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

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Key words: *sepsis-associated encephalopathy, astroglial reactivity, aquaporin-4*
Ключові слова: *сепсис-асоційована енцефалопатія, астрогліальна реактивність, аквапорин-4*

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.*

Реферат. Експресія мозкового аквапорину-4 в моделі сепсису в щурів (імуногістохімічне дослідження). Шулятнікова Т.В., Туманський В.О. *Дослідження, що було спрямоване на визначення експресії аквапорину-4 в різних ділянках мозку, проведено з використанням щурів лінії Вістар, що піддавалися септичній моделі перев’язки й пункції сліпої кишки (ППСК). Імуногістохімічне дослідження аквапорину-4 проводили в сенсомоторній корі, білій речовині, гіпокампі, таламусі та хвостатому ядрі/шкаралупі між 20 і 38 год. після ППСК. Починаючи з 12-ї години після ППСК у всіх тварин спостерігалося прогресуюче погіршення ознак сепсису, у зв’язку з чим 9 щурів були евтаназовані між 20-38 годинами (ППСК-Б; загиблі); 11 тварин вижили до 48 год. (ППСК-А; ті, що вижили). Після операції в групі ППСК-Б відмічено регіонально-специфічне динамічне підвищення експресії аквапорину-4 в головному мозку, переважно локалізованої в астрогліальних капілярних кінцьових ніжках: на 23 годині в корі – на 234,15%, на 24 годині в таламусі – на 129,47% та гіпокампі – на 101%, на 30 год у білій речовині – на 135,31 % і на 38 год. у хвостатому ядрі/шкаралупі – на 92,85%; з найбільшим збільшенням у корі: у 3,34 раза. Неоднорідне та гетерохронне підвищення аквапорину-4 серед регіонів мозку вказує на території, більшою та меншою мірою вразливі до системного токсичного впливу при сепсисі, а також вказує на неоднорідність реактивної відповіді місцевих астрогліальних популяцій протягом певного періоду часу після ППСК. Більш високі показники аквапорину-4 в корі загиблих тварин у моделі ППСК вказують на суттєве значення підвищення експресії мозкового аквапорину-4 в механізмах декомпенсації сепсису.*

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 due 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 reviewed 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 vasogenic 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 heterogeneity 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 Experimental 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 as periorbital exudation, piloerection, diarrhea, fever/hypothermia, social isolation, deep lethargy and severe respiratory disorders progressively aggravated. 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 conducted according to conventional steps [10]. IHC study involved detection of immunopositive labels using rabbit polyclonal anti-AQP4 primary antibody (Thermo Scientific, USA) and Ultra Vision Quanto Detection imaging system with diaminobenzidine (Thermo 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 Videotest-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, thalamus 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. JPZ804I382130ARCN10-J) with evaluating 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 infiltration 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 abundance 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 (3.27; 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).

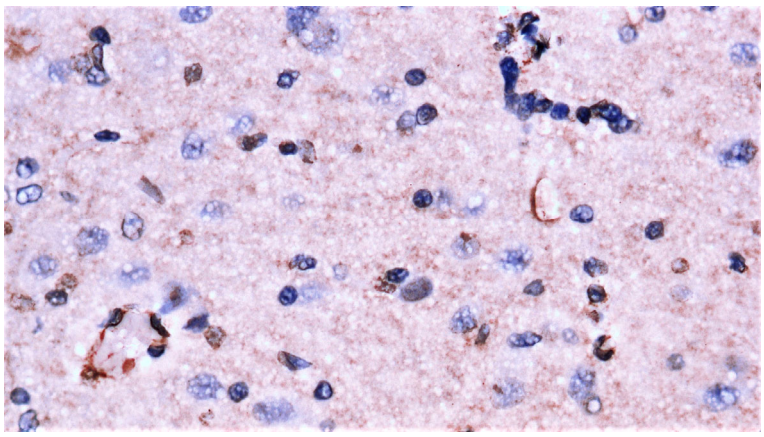

Fig. 1. AQP4 expression in the cortex of the control rat (CLP-C group) 48 h after the sham procedure (anti-AQP4, Thermo Scientific, USA). X400

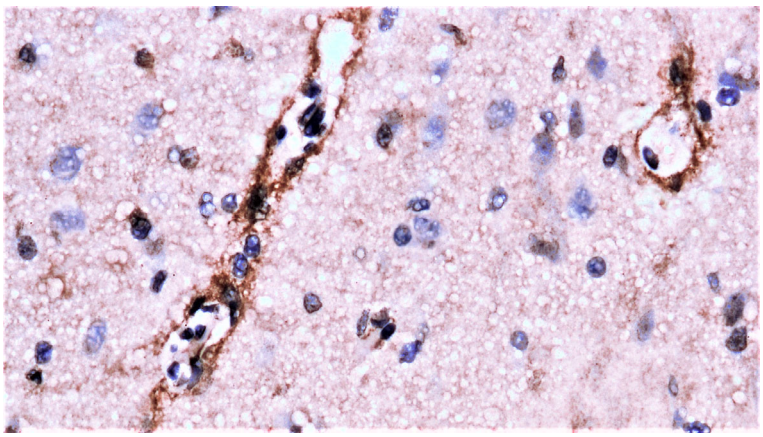

Fig. 2. AQP4 expression in the cortex of the non-survived rat (CLP-B group) 38 h after the CLP-procedure (anti-AQP4, Thermo Scientific, USA). X400

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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