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Nestin, CD44, Ki-67, GS and AQP4 expression in the brain neurogenic niches of deceased patients with liver cirrhosis of different degree

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Key words: hepatic encephalopathy, neurogenesis, astrocytogenesis, stem cells, Nestin, CD44, Ki-67, GS, AQP4.

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The aim of the study. Immunohistochemical study of Nestin, CD44, Ki-67, GS, AQP4 expression in the subventricular zone of the lateral ventricle and hippocampus of deceased patients with liver cirrhosis depending on the age and Child–Pugh score.

Materials and methods. The brains of 90 deceased patients aged 65 ± 3 years with non-alcoholic liver cirrhosis (LC) Child–Pugh class A, B and C were studied, which comprised 3 groups: group "A" – 30 deceased patients with compensated LC; "B" – 30 deceased patients with subcompensated LC ("mild decompensation"); "C" – 30 deceased patients with decompensated LC. Control group included brains of 30 patients died from acute cardiovascular failure and did not have liver disease. Each group was divided into 2 subgroups: patients ≤59 y. o. and patients ≥60 y. o. Grade 1–4 hepatic encephalopathy was detected in 59 out of 90 (65.55 %) patients with LC. The immunohistochemical levels of Nestin, CD44, Ki-67, GS and AQP4 were evaluated in paraffin tissue sections of the subventricular zones (SVZ) of the anterior and lower horns of the brain lateral ventricles, as well as the subgranular zone (SGZ) of the dentate gyrus (DG) and other structures of hippocampus in standardized fields of view of the microscope Scope A1 Carl Zeiss (Germany) using Videotest-Morphology 5.2.0.158 software.

Results. In SVZ of control subgroups, Nestin+ astrocyte-like stem cells were localized mainly in subventricular glial nodules (SGN) and to a lesser extent in astrocytic ribbon. In brains of patients with compensated and subcompensated LC, there was increased Nestin expression compared to control (by 61.36 % and 208.74 %, respectively) due to increased numbers of Nestin+ cells in astrocytic ribbons. In the hippocampus of control and cirrhotic patients, Nestin expression was determined mainly in astrocyte-like cells of the *fimbria-fornix*, "glial plates" around the blood vessels entering the choroid plexus and subpial zone. In the SVZ of patients with subcompensated LC, the expressions of Nestin, CD44, and Ki-67 were maximally increased (by 208.74 %, 37.83 %, and 3 times, respectively), moreover, in the areas of periventricular reparative astrogliosis, in small foci of encephalolysis in the head of caudate nucleus, among GS+ and CD44+ astrocytes clusters of astrocyte-like Nestin+ and CD44+ cells were detected. In patients with decompensated LC, a significant decrease in Nestin and CD44 expression and absence of Ki-67 were observed in the SVZ, with a simultaneous maximum increase in the expression of GS and AQP4.

Conclusions. In the neurogenic niches of the lateral ventricles and hippocampus of patients with compensated and subcompensated LC, there are signs of activation of neural stem cells and niche astrocytes with increased expression of Nestin, CD44, and Ki-67, which reaches maximum in subcompensated LC. Clusters of astrocyte-like Nestin+ and CD44+ cells appear in foci of periventricular repair, which probably migrate from active adjacent subventricular niche. In the brains of the patients with decompensated LC and severe Grade 3–4 hepatic encephalopathy, deep astrocytic dysmetabolic dystrophy is associated with substantial decrease in the activity of subventricular stem niche and expected astrocytogenesis.

Ключові слова: печінкова енцефалопатія, нейрогенез, астроцитогенез, стовбурові клітини, Nestin, CD44, Ki-67, GS, AQP4.

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Характеристика експресії Nestin, CD44, Ki-67, GS і AQP4 у стовбурових нейрогенних нішах головного мозку померлих хворих на цироз печінки різних ступенів тяжкості

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Мета роботи – імуногістохімічне дослідження експресії Nestin, CD44, Ki-67, GS, AQP4 у субвентрикулярній зоні бічного шлуночка та гіпокампі померлих хворих на цироз печінки різних вікових груп залежно від стадії захворювання за Чайлд–П'ю.

Матеріали та методи. Дослідили головний мозок (ГМ) 90 померлих (вік – 65 ± 3 роки) хворих на неалкогольний цироз печінки (ЦП) класу А, В і С за Чайлд–П'ю. Сформували 3 групи спостережень: група «А» – 30 померлих хворих на компенсований ЦП; «В» – 30 померлих хворих на субкомпенсований ЦП; «С» – 30 померлих хворих на декомпенсований ЦП. У групі контролю – ГМ 30 померлих від гострої серцево-судинної недостатності, які не мали захворювань печінки. Кожну групу поділили на 2 підгрупи за віком: ≤59 років і ≥ 60 років. У 59 із 90 (65,55 %) хворих на ЦП визначили симптоматику печінкової енцефалопатії I–IV ступенів. У парафінових зрізах тканини субвентрикулярних зон (SVZ) переднього та нижнього рогів бічних шлуночків ГМ, а також субгранулярної зони (SGZ) зубчастої звивини (DG) та інших структур гіпокампа визначили рівень імуногістохімічної експресії Nestin, CD44, Ki-67, GS і AQP4 у стандартизованих полях зору мікроскопа Scope A1 Carl Zeiss (Germany) з програмою Відеотест-Морфологія 5.2.0.158.

Результати. У SVZ ГМ контрольних підгруп Nestin+ астроцитоподібні стовбурові клітини локалізувалися здебільшого в субвентрикулярних гліальних вузликах (SGN), менше – в астроцитарних стрічках. У ГМ хворих на компенсований і субкомпенсований ЦП встановили підвищену експресію Nestin щодо контролю (на 61,36 % і 208,74 % відповідно) внаслідок підвищення кількості Nestin+ клітин в астроцитарних стрічках. У гіпокампі контрольних пацієнтів і хворих на ЦП експресію Nestin визначали передусім в астроцитоподібних клітинах бахромки-склепіння, «гліальних пластинок» основи судин хоріоїдного сплетення та субпіальної зони. У SVZ ГМ хворих на субкомпенсований ЦП експресія Nestin,

CD44 і Ki-67 максимально підвищувалася (на 208,74 %, 37,83 % і втричі відповідно), а в ділянках перивентрикулярного репаративного астрогліозу в дрібних вогнищах енцефалолізису голівки хвостатого ядра серед GS+ і CD44+ астроцитів визначили скупчення астроцитоподібних Nestin+ і CD44+ клітин. У хворих на декомпенсований ЦП у SVZ ГМ спостерігали значне зниження експресії Nestin і CD44, відсутність експресії Ki-67 при одночасному максимальному підвищенні експресії GS і AQP4.

Висновки. У хворих на компенсований і субкомпенсований ЦП у нейрогенних нішах бічних шлуночків і гіпокампа виявили ознаки активації нейральних стовбурових клітин і нішевих астроцитів з підвищенням експресії Nestin, CD44 і Ki-67, що досягає максимальних значень при субкомпенсації ЦП. У вогнищах перивентрикулярної репарації з'являються скупчення астроцитоподібних Nestin+ і CD44+ клітин, що, ймовірно, мігрують з активних субвентрикулярних ніш. У ГМ хворих на декомпенсований ЦП і тяжку Grade3–4 печінкову енцефалопатію на фоні глибокої астроцитарної дисметаболічної дистрофії зафіксували виразне зниження активності субвентрикулярних стовбурових ніш і астроцитогенезу.

Hepatotoxic brain damage in liver cirrhosis (LC) is characterized by a progressive amplification of astrocyte deficiency with the development of astroasthenia, and subsequently decompensated edema and death of these cells [1]. High concentrations of plasma and intracerebral ammonia, along with other neurotoxins, disrupt the astroglia functionality leading to dysfunction and damage of neurons, that clinically manifest as hepatic encephalopathy [2]. Persistent brain edema of varying degree and accompanied microcirculation disorders leads to ischemic damage and mosaic-to-focal neuronal death in the cerebral cortex, thalamus, striatum, cerebellum and hippocampus. Furthermore, episodes of coma in cirrhotic patients associated with laminar necrosis in deep layers of the cerebral cortex. The loss of a significant number of astrocytes and neurons creates a need for neurogenesis to restore the lost of cellular elements of a single neurogliovascular unit.

Neurogenesis in the adult human brain is still one of the most debated issues. In a broad sense, neurogenesis is the generation of brain cells from neural stem cells (NSCs) which mostly localized in the so called canonical neurogenic niches: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [3]. It is believed that multipotent NSCs are astrocytic in nature; in the course of proliferation and differentiation, they are able to give rise to new generations of neurons, astrocytes, and oligodendrocytes [3]. In contrast to rodents, the main human neurogenic niche, which retains weakly proliferating stem cells throughout life, is the SVZ of the lateral ventricles, and to a lesser extent, the SGZ DG [4].

Quiñones-Hinojosa A. group described cytoarchitecture and ultrastructure of four SVZ layers in lateral walls all horns of human lateral ventricles [5]: I – ependymal cells monolayer; II – hypocellular gap (distinguishing feature of the human SVZ from rodents and non-human primates); III layer – astrocytic ribbon (GFAP+ astrocytes); IV layer – transitional zone, passing into the underlying nervous tissue. Authors demonstrated that some astrocytes and single GFAP+ cells of hypocellular gap retained signs of PCNA+ and Ki-67+ proliferative activity. Moreover, it is expected the existence of alternative neurogenic niches and reparative cellular transformations in neurodegenerative diseases, epilepsy, and cerebrovascular disease [6,7,8].

Among the most commonly used markers to identify human NSCs are Nestin, EGFR, NGFR, Sox2, GFAPα/δ, and Musashi-1. Proliferating NSCs express GFAP, Ki-67, and PCNA; the resulting progenitor cells retain the expression of Nestin, Ki-67, PCNA and reduced expression of GFAP additionally expressing transcription factors (Mash1). Cells of specific differentiation emerging from multipotent cells express proteins specific for astrocytes, oligodendrocytes, and neurons during migration and maturation. To identify maturing and mature astrocytes, commonly used markers are CD44, GFAP / GS, AQP4 [9].

Activation of adult human neurogenesis was shown in a limited list of brain pathologies including brain infarction [10], traumatic brain injury [11], Huntington disease [12], Alzheimer's disease [13]. To our knowledge, there are no studies on adult human neurogenesis, including astrocytogenesis, under conditions of chronic hepatogenic intoxication, as well as data on how the immunocytochemical profile and proliferative activity of neurogenic niche cells change during their differentiation in the brain of cirrhotic patients. Analysis of astrocytogenesis in patients with hepatogenic neurotoxicity opens new perspectives for potential therapies aimed at restoration of astrocytes, the primary targets of this pathology.

Aim

Immunohistochemical study of Nestin, CD44, Ki-67, GS, AQP4 expression in the subventricular zone of the lateral ventricle and hippocampus of deceased patients with liver cirrhosis depending on the age and Child–Pugh score.

Materials and methods

The study was performed on the brain of 90 deceased patients aged 46 to 83 y. o. (mean age 65 ± 3 y. o.) who suffered from non-alcoholic cirrhosis of classes A, B and C according to the Child–Pugh scoring [14], which comprised 3 groups: group "A" (n = 30, compensated LC), group "B" (n = 30, subcompensated or "mild decompensation" LC) and group "C" (n = 30, decompensated LC). Each group was divided into 2 age subgroups: patients ≤59 y. o. (46–57 y. o.) ["x1"] and patients ≥60 y. o. (61–83 y. o.), ["x2"] – groups "A1" (n = 23), "A2" (n = 7), "B1" (n = 19), "B2" (n = 11), "C1" (n = 9), "C2" (n = 21). Cases of endocrine pathology, cancerous and alcoholic nature of liver pathology were excluded from the study. In the control group ("Cntr"), the brain of 30 patients aged 40 to 81 y. o. who died from acute cardiovascular insufficiency (mean age 59.0 ± 2.5 y. o.), who did not suffer from liver pathology and other toxic-metabolic diseases, was studied. By age, the group was also divided into 2 subgroups: "Cntr 1" (n = 12), "Cntr 2" (n = 18).

In 59 of 90 (65.55 %) cirrhotic patients, symptoms of HE Grade 1–4 were clinically diagnosed. In group "A",

8 (26.66 %) patients had symptoms of HE Grade 1, in group "B", 21 (70.00 %) patients had HE Grade 1 and 2, in group "C", HE Grade 2–3 occurred in 23 (76.66 %) patients, and Grade 4 HE (hepatic coma) before death was registered in 7 (23.33 %) patients.

During autopsies which were performed 6–20 h. after death, samples of the liver (to verify LC), subventricular zone of the lateral wall of the brain lateral ventricle anterior horn with the adjacent tissue of the caudate nucleus head as well as the hippocampus were collected. The choice of these brain regions was argued by evidence for their neurogenic potential in the adult human brain [3]. The autopsy material was fixed in 10 % buffered formalin with further embedding in paraffin blocs. Serially slides of 4 μm thickness prepared on a precision rotary microtome HM 3600 ("MICROM Laborgerate GmbH", Germany), after deparaffination were treated with hematoxylin and eosin for general assessment of microscopic changes. For immunohistochemical (IHC) detection of NSCs, Nestin was chosen as a marker most specifically detecting these cells and most often used by other authors in NSCs studies [15]. For assessing cell population with astrocytic phenotype, more astrocyte-restricted markers were used: involved in glutamate and glutamine metabolism (glutamine synthetase, GS); water channels (aquaporin-4, AQP4); hyaluronan receptor (CD44). Ki-67 was chosen to detect proliferating cells.

IGH studies were performed according to the standard protocol of the antibody manufacturer using primary antibodies: mouse anti-human Nestin monoclonal antibody (Clone 10C2, ready-to-use MAD-000719QD-3, Master Diagnostica S. L., Vitro, Spain), rabbit polyclonal anti-CD44 (PA5-21419, Thermo Scientific, USA), rabbit polyclonal anti-GS (Thermo Scientific Inc., USA), rabbit polyclonal anti-AQP4 (Thermo Scientific Inc., USA), Ki-67 recombinant rabbit monoclonal antibody (Clone SP6, Thermo Scientific Inc., USA) and the Ultra VisionQuanto Detection imaging system with diaminobenzidine (Thermo Scientific Inc., USA).

For statistical analysis, IHC levels of Nestin, CD44, Ki-67, GS, AQP4 were determined in the hippocampal DG and SVZ of both anterior and inferior horns of the lateral ventricles (SVZs). When studying expression of Nestin, CD44, GS, AQP4 in the SVZs of both locations and hippocampal DG, 10 random, standardized fields of view (SFV) of the microscope Scope A1 "Carl Zeiss" (Germany) with Jenoptik Progres Gryphax 60N-C1' '1.0x426114 (Germany) camera were studied at magnification of x200 using Videotest-Morphology 5.2.0.158 (VideoTest) software. Nestin, CD44, GS, AQP4 levels were expressed as a percentage of the relative area (Srel., %) that occupies immunopositive material to the total area of SFV. Ki-67 level in DG and SVZs was evaluated in 20 SFV of each of two regions, covering adjacent neural tissue at least 600 μm below the astrocytic ribbon (for the SVZs) at mag.×200, and expressed as the mean number of immunopositive cells per 1 mm^2 tissue section area in each autopsy case.

Data analyzed using the Statistica® package for Windows 13.0 (StatSoft Inc., License No. JPZ804I382130ARCN10-J). Results were expressed as median (Me) with range (Q1; Q3). The Mann–Whitney U test was used to compare two groups, and the Kruskal–Wallis test was used to compare more than two groups. The results were considered statistically significant at the level of 95 % ($p < 0.05$).

Results

IHC patterns of neurogenesis and astrocytogenesis markers (Nestin, CD44, Ki-67, GS i AQP4) in SVZ of anterior and inferior lateral ventricular horns and hippocampal DG revealed heterogeneity of immunohistochemical cytoarchitecture of these zones.

Nestin expression. In patients of all studied groups, in DG and SVZs was revealed territorial heterogeneity in distribution of Nestin-expressing cells (NECs) due to different abundance of NSCs, as well as different density and trajectory of the local vasculature.

In SVZs and hippocampal DG of control group cytoplasmic Nestin expression was found in the endothelium of all vessels, including well developed subventricular vasculature. In SVZs, extravascular accumulations of Nestin+ cells (*Fig. 1*) was detected mainly in the so-called subventricular glial nodules (SGNs) – cellular clusters of subependymal layer protruding into the ventricular lumen and often without ependymal coverage. In astrocytic ribbon, cytoplasmic Nestin expression was defined in the bodies and short, thin processes of moderate number of slightly elongated NSCs-astrocyte-like cells, localized around vessels and oriented parallel to ependymal layer (*Fig. 2*). Outside the DG zone of the hippocampus, NECs of multipolar slightly elongated morphology were found, similar to those in SVZ of both horns. The highest prevalence of these NECs was determined in the hippocampal *fimbria-fornix* including locations near the thin septum separating the subarachnoid space of the choroid fissure of the *cistern ambiens* and the cavity of the lower horn of the lateral ventricle; "glial plates" around the base of the choroid plexus (CP) vessels. From there, the flow of NECs was divided into two branches: 1) subpially and up to the subiculum; 2) to a lesser extent – along the entire SVZ with an extension in the form of a weakly positive periventricular white matter around the tail of the inferior ventricular horn. This feature of Nestin expression in the hippocampus outside DG was equally characteristic of both age subgroups.

Between two age control "Cntr1" / "Cntr2" subgroups, differences in Nestin expression by DG and SVZ cells was not detected.

In compensated cirrhotic patients of age subgroups "A1" and "A2", Nestin levels in DG did not differ from DG values in control "Cntr1" and "Cntr2" subgroups, however, in the SVZs, Nestin was significantly increased compared to control "Cntr1" and "Cntr2" (by 48.95 % and 73.78 %, respectively) by increasing the amount of NECs in the astrocyte ribbon. When comparing age subgroups "A1" and "A2" in both SVZ and DG, Nestin levels did not show differences ($p > 0.05$) (*Table 1*).

In subcompensated LC age subgroups "B1" and "B2", the level of Nestin in DG exceeded by 79.02 % and 68.91 % similar parameters of control "Cntr1" and "Cntr2"; and in SVZs, Nestin was higher by 257.10 % and 160.39 % (respectively) of the similar parameters of

Table 1. Nestin, CD44, GS, AQP4 levels in the DG and SVZs of cirrhotic patients and control group expressed as Srel. (%) and numbers/mm^2 for Ki-67

Parameter	Group "A"		Group "B"		Group "C"		Control group	
	≤59 y. o. ("A1")	≥60 y. o. ("A2")	≤59 y. o. ("B1")	≥60 y. o. ("B2")	≤59 y. o. ("C1")	≥60 y. o. ("C2")	≤59 y. o. ("Cntr1")	≥60 y. o. ("Cntr2")
Dentate gyrus of hippocampus								
Nestin	4.13 (3.56; 4.26)	4.05 (3.34; 4.58)	7.34 (6.47; 8.39)*#	5.27 (4.83; 6.65)*#	5.45 (4.14; 5.53)†	3.17 (2.75; 4.62)†	4.10 (3.49; 4.94)	3.12 (2.45; 4.28)
CD44	11.48 (10.18; 12.49)	15.76 (13.77; 16.95) §	17.39 (16.78; 18.15)*#	22.47 (19.35; 25.32)*#§	11.96 (10.88; 13.47)†	14.58 (14.19; 16.63)†§	11.26 (10.53; 12.24)	14.37 (13.34; 15.28)
Ki-67	No	No	2.00 (1.00; 3.00)*#†§	No	No	No	No	No
GS	3.48 (3.21; 5.37)*	3.79 (3.14; 5.58)*	6.26 (5.95; 7.48)*#	6.67 (5.83; 7.97)*#	8.23 (7.93; 8.75)*#†	8.84 (8.14; 9.11)*#†	2.25 (1.45; 2.72)	2.36 (1.23; 2.84)
AQP4	7.38 (7.12; 8.39)*	8.47 (7.95; 9.76)*	12.16 (11.39; 14.53)*#	14.34 (13.48; 15.83)*#	15.36 (14.15; 16.65)*#	16.23 (14.37; 18.14)*#	4.58 (3.29; 6.92)	4.14 (3.45; 6.74)
Subventricular zones of lateral ventricle								
Nestin	6.39 (5.84; 6.81)*	6.10 (5.83; 7.65) *	15.32 (7.15; 11.32)*#	9.14 (8.26; 10.31)*#§	5.45 (4.23; 6.28)†	4.47 (3.92; 6.12)†	4.29 (3.54; 5.72)	3.51 (3.18; 5.11)
CD44	16.08 (15.72; 16.58)*	18.96 (18.45; 19.28) *§	19.45 (18.36; 21.79)*#	24.26 (22.84; 27.58)*#§	16.59 (15.88; 17.63)*†	19.12 (18.89; 19.63)*†§	14.37 (13.48; 15.14)	17.29 (17.04; 18.15)
Ki-67	2.00 (1.00; 4.00)	No	6.00 (5.00; 7.00)*#†§	No	No	No	2.00 (1.00; 3.00)	No
GS	6.56 (5.11; 7.27)*	7.29 (7.03; 8.04)*	8.73 (7.92; 9.16)*#	9.34 (8.43; 9.97) *#	10.12 (9.33; 10.68)*†	7.16 (6.84; 7.43)*†§	3.47 (2.79; 4.93)	4.74 (3.54; 6.72)
AQP4	12.36 (11.46; 15.58)*	14.43 (13.67; 15.23)*	16.64 (16.15; 17.59)*#	17.52 (16.29; 18.76) *#	18.38 (17.29; 19.14)*#	20.27 (18.34; 22.63)*#	5.34 (4.28; 9.54)	7.30 (5.72; 10.63)

***:** significant difference compared to control ($p < 0.05$); **#:** significant difference compared to group "A" ($p < 0.05$); **†:** significant difference compared to group "B" ($p < 0.05$). These comparisons were made between indicators of the same age group and within the same brain region. **§:** significant difference between age groups within the same LC class ($p < 0.05$). All data are presented as a median with the lower and upper quartiles.

"Cntr1" and "Cntr2". At the same time, Nestin level in DG and SVZs of subcompensated subgroups "B1" and "B2" was higher than levels in compensated subgroups "A1" and "A2" ($p < 0.05$) (*Table 1*).

When comparing age subgroups "B1" and "B2" of subcompensated LC, the difference in Nestin level occurred only in SVZs, where in older "B2" subgroup indicators were significantly lower by 167.61 % ($p < 0.05$) than in younger "B1" subgroup (*Table 1*). Increased Nestin level in DG is explained by the appearance of immunopositive reactive astrocytes in the molecular layer, while in SVZs – by increased numbers of Nestin+ slightly elongated astrocyte-like cells in the astrocyte ribbon outside SGNs, as in compensated LC. In the head of the caudate nucleus adjacent to SVZ in patients with subcompensated LC of both age subgroups, Nestin expression was also determined in the cytoplasm and short processes of astrocyte-like cells, as well as in the bodies and elongated thin processes of reactive astrocytes, localized in areas of astrogliosis, which sometimes encountered around small encephalolysis foci (*Fig. 3*). In other parts of the hippocampus, elevated Nestin level was due to an increased number of NECs and Nestin+ processes in the *fimbria-fornix*, "glial plates" around the base of CP vessels (*Fig. 4*), and subpial zone.

In patients with decompensated LC age subgroups "C1" and "C2", Nestin level was lower than in patients with subcompensated LC subgroups "B1" and "B2": in DG – lower by 34.67 % and 66.24 % respectively; in SVZs – lower by 181.10 % and 104.47 % ($p < 0.05$) (*Table 1*). At the same time, Nestin expression in DG and SVZs did not differ from the indices of "A1" and "A2" subgroups of compensated LC and control subgroups "Cntr1" and "Cntr2" ($p > 0.05$) (*Table 1*). In the SVZs of subgroups "C1" and "C2", areas of perivascular rarefaction of the subependymal tissue were often observed, as well as more often determined foci of encephalolysis adjacent to the SVZ, however, the latter were surrounded by reactive astrocytes with reduced or lost Nestin expression. In the hippocampus outside the DG, Nestin expression was proportionally reduced in all previously described structures and retained relatively pronounced only in the elongated multipolar NECs of "glial plates" around the bases of the CP vessels. Nestin levels in DG and SVZs in subgroups "C1" and "C2" did not show significant differences ($p > 0.05$) (*Table 1*).

CD44 expression. In DG and SVZs in control subgroups "Cntr1" and "Cntr2", CD44 expression was determined in the cell membranes of fibrous-like astrocytes and their long, straight perpendicularly directed processes with small varicosities. In SVZ of anterior horn, CD44+ processes localized directly under ependymal monolayer and spread up to the top layers of underlying caudate nucleus head. In DG, CD44+ astrocytes determined predominantly in SGZ and *hilus*. Outside DG, weak to moderate CD44 expression was detected in astrocytes of *alveus*, *stratum lacunosum, fimbria-fornix* and in interlaminar astrocytes of the subpial zone. CD44 levels in DG and SVZs of older subgroup "Cntr2" exceeded by 27.61 % and 20.32 % (respectively) indicators of younger subgroup "Cntr1" (*Table 1*).

In patients with compensated LC of subgroups "A1" and "A2", CD44 level in DG did not differ from control subgroups, however, in SVZs, CD44 levels significantly exceeded the indices of "Cntr1" and "Cntr2" – by 11.89 % and 9.65 %, respectively. Growth of CD44 expression is explained by expansion of the area of immunopositive cells and their processes in the same hippocampal regions and SVZs as in the control. In older subgroup "A2" of patients with compensated LC, both in DG and SVZs,

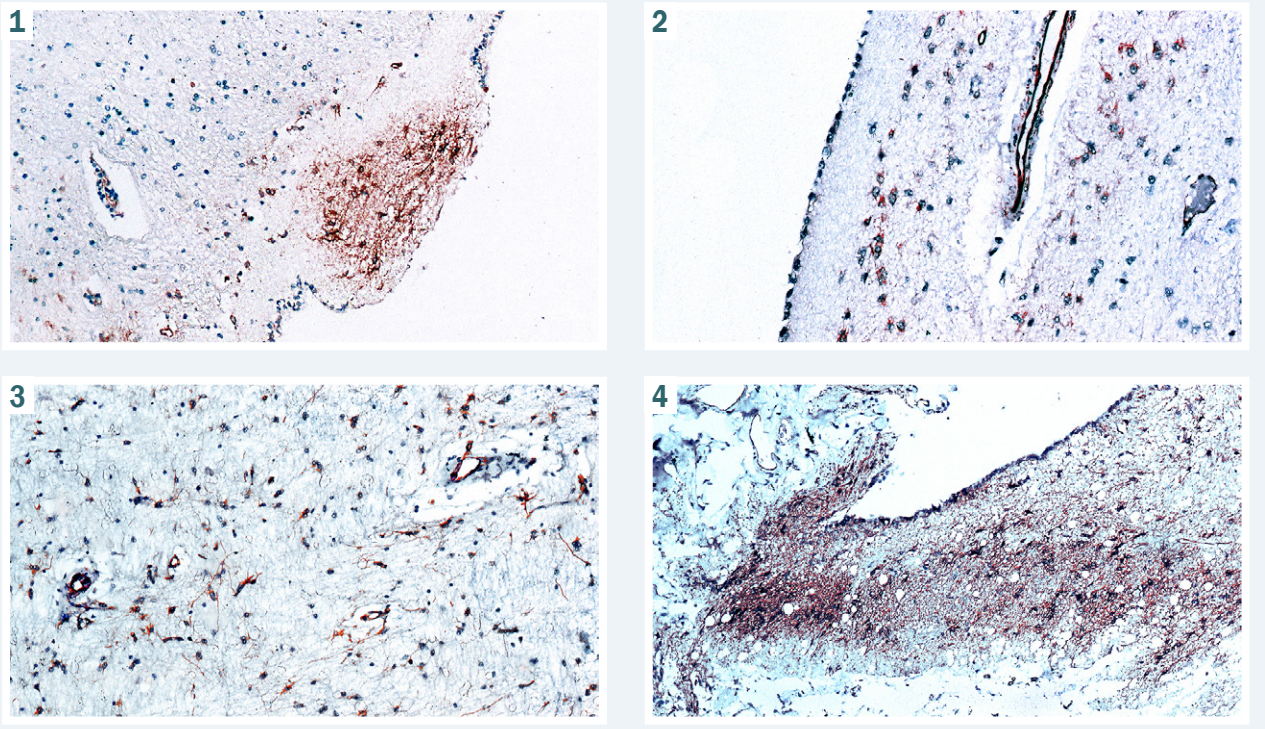


Fig. 1. Cytoplasmatic Nestin expression in cell bodies and processes within the subventricular glial nodule in the anterior horn of the brain lateral ventricle in 63 y. o. deceased patient of control group (subgroup "Cntr2"). Mo monoclonal Nestin, clone 10C2, Master diagnostica. Mag. ×100.

Fig. 2. Nestin expression in the bodies and processes of astrocyte-like cells and in the vascular endothelium of the astrocytic ribbon in SVZ of the lateral ventricle anterior horn in 67 y. o. deceased patient of the control group (subgroup "Cntr2"). Mo monoclonal Nestin, clone 10C2, Master diagnostica. Mag. ×200.

Fig. 3. Nestin expression in the cytoplasm and processes of astrocyte-like cells and in the vascular endothelium of the emerging glial scar near the SVZ of the lateral ventricle anterior horn in 63 y. o. deceased patient with subcompensated LC (subgroup "B2"). Mo monoclonal Nestin, clone 10C2, Master diagnostica. Mag. ×100.

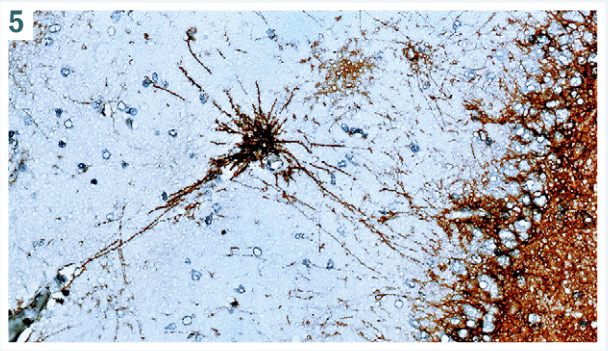
Fig. 4. Nestin expression in the cytoplasm and processes of astrocyte-like cells in the fimbrial region of the hippocampus near the base of the choroidal vessels in 72 y. o. deceased patient with subcompensated LC (subgroup "B2"). Mo monoclonal Nestin, clone 10C2, Master diagnostica. Mag. ×100.

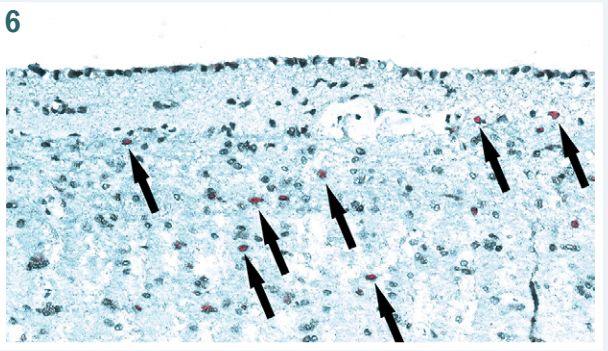
a higher level of CD44 was determined: by 37.28 % and 17.91 %, respectively ($p < 0.05$), compared to younger subgroup "A1" (*Table 1*).

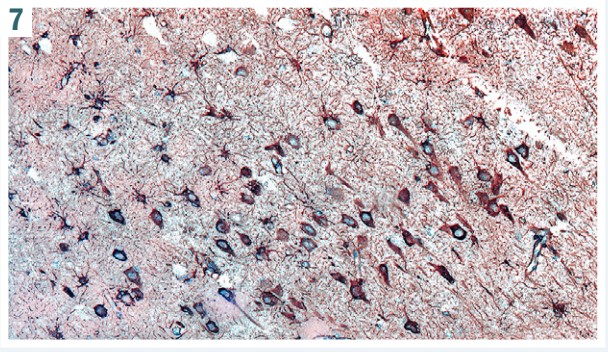
In patients with subcompensated LC of subgroups "B1" and "B2", CD44 level exceeded parameters of control subgroups "Cntr1" and "Cntr2": in DG – by 54.44 % and 56.36 %; in SVZs – by 35.35 % and 40.31 %, respectively ($p < 0.05$), as well as values in compensated LC of subgroups "A1" and "A2": in DG – by 51.48 % and 42.57 %; in SVZs – by 20.95 % and 27.95 %, respectively ($p < 0.05$). In both DG and SVZs, higher values were noted in older subgroup "B2" compared to younger "B1": by 29.21 % and 24.73 %, respectively ($p < 0.05$) (*Table 1*). In DG, increased CD44 expression was determined mainly in a dense network of CD44+ fibers in the SGZ and *hilus,* as well as around the cell membranes of neurons in the granular layer. Determining whether this expression is neuronal-membrane is problematic without double labeling technique. In the molecular layer of DG, single CD44+ astrocytes often connected by their processes the membranes of granular neurons of *stratum granulosum* and vessel walls of the molecular layer (*Fig. 5*). In "B1" and "B2" subgroups of subcompensated LC, CD44+ astrocyte-like cells and a dense network of long CD44+ processes were found in increased amounts in *the alveus*, *stratum radiatum*, *stratum lacunosum*, *fimbria-fornix*, subpial zone (interlaminar astrocytes), "glial plates" around the base of the CP vessels, partially coinciding in localization with the highest expression of Nestin in the same subgroups.

In the SVZ of the anterior ventricular horn, increased CD44 expression was determined in its layers II, III, and IV. In areas of astrogliosis of the head of the caudate nucleus in subcompensated LC brains, there was also a significant expression of CD44 in a network of elongated straight astrocytic varicose processes and their fragments, as well as in the clusters of small rounded-shaped stem-like cells with 1–2 short thin processes. In addition to fibrous astrocytes, CD44 expression was also determined in protoplasmic astrocytes with bushy appearance. Often, the bodies of CD44+ astrocytes localized near large vessels sending a smaller part of the processes to the vascular walls and most of them directing radially from the vessels, forming characteristic, asymmetric perivascular radiances.

In decompensated LC of subgroups "C1" and "C2", CD44 level was reduced relative to compensated subgroups "B1" and "B2": in DG – by 45.40 % and 54.11 %; in SVZs – by 17.23 % and 26.88 %, respectively ($p < 0.05$) (*Table 1*). Despite the general trend towards a decrease in CD44 expression in decompensated patients, CD44 rates in DG and SVZs in older "C2" subgroup exceeded







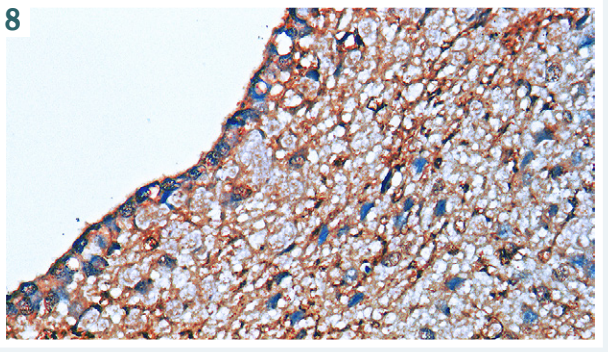


Fig. 5. CD44 expression in the granular and subgranular layer of DG, as well as in the fibrous-like astrocyte of the molecular layer, sending its direct processes to the granular layer and to the vascular wall, in deceased 67 y. o. patient with subcompensated LC (subgroup "B2"). Rabbit polyclonal anti-CD44. Mag. ×200.

Fig. 6. Ki-67 expression by single cellular nuclei within the hypocellular gap, astrocytic ribbon, and also in the head of the caudate nucleus adjacent to the SVZ in deceased 45 y. o. patient with subcompensated LC (subgroup "B1"). Ki-67 rabbit monoclonal antibody, clone SP6. Mag. ×200.

Fig. 7. GS expression in the cytoplasm and dendrites of neurons, perineuronal gliocytes, astrocytes and their processes near the focus of encephalolysis in the caudate nucleus adjacent to the SVZ of the lateral ventricle anterior horn in deceased 73 y. o. patient with decompensated LC (subgroup "C2"). Rabbit polyclonal anti-GS. Mag. ×50.

Fig. 8. AQP4 expression in ependymocytes and astrocytic processes within hypocellular gap and astrocytic ribbon in the SVZ of the lateral ventricle anterior horn in deceased 45 y. o. patient with subcompensated LC (subgroup "B1"). Rabbit polyclonal anti-AQP4. Mag. ×400.

those in younger "C1" subgroup: by 21.90 % and 15.25 %, respectively ($p < 0.05$). In foci of encephalolysis of different age in the head of the caudate nucleus adjacent to the SVZ, membrane expression of CD44 was often found in the body and elongated processes of reactive astrocytes that form glial scars.

Ki-67 expression. In the DG, nuclear Ki-67 expression was determined in single cells of the subgranular zone only in the younger "B1" subgroup in subcompensated LC (*Table 1*), however, the method of this IHC study does not identify the phenotype of Ki-67+ cells.

In the SVZs of control subgroup "Cntr1", Ki-67+ nuclei were found in single cells of the astrocytic ribbon in the anterior and lower horn of the lateral ventricle, as well as in layer II directly under the ependyma. In younger patients with compensated LC of "A1" subgroup, Ki-67 expression was observed in single cells of the same localization. In younger patients with subcompensated LC of "B1" subgroup, a 3-fold higher number of Ki-67+ cells were determined in SVZs compared to control "Cntr1", and single Ki-67+ cells were found in the caudate nucleus directly under the astrocytic ribbon (*Fig. 6*). At the same time, in patients with decompensated LC of both age subgroups "C1" and "C2", no Ki-67+ cells were detected at all in SVZs. In patients with subcompensated and decomensated LC, Ki-67 expression was absent in small areas of the reparative astrogliosis around encephalolysis foci adjacent to the SVZs.

Both in control patients and in all cirrhotic groups older than 60 y. o., in DG and SVZs Ki-67+ cells were absent (*Table 1*).

GS expression. In control patients, GS-antibody labeled cytoplasm of astrocytic bodies and initial segments of processes in DG SGZ, in the hippocampal *fimbria-fornix* and in the "glial plates" of the CP vessels, as well as in astrocytes of astrocytic ribbons of SVZs, in protoplasmic-like astrocytes of the caudate nucleus adjacent to the SVZ. Weak GS expression was found in the cytoplasm of ependymal cells of lateral ventricles. GS level had no significant differences between age subgroups "Cntr1" and "Cntr2" (*Table 1*).

In compensated LC of subgroups "A1" and "A2", GS level exceeded control "Cntr1" and "Cntr2": in DG – by 54.66 % and 60.59 %; in SVZs – by 89.04 % and 53.79 %, respectively ($p < 0.05$) (*Table 1*). Elevated GS expression was due to an increased number of GS+ cells and their processes in the same localizations as in control. GS level in DG and SVZs did not differ between the age subgroups "A1" and "A2".

In subcompensated LC of subgroups "B1" and "B2", GS level exceeded not only control values, but also indices of compensated subgroups "A1" and "A2": in DG – by

79.88 % and 75.98 %; SVZs – by 33.07 % and 28.12 %, respectively ($p < 0.05$) (*Table 1*). In subcompensated LC, in the foci of postnecrotic astrogliosis of the caudate nucleus head adjacent to the SVZ, as well as in the white matter of hippocampus, GS+ hypertrophied fibrous astrocytes, GS+ binuclear astrocytes and astrocyte-like cells were found, as well as weakly GS-positive perivascular astrocytic endfeet. In the "glial plates" of the base of the CP vessels, an increased number of GS+ astrocytes were also found.

In decompensated LC of subgroups "C1" and "C2", an increased GS level was determined in DG, which significantly ($p < 0.05$) exceeded that in compensated and subcompensated LC subgroups (*Table 1*). The highest GS level compared to control in decompensated LC was observed in the SVZ astrocytic ribbon and brain tissue adjacent to the SVZ. Interestingly, SVZs of younger decompensated patients ("C1") was characterized by significantly higher GS levels than compensated and subcompensated patients of the same age subgroups ("A1" and "B1") ($p < 0.05$), however, in older decompensated patients ("C2"), it decreased to the values of compensated ("A2") and became less than subcompensated ("B2") by 30.44 % ($p < 0.05$) (*Table 1*).

GS expression in DG and SVZs did not differ between the groups "A1" / "A2" and "B1" / "B2"; however, for SVZs, GS level in older "C2" subgroup was significantly lower than in younger "C1" by 41.34 %. Despite the trend towards a decrease in GS level in "C2", in the subventricular tissue of the caudate nucleus and in hippocampus, separate groups of neurons were identified to selectively express GS in their cytoplasm and dendrites, as well as in the initial parts of axons (*Fig. 7*). Additionally, nearby acute and healing small foci of encephalolysis, polyphenotypic GS appearance were defined in the satellite oligodendrocyte-like glia around GS- and GS+ neurons, in firbrose-like astrocytes, perivascular astrocytic endfeet, as well as in individual chains of oligodendrocytes.

AQP4 expression. In DG and SVZs of control subgroups "Cntr1" and "Cntr2", AQP4 expression of was determined in the plasma membranes of ependymocytes, plasmalemmas of astrocytic bodies and perivascular endfeet, as well as in the parenchymal processes forming a network of intersecting immunopositive thin strands in the neuropil of the SVZ hypocellular gap; in astrocytes of astrocytic ribbons; astrocytes of DG SGZ and hippocampal *hilus*, as well as in the astrocytes of the "glial plates" of the base of the CP vessels. AQP4 level in both DG and SVZ had no differences between subgroups "Cntr1" and "Cntr2" (*Table 1*).

With progression of LC from compensated to subcompensated and decompensated stage, the same aforementioned cell populations of DG and SVZs showed an increase in AQP4 expression (*Fig. 8*) with significant difference between subgroups "A1" / "B1" and "A2" / "B2". However, in patients with decompensated LC, AQP4 expression in both DG and SVZs stops its growth having no differences between the subgroups "B1" / "C1" and "B2" / "C2". AQP4 levels between age subgroups of the same LC class ("A1" / "A2", "B1" / "B2", "C1" / "C2") did not have a significant difference ($p < 0.05$) (*Table 1*).

Discussion

It is believed that mammalian NSCs are specialized cells capable of self-renewal, proliferation, and differentiation in new lineages, which, however, almost completely cease to realize their neurogenic potential soon after birth under physiological conditions [16]. Quiescence, reactivation, subsequent proliferation, and selection further differentiation pathway are finely regulated by factors that are synthesized by niche astrocytes [17], by NSCs as well as controlled by activity nearby neurons and composition of CSF containing numerous growth factors [4]. According to E. Akdemir et al. and S. Clavreul et al. [9,18], during animal embryogenesis, glial differentiation of progenitor cells are finely tuned by the Notch signaling pathway and dynamic expression of transcription factors (sex-determining region Y-box 9 [Sox9]; brain-specific homeobox/POU domain protein 2 [Brn2]; nuclear factor-I A [NFIA]; zinc finger and BTB domain-containing protein 20 [Zbtb20]), which suppress neuronogenesis and promote switching of differentiation program to the path of astrocytogenesis. It is believed, that under physiological conditions in vertebrates, the proliferation of astrocytes derived from niche NSCs ends in the early postnatal period [19,20], and in adults, a low-level proliferation is determined only in mature astrocytes [9].

Specific factors and pathological processes causing stimulation, activation and proliferation of niche NSCs in adults still need to be established, and the mechanisms of adult astrocytogenesis are extremely poorly described even in animals [9,18].

Nestin, an intermediate filament protein Class VI, still considered the main marker of NSCs and NPCs, its expression is suppressed during subsequent differentiation of NPCs into neurons or glia [21]. Nevertheless, Nestin expression, which depends on the ubiquitin proteasome system, can also be determined in mature reactive astrocytes, in which it is presumably involved in the processes of mitosis, differentiation and migration [21].

In our study, Nestin expression was determined in multi- and bipolar SVZs cells of the anterior horn of the lateral ventricles of the GM, which morphologically resembled niche astrocyte-like NPCs and neuroblasts described by C. Wang et al. [22]. In the SVZs of control cases Nestin+ cells were localized mainly in subventricular glial nodules, which is consistent with the data of S. de Sonnaville et al. [4]. The low Nestin level in SVZs of control patients in both age groups indicates that, outside of pathological stimulation, the basal amount of Nestin+ NSCs in niche areas is largely unchangeable.

In patients with compensated cirrhosis, the SVZs astrocytic ribbons were found to have significantly increased Nestin+ level compared to control, and even more so in elderly patients. SVZs of subcompensated cirrhosis was characterized by maximal Nestin expression, associated with 3-fold increase number of Ki-67+ cells in patients less than 60 y. o., which significantly exceeds the indicators of elderly patients with more pronounced destructive processes in the periventricular tissue, and indicates a greater degree of reactivity of niche cells in response on less pronounced damage to the nervous tissue in younger patients.

Herewith, we failed to detect Nestin+ cells outside the vascular walls in the DG SGZ of the hippocampus, which indicates a lower significance of the DG SGZ as a niche of permanent adult neurogenesis in a conditionally intact brain, as well as in response to chronic hepatogenic intoxication and that is partially confirmed by other studies [16]. We found that in control groups as well as in compensated and subcompensated cirrhotic patients, hippocampal NECs and Nestin+ fibers were maximally localized in the *fimbria-fornix,* "glial plates" of the base of the CP vessels, SVZ, subpial zone up to *subiculum*. A similar localization of NECs in the hippocampus of healthy adults and patients with epilepsy has been described by J. Liu et al. [15]. Mentioned Nestin+ cells were morphologically similar astrocyte-like NECs found in the astrocytic ribbon of the anterior ventricular horn. In compensated and subcompensated cirrhotic patients, named areas of the hippocampal formation were characterized by increased expression of Nestin+, CD44+, GS+, including that in astrocyte-like cells. This suggests the potential role of the lower ventricular horn SVZ, the *fimbria fornix*, "glial plates" around the blood vessels entering the CP, and hippocampal subpial zone as a possible alternative neurogenic niche of the hippocampal formation.

As was indicated by R. Bihlmaier et al. [23] "glial plate's" astrocytes are surrounded by specific microenvironment and are highly susceptible to aggressive and trophic factors, which exchange in CP is persistently enhanced. According to J. Passarelli et al., they may directly influence neurogenesis, being in close relationship with subependymal niche astrocytes [8]. It should be noted that NECs in the studied SVZs and hypothetical alternative neurogenic niches morphologically correspond to the proliferative NSCs described by other researchers [15,24]. Thus, we assume that revealed growth of Nestin+, CD44+, GS+ expression in astrocyte-like cells of SVZs and hypothetical alternative neurogenic niches during compensated and subcompensated cirrhosis is due to the activation of astrocytogenesis programs in response to widespread dysmetabolic astrodystrophy and astrocyte loss developing in patients with cirrhosis [1].

In the brain of patients with compensated and subcompensated cirrhosis, we identified areas of reparative astrogliosis around single small foci of encephalolysis in the head of the caudate nucleus adjacent to the SVZ of the anterior horn, which contained astrocyte-like NECs with niche morphology, most likely migrating from the nearby SVZ niche. Migration is also supported by the absence of Ki-67 expression in areas of astrogliosis with the simultaneous presence of single Ki-67+ cells in the nearby SVZ.

We found that with the progression of cirrhosis, astrocytes of SVZs and SGZ DG significantly increases GS expression, and this sharply decrease in elderly patient's SVZ with decompensated cirrhosis, as well as in other brain regions [1]. This most likely indicates an adaptive increase in GS synthesis by periventricular, including fibrous astrocytes in response to high levels of ammonia in the brain tissue, which is accompanied by the activation of neurogenesis programs in niches and an increased Nestin level.

In older patients of control groups and of cirrhotic groups of all three Child–Pugh classes, in both SVZs and DG SGZ we established significantly increased CD44 expression, the main receptor for hyaluronic acid (HA), compared to younger patients, which is in line with idea of the age-related accumulation of HA in the brain and increased expression of CD44 [25]. Membrane CD44 is involved in intercellular adhesion, cell migration and signaling [26]. In human brain, the main cells expressing CD44 are white matter fibrous astrocytes and, to lesser extent, oligodendrocytes, neurons, and microglia [26]. In our study, the location of CD44+ cells with their characteristic long straight processes coincides with the localization of Nestin+ cells in the SVZs and beyond, including areas of periventricular reactive astrogliosis. Liu Y. et al. [27] have shown that overexpression of CD44 in glial progenitor cells inhibits oligodendrocyte differentiation and promotes astrocytic lineage differentiation. Moreover, recent studies [28] evidenced that Nestin expression in adult niche astrocytes negatively regulates neurogenesis through Notch signaling switching the differentiation of new generations of NSCs to the astrocytic lineage. It can be assumed that the simultaneous increase in the expression of Nestin and CD44 in subventricular neurogenic niches in patients with compensated and subcompensated cirrhosis is an adaptive attempt to activate NSCs, including subsequent switching towards astrocytogenesis (which is indirectly confirmed by the presence of clusters of Nestin+ NSC-like cells in areas of periventricular astrogliosis).

Cirrhosis decompensation leads to a dysfunctional state of the entire astrocytic population, edematous changes and clasmatodendrosis of astrocytes [1]. In patients with decompensated liver cirrhosis and severe Grade 3–4 hepatic encephalopathy, associated with the maximal ammonia accumulation in the brain and aggravation of astrodystrophy [2], compensatory astrocytogenesis in the neurogenic niches drops, characterized by significant decrease in Nestin and CD44 expression, and the absence of Ki-67 expression in all brain regions studied.

Conclusions

1. In patients with compensated and subcompensated liver cirrhosis, subventricular neurogenic niches (subventricular zone of the anterior and inferior horns of the lateral ventricles), as well as in the *fimbria-fornix*, "glial plates" of the base of the choroid plexus vessels, subpial zone of the hippocampus, demonstrate signs of activation of neural stem cells and niche astrocytes in the form of increased expression of Nestin, CD44, Ki-67, and GS.
2. In subventricular zones of control patients, the largest number of Nestin+ cells are localized in subventricular glial nodules, while in cirrhotic patients, an increase in Nestin-positive cells is observed mainly in the astrocytic ribbons.
3. In cirrhotic patients, cells of the subgranular zone of the hippocampal dentate gyrus, which is considered the second canonical neurogenic niche of mammals, show insignificant expression of Nestin, which indicates less involvement of this zone in the processes of adult neurogenesis in chronic hepatogenic neurotoxicity.

4. During subcompensated liver cirrhosis, periventricular reparative astrogliosis around encephalolytic foci in the head of caudate nucleus, beside GS+ and CD44+ astrocytes include clusters of astrocyte-like Nestin+ and CD44+ cells, which may indicate the migration to these reparative foci of astrocytes that differentiate from stem cells of the nearby active subventricular niche.

5. The patterns of Nestin, CD44, GS, AQP4 and Ki-67 expression in patients with decompensated liver cirrhosis with severe hepatic encephalopathy Grade 3–4 and deep astrocytic insufficiency reflect significant decrease in the activity of subventricular neurogenic niches and inhibition of astrocytogenesis in periventricular foci of astrogliosis.

Prospects for further research. Further studies are needed to evaluate more astrocyte-specific progenitor markers to confirm or refute the hypothesis of activation of the adult astrocytogenesis from niche stem cells in health and hepatogenic neurotoxicity to search the methods of control the regenerative programs that are expected to exist throughout human life.

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