the progressive impairment of sepsis signs and therefore, 9 rats were euthanized between 20-38 h ("CLP-B", non-survived); 11 animals survived up to 48 h (constituted "CLP-A", survived). After operation, CLP-B group displayed regionally-specific dynamic increase in aquaporin-4 level in the brain mostly associated with astroglial capillary endfeet: by 23rd h in the cortex – 234.15%, by 24th h in the thalamus –129.47% and hippocampus – 101.36%, by 30th h in the white matter – 135.31% and by 38 h in the caudate/putamen – 92.85%; with the highest increase in cortex: by 3.34 times. Heterogeneous and heterochronous aquaporin-4 elevation among brain regions indicates territories more and less susceptible for systemic toxic exposure in sepsis as well as points to diverse reactive responsiveness of local astroglial populations during specific time-period after CLP. The higher rates of aquaporin-4 in the cortex of non-survived animals in CLP model reflects the importance of aquaporin-4 increase in the mechanisms of sepsis decompensation.

Key words: sepsis-associated encephaopathy, astroglial reactivity, aquaporin-4

Sepsis-associated encephalopathy (SAE) represents one of the most adverse complications of sepsis with rates up to 70% in ICU septic patients [1]. Assumed pathophysiology of SAE involves multifactorial systemic influence of microbial components, factors of dysregulated host immunity, byproducts of impaired metabolism due to multiple organ failure, hypoperfusion, as well as intracerebral events including hypoxic and ischemic lesions, blood-brain barrier breakdown, abnormal glial reactivity, neuroinflammation, neurotransmitter imbalance, neuronal death and brain edema [2]. Vasogenic and cytotoxic edematous changes in SAE brains were previously established in animal models and postmortem human studies, however there is still no common view on the precise mechanisms that prevail in its development.
due to variability of brain lesions in SAE as well as
to differences in research designs [3]. Astrocytes,
being central homeostatic cell population in the brain,
are key players responsible for water and ion
homeostasis. Aquaporin-4 (AQP4) is the main water
channel protein in the brain located at the astrocytic
end-feet [4]. A. Vandebroek et al. has recently re-
viewed that at different disorders AQP4 can act in a
specific manner. In particular, cerebral ischemia
stimulates increase in AQP4 expression accompanied
by cerebral edema, herewith AQP4 inhibition leads to
edema decline under the same conditions [5, 6].
Nevertheless, AQP4 loss in the condition of vaso-
genic edema causes impairment of the pathology
conditioned by decreased water drainage from the
brain [5, 7]. Brain AQP4 expression during sepsis is
reviewed only partially and does not reflect hetero-
genosity in astroglial reactivity in respect to this key
protein, which suppose providing such research using
animal septic model. Thus, the purpose of the present
study was determining the immunohistochemical
level of aquaporin-4 expression in different brain
regions in the experimental sepsis in rats.

MATERIALS AND METHODS OF RESEARCH
The study was performed in Wistar rats of 200-
300 g body weight. All procedures were ruled in
accordance with the European Convention for the
Protection of Vertebrate Animals Used for Experi-
mental and other Scientific Purposes (Strasbourg,
18 March 1986; ETS No. 123), the Directive
2010/63/EU and approved by the Bioethics committee
of Zaporizhzhia State Medical University (protocol
No. 1 on January 12, 2022). Rats were subjected to
the traditional cecal ligation and puncture (CLP)
model of sepsis in rodents [8], dividing animals into
2 groups: CLP (n=20) and sham-operated (control),
n=5. All experimental stages were conducted in
accordance with the previously described technique
[9]. After CLP, rats were observed up to 48 h.
Beginning from the 12th h the following clinical signs
were noted: periorbital edema, piloerection, diarrhea,
fever/hypothermia, social isolation, deep lethargy and
severe respiratory disorders progressively aggra-
vated. During 20-38 h after CLP, 9 rats showed
highly expressed mentioned clinical symptoms and
were euthanized ("CLP-B" – non-survived); 11 animals expressed less pronounced suffering until
48 h – end-point of the experiment – they constituted
"CLP-A" group (survived). Control rats ("CLP-C")
showed no lethal outcomes. All CLP-A and CLP-C
animals were sacrificed 48 h after CLP by sodium
thiopental overdosing. Brain and liver samples were
processed according to standard steps with formation
of paraffin blocks. Histopathological analysis was
performed using hematoxylin-eosin stained sections.

Immunohistochemical (IHC) procedures were con-
ducted according to conventional steps [10]. IHC
study involved detection of immunopositive labels
using rabbit polyclonal anti-AQP4 primary antibody
(Termo Scientific, USA) and Ultra Vision Quanto
Detection imaging system with diaminobenzidine
(Termo Scientific Inc., USA). Results of IHC
reaction were assessed at x200 in a standardized field
of view (SFV) of the microscope Scope. A1 “Carl
Zeiss” (Germany) using Jenoptik Progres Gryphax
60N-C1”1,0x426114 (Germany) camera and Video-
test-Morphology 5.2.0.158 (Video Test LLC, RF)
program. AQP4 level was assessed as a percentage of
the relative area (S rel., %) of immunostained labels
to the total area of the section in SFV. Sensorimotor
cortex, subcortical white matter, hippocampus, thal-
amus and caudate nucleus/putamen regions were
harvested for the analysis of the AQP4 staining.
Five SFV of each mentioned region were analyzed
for each animal. Data were processed by Statistica®
for Windows 13.0 (StatSoft Inc., license No. JPY8041382130RCN10-J) with evalu-
ating median (Me), lower and upper quartiles (Q1;
Q3). For comparison between groups Mann-Whitney
and Kruskal-Wallis tests were used. The results were
considered significant at 95% (p<0.05) [11].

RESULTS AND DISCUSSION
Control rats demonstrated diverse AQP4 staining
among studied brain regions with the highest values
in the cortex – 2.43 (2.10; 3.67)% and the lowest ones
in the subcortical white matter – 0.56 (0.35; 1.12)%
(Table). AQP4 labeling in all brain regions in CLP-C
was predominantly associated with astroglial
vascular end-feet and in lesser extend belonged to
parenchymal astroglial processes (Fig. 1).

Beginning from the 23rd h, in non-survived, as
well as at 48 h in survived animals, histopathological
examination of the liver revealed signs of spread
balloon dystrophy and selective necrosis of
centrilobular individual hepatocytes, as well as small
focal centrilobular necrosis, focal neutrophilic infiltr-
ation and signs of moderate cholestasis aggravated
over time after CLP in non-survived animals. ICH
examination of the samples from 5 studied brain
regions of CLP-rats revealed increased AQP4 abun-
dance predominantly related to capillary astroglial
end-feet as well as in glia limitans processes in the
cortical region. However, neuropil of all studied
regions either showed moderate-to-weak AQP4
staining (Fig. 2). Both in CLP-A and CLP-B rats the
alteration of AQP4 expression appeared to be diverse
among different brain regions and time-points of the
experiment, likewise, more pronounced elevation of
the indicators was found in non-survived rats.
Brain aquaporin-4 expression in different experimental groups expressed in the percent of immunopositive labels in the SFV. Data are represented as median and lower, upper quartiles: Me (Q1; Q3)

| Brain region          | CLP-A          | CLP-B          | CLP-C          |
|-----------------------|----------------|----------------|----------------|
| Cortex                | 5.34 (4.36; 6.25) *† | 8.12 (6.74; 8.32) *† | 2.43 (2.10; 3.67) |
| Subcortical white matter | 1.25 (1.20; 1.89) * | 1.32 (1.25; 2.15) * | 0.56 (0.35; 1.12) |
| Hippocampus           | 4.20 (3.15; 4.89) * | 4.43 (3.37; 5.15) * | 2.20 (2.10; 3.06) |
| Thalamus              | 2.06 (1.94; 2.54) * | 2.18 (2.05; 2.84) * | 0.95 (0.63; 1.76) |
| Caudate/putamen       | 2.10 (1.90; 2.72) * | 2.16 (1.95; 2.93) * | 1.12 (0.43; 1.82) |

Notes: reliable differences in indicators compared to the control animals (p<0.05) are marked with an asterisk (*); reliable differences between CLP-A and CLP-B groups in the same brain region (p<0.05) are marked with the dagger (†). The highest increase in AQP4 level compared to control was revealed in the cortical region of non-survived rats: 8.12 (6.74; 8.32)% vs 2.43 (2.10; 3.67)%, p<0.05, which corresponded to 234.15% or 3.34-fold increase comparing medians of the noted indicators. Moreover, AQP4 cortical elevation showed reliably higher rates in CLP-B compared to CLP-A: 8.12 (6.74; 8.32)% vs 5.34 (4.36; 6.25)%, p<0.05, unlike other brain regions displayed no statistical difference between CLP-A and CLP-B groups (p>0.05) (Table). Subcortical white matter, thalamic and hippocampal regions of CLP-B group showed the most substantive elevation in AQP4 staining, respectively: by 135.71% (2.35-fold), by 129.47% (2.29-fold), by 101.36% (2.01-fold), comparing medians to control values (p <0.05). The least significant reliable increase in AQP4 staining was detected in the caudate nucleus/putamen of CLP-B rats: by 92.85% (1.92-fold increase) (Table).

Fig. 1. AQP4 expression in the cortex of the control rat (CLP-C group) 48 h after the sham procedure (anti-AQP4, Thermo Scientific, USA). X400

Fig. 2. AQP4 expression in the cortex of the non-survived rat (CLP-B group) 38 h after the CLP-procedure (anti-AQP4, Thermo Scientific, USA). X400
After CLP-procedure, non-survived animals displayed dynamic heterochronous increase in AQP4 indicators among studied brain regions with the highest values 38 h after operation. The earliest reliable elevation in AQP4 values was detected in the cortical region – at 23rd h, hippocampal and thalamic regions displayed significant difference by 24th h, white matter – by 30th h, whereas caudate/putamen elevation reached statistical validity compared to control only by 38th h after operation.

Upregulation of the brain AQP4 in our present research partially confirms previously established data on the special role of AQP4 in septic encephalopathy and SAE both associated with vasogenic edema development, where alteration of AQP4 expression has been shown to be mediated by invading immune cells as well lipopolysaccharide (LPS) exposure [3]. Furthermore, AQP4 was recently proposed as the novel serum biomarker of SAE, as it was established in astrocyte-derived exosomes in this condition [12]. As it was mentioned previously, AQP4-null experiments performed more favorable outcomes in cytotoxic edema, herewith, appeared to be disadvantageous in case of vasogenic mechanisms [13]. Ischemic as well as hypoxic brain damage, both characteristic for SAE and signed by cytotoxic and vasogenic edema, are also linked to AQP4 alteration, however the precise role of the latter is still unclear. In particular, it was reported on the opposite or biphasic elevation changes in AQP4 levels over time after hypoxic and ischemic events respectively [13]. Meli R. and coauthors have recently reviewed that inflammatory medium which is widely recognized for SAE condition, also involves AQP4-dependent links. Thus, earlier studies have revealed that AQP4-knockout animals displayed less pronounced neuroinflammation, astrocyte swelling and release of proinflammatory cytokines than wild type animals after intracerebral LPS administration [14]. Moreover, it was established that AQP4 is intimately linked to the intercellular astroglial/microglial interactions in the inflammatory and edematous mechanisms [15]. As SAE is a result of the primary multifactorial peripheral influence on the CNS, the products of dysregulated metabolism in other organs might contribute significantly. Among the central vital organs, failure of the liver has one of the highest values in sepsis. In our recent partially published studies, we have revealed increase in glutamine synthetase (GS) and AQP4 levels in the same 5 brain regions in the acetaminophen-induced liver failure in rats, where AQP4 alterations preceded GS ones in two regions out of five. AQP4 elevation can be reasoned by hyperosmolarity [16] and in case of sepsis complicated by liver failure, such elevation could be stimulated by glutamine accumulation in the astrocytes, as well as by independent factors. The latter can be indirectly confirmed in the present study by first morphological changes in the liver at 23 h, which appeared simultaneous to the earliest increase in the cortical AQP4 in the same animals and found later in other brain regions. The earliest and highest AQP4 increase in the cortex indicates a region the most susceptible to systemic factors in sepsis and suggests it as one of the principal territories for SAE mechanisms involving AQP4 alteration. Heterogeneous AQP4 elevation points to region-specificity of the local astroglial populations and their reactivity in SAE conditions. The higher AQP4 levels in non-survived animals compared to survived ones indicates the significance of the brain AQP4 elevation in the pathophysiology of sepsis aggravation.

CONCLUSION

Cecal ligation and puncture septic model stimulates dynamic increase in aquaporin-4 level in the cortex by 23rd h, hippocampus and thalamus – by 24th h, in the white matter – by 30 h and in the caudate nucleus/putamen – by 38th h with the highest elevation in the cortex. Heterogeneous aquaporin-4 elevation among brain regions indicates brain territories differently susceptible for systemic toxic exposure in sepsis as well as points to heterogeneous reactive responsiveness of local astroglial populations during specific time-period after cecal ligation and puncture procedure. The higher rates of aquaporin-4 expression in the cortex of non-survived animals in cecal ligation and puncture model reflects the importance of the brain aquaporin-4 increase in the mechanisms of sepsis decompensation.

Contributors:
Shulyatnikova T.V. – conceptualization, methodology, resources, investigation, formal analysis, validation, visualization, writing – original draft; Tumanskiy V.O. – conceptualization, methodology, supervision, project administration, writing – review & editing.

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Conflict of interests. The authors declare no conflict of interest.
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