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Research article

A MORPHOMETRIC ANALYSIS OF THE POSTANATAL DEVELOPMENT OF THE CHOROID PLEXUS EPITHELIUM IN THE MALE AND FEMALE RAT

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Morphometric parameters of the lateral ventricle choroid plexus epithelial cells (average area, perimeter, bounding rectangle area, average nuclear area, nuclear perimeter, nuclear circularity and average nucleocytoplasmic ratio) were studied in postnatal and juvenile (10th, 16th and 38th postnatal days) 15 male and 15 female rats. The results were statistically analyzed by factorial ANOVA.

Mean values of epithelial cells area, bounding rectangle area and perimeter were significantly higher in 16 days old, than in 10 and 38 days old rats. Opposite to this, the nucleocytoplasmic ratio was lower in the 16 days old, than in 10 and 38 days old rats. Average nuclear area and perimeter showed similar trends, while nuclear circularity increased from the 10th to the 38th day. Significant sex differences were in the epithelial cells area, bounding rectangle area and perimeter, being higher in males than in females in both 16 and 38 days groups. Nucleocytoplasmic ratio was higher in 10 days old male rats, but lower in 16 and 38 days old male rats.

Generally, choroid epithelial cells size increased on the 16th and then decreased on the 38th day, but still remained higher compared to the 10th day. Nuclear size after increasing on day 16, also decreased on day 38, but to values lower than on day 10. The general decrease of nucleocytoplasmic ratio which accompanied these changes indirectly suggests a functional decrease. In the investigated period the male rat choroid epithelial cells were larger, but their nucleocytoplasmic ratio, which suggests the functional status, was lower than in females, indicating sex differences in the growth dynamics of the rat choroid plexus.

Key words: choroid plexus, lateral ventricle, morphometry, postnatal development, rat, sex differences

INTRODUCTION

The choroid plexus (CP), as the main site for cerebrospinal fluid production [1], is lined by non-nervous epithelium consisting of cuboidal glandular cells, located in the

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extensive folds. These folds exist in order to increase the surface area. The single layer of epithelial cells represents a persistence of the neural tube in its embryonic form [2]. The choroidal plexus epithelial cells (CPEC) contain a large central spherical nucleus, abundant cytoplasm and numerous mitochondria needed to maintain their high respiratory metabolism and energy requirements [3]. The tight junctions between epithelial cells physically restrict the movement of substances to and from the CSF (i.e. BCSFB) [3] and are functionally mature from very early in the development [4]. As the main gatekeeper of the brain's internal homeostasis the CP also has transport, excretory, secretory, neuroimmune [5-7], and detoxification functions [1,3,8,9]. These functions were attributed mainly to its epithelium and the alterations of CPEC functional status might be related to development and especially to aging [10,11], as well as to pathological processes.

During the development of the CNS the CP plays a critical role in the morphogenesis, functioning and stability [3]. Our previous studies have indicated the importance of the postnatal period in the development of the central nervous system [12-15]. In the postnatal periods in the rat CP as BCSFB continues to grow to reach its maturity [16], but the period of this maturation differs considerably among mammalian species [17]. However, the longstanding belief in the immaturity of barriers in the developing brain has led to poor experimental design, and to misinterpretations of clinical situations [4].

The goal of our study was to obtain and to analyze morphometric parameters of CPEC in neonatal (P10), early juvenile (P16), and late juvenile rats (P38), including potential sex differences.

MATERIAL AND METHODS

Neonatal female (15) and male (15) Wistar rats used in this study were kept at constant temperature conditions with food and water *ad libitum*. The rats were killed by ether anesthesia at P10 (5 male and 5 female- neonatal), P16 (5 male and 5 female- early juvenile) and P38 (5 male and 5 female- late juvenile). Their brains were removed, fixed in Bouine solution and processed using paraffin embedding. Serial 5 μm-thick coronal sections were stained by standard hematoxylin and eosin method [18]. The same region of the CP plexus located in the central part of the lateral ventricle was analyzed. Typical areas were photographed under magnification (x528). This investigation was approved by the Ethical Committee of the Faculty of Medical Science (University of Kragujevac) No. 01-11037/1.

Morphometric analysis

Randomly selected CP areas were captured under magnification (objective x40, projective x3.3 and photographic 4) x528 (digital camera MOTICAM 1000 on trinocular microscope Motic BA 210). Digital images were processed using ImageJ program (NIH, Bethesda, USA). Morphometric analysis of CPEC included measurement of

their average area (A_E), perimeter (B_E), bounding rectangle area (A_{BR}), average nuclear area (A_N), nuclear perimeter (B_N), nuclear circularity and, average nucleocytoplasmic ratio (N/C). Five fields of vision were analyzed in each of the five cases in every group of animals. Ten epithelial cells were measured in each field of vision. Thus, 50 epithelial cells were measured in each analyzed case, 250 in each group. A total of 750 epithelial cells were analyzed in male rats and 750 epithelial cells in female rats. The shape descriptors used for the epithelial cells and their nuclei are A_{BR} and nuclear circularity, respectively.

Statistical analysis

The sample size was calculated according to the statistical program *G*Power* 3.1 [19]. After descriptive statistics, factorial ANOVA was used to establish the effects of age and gender on CPEC morphometric characteristics. Equality of variance was evaluated with Levene's test and Games – Howell's test was used for multiple comparison analysis [20]. Statistical software used for analysis was SPSS (version 16).

RESULTS

In all investigated groups CP tissue showed a frond like appearance with numerous villi composed of cubic or low cylindrical epithelial cells and thin connective tissue stroma containing sparse spindle shaped nuclei and voluminous blood vessels. Tightly packed epithelial cells contained round, or slightly oval nuclei, located in the central part or near the basal pole of cell (Figures 1,2,3). Female 38 days old rats (P38) occasionally contained vacuoles in the CPEC cytoplasm near their nuclei, while such structures were not observed in the males of corresponding age.

Values of the measured morphometric parameters are presented in Table 1, where section "total" contains the obtained values when both sexes were evaluated together. In that case a linear increase from P10, through P16, to P38 was present only for nuclear circularity and nuclear perimeter of rat CPEC. All other parameters generally increased from P10 to P16, and then decreased on P38 day of life. Factorial ANOVA was used to establish the effects of age and sex on the investigated parameters (Table 2). Single effect of sex for A_E ($p=0.031$) and A_{BR} ($p=0.003$), was statistically significantly higher in male than in female rats (Tables 1 and 2), but with low values of the effect size (eta) for both parameters (0.003 and 0.006 respectively) (Table 2). Single effect of the age was significant in all evaluated parameters (Table 2). Levene's test showed significantly different ($p<0.05$) variances of the evaluated groups. Hence, the Games – Howell test was used for multiple comparison analysis of the mean values of evaluated parameters presented in Table 1 (section "total"). Values of A_E significantly ($p<0.05$) increased on day P16, then significantly ($p<0.05$) decreased on day P38 in relation to day P16, but still remained significantly higher ($p<0.05$) than on day P10. Values of A_{BR} and B_E were significantly higher ($p<0.05$) on day P16 than on P10. However, while A_{BR} on day

P38 was significantly higher ($p<0.05$) than P16 and P10, B_E on P38 was higher than on P10 and lower than on day P16, but these differences were not significant ($p>0.05$). Mean values for A_N and of B_N significantly increased ($p<0.05$) on P16 in relation to P10 and then decreased significantly ($p<0.05$) on day P38 in relation to days P10 and P16. Average nuclear circularity significantly increased ($p<0.05$) on P38, in relation to day P10 and P16. Finally, mean nucleocytoplasmic ratio (N/C), parameter indicating

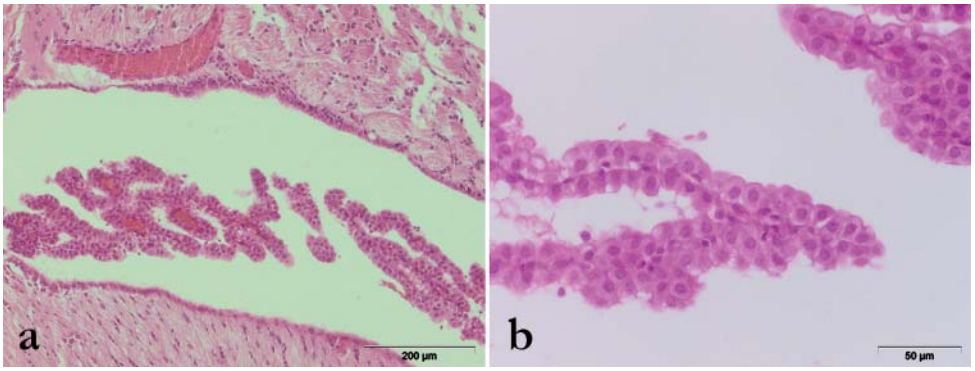

Figure 1. 10th postnatal day female rats: A- HE, x200; B- HE, x600

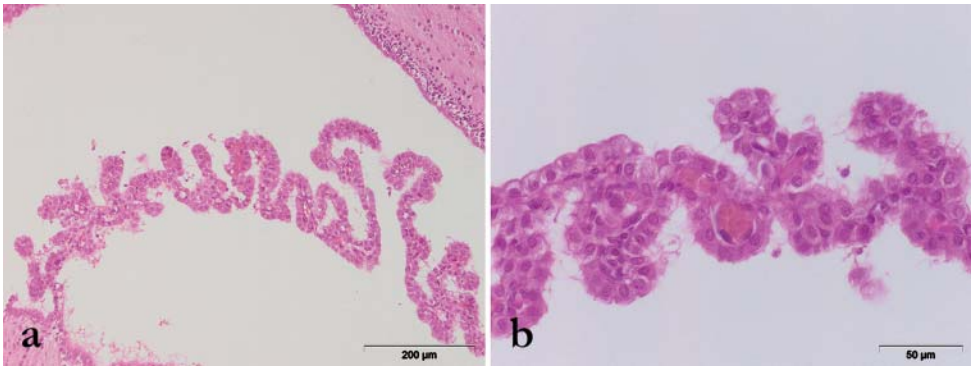

Figure 2. 16th postnatal day female rats: A- HE, x200; B- HE, x600

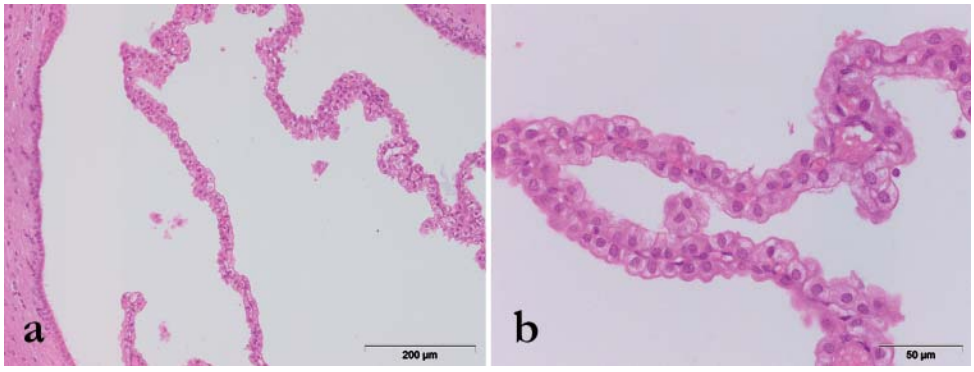

Figure 3. 38th postnatal day male rats: A- HE x200; B- HE, x600

Table 1. Morphometric parameters of the choroid plexus epithelial cells of the evaluated male and female cases in the 10th, 16th and 38th day of life

Variable		N	A_E (μm²)		B_E (mm)		A_{BR} (μm²)		A_N (μm²)		B_N (mm)		Nuclear circularity		N/C	
Gender	**Age**		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Male	Day 10	250	115.03	27.88	40.16	4.89	164.38	41.34	36.50	7.02	22.43	2.15	0.903	0.034	0.53	0.23
	Day 16	250	131.77	24.39	43.12	4.23	183.71	36.75	36.97	6.70	22.56	2.10	0.905	0.029	0.42	0.16
	Day 38	250	126.61	35.68	41.98	5.93	179.49	55.77	34.53	5.99	21.65	1.85	0.919	0.026	0.44	0.20
	Total	750	124.47	30.47	41.76	5.20	175.86	46.05	36.00	6.66	22.21	2.07	0.909	0.030	0.46	0.20
Female	Day 10	250	119.85	35.02	41.11	5.73	167.29	44.05	35.97	7.41	22.18	2.29	0.909	0.027	0.49	0.21
	Day 16	250	124.61	32.25	41.81	5.34	173.86	47.24	37.73	8.07	22.71	2.37	0.908	0.033	0.49	0.21
	Day 38	250	118.71	26.84	40.85	4.69	165.53	42.18	34.39	5.24	21.65	1.62	0.917	0.027	0.45	0.17
	Total	750	121.06	31.62	41.26	5.28	168.89	44.62	36.03	7.13	22.18	2.16	0.911	0.029	0.48	0.20
Total	Day 10	500	117.44	31.71	40.64	5.34	165.83	42.70	36.24	7.22	22.31	2.22	0.906	0.031	0.51	0.22
	Day 16	500	128.19	28.79	42.47	4.86	178.78	42.56	37.35	7.42	22.64	2.24	0.907	0.031	0.46	0.19
	Day 38	500	122.66	31.79	41.42	5.37	172.51	49.89	34.46	5.62	21.65	1.73	0.918	0.026	0.44	0.19
	Total	1500	122.76	31.09	41.51	5.25	172.37	45.46	36.02	6.90	22.20	2.12	0.910	0.030	0.47	0.20

Table 2. Results of the factorial ANOVA analysis, of the evaluated male and female epithelial cells morphometric parameters in the 10th, 16th and 38th day of life

Variable and Source	Type III SS	df	MS	F	p	η^2
			A_E (μm²)			
Gender	4364.82	1	4364.82	4.65	0.031	0.003
Age	28924.77	2	14462.39	15.40	0.000	0.020
Gender*Age	12749.72	2	6374.86	6.79	0.001	0.009
Error	1402608.82	1494	938.83			
R Squared = 0.032 (Adjusted R Squared = 0.029)						
			B_E (μm)			
Gender	93.26	1	93.26	3.49	0.062	0.002
Age	842.98	2	421.49	15.77	0.000	0.021
Gender*Age	398.67	2	199.33	7.46	0.001	0.010
Error	39920.48	1494	26.72			
R Squared = 0.032 (Adjusted R Squared = 0.029)						
			A_{BR} (μm²)			
Gender	18183.42	1	18183.42	9.00	0.003	0.006
Age	41956.82	2	20978.41	10.38	0.000	0.014
Gender*Age	19337.45	2	9668.73	4.79	0.008	0.006
Error	3018077.76	1494	2020.13			
R Squared = 0.026 (Adjusted R Squared = 0.022)						
			A_N (μm²)			
Gender	0.39	1	0.39	0.01	0.927	0.000
Age	2120.66	2	1060.33	22.92	0.000	0.030
Gender*Age	109.46	2	54.73	1.18	0.307	0.002
Error	69116.50	1494	46.26			
R Squared = 0.031 (Adjusted R Squared = 0.028)						
			B_N (μm)			
Gender	0.44	1	0.44	0.10	0.750	0.000
Age	254.42	2	127.21	29.46	0.000	0.038
Gender*Age	10.35	2	5.18	1.20	0.302	0.002
Error	6450.59	1494	4.32			
R Squared = 0.039 (Adjusted R Squared = 0.036)						
			Nuclear circularity			
Gender	0.00	1	0.00	2.18	0.140	0.001
Age	0.04	2	0.02	26.17	0.000	0.034
Gender*Age	0.00	2	0.00	2.44	0.088	0.003
Error	1.28	1494	0.00			
R Squared = 0.038 (Adjusted R Squared = 0.035)						
			(N/C)			
Gender	0.08	1	0.08	2.12	0.146	0.001
Age	1.08	2	0.54	13.81	0.000	0.018
Gender*Age	0.74	2	0.37	9.45	0.000	0.012
Error	58.56	1494	0.04			
Total	390.14	1500				
R Squared = 0.032 (Adjusted R Squared = 0.028)						

functional status of epithelial cells, was significantly lower ($p<0.05$) on days P16 and P38 than in day P10.

The interaction between sex and age was statistically significant for mean values of A_E, B_E, B_N and N/C (Table 2). Simple effect post hoc analysis showed a significantly higher area of male rats epithelial cells in P16 ($p=0.005$) and P38 ($p=0.005$), than in female rats (Table 2). Values of A_{BR} in male rats were significantly higher on P16 ($p=0.01$) and P38 day ($p=0.002$) than in females. Mean B_E of the male rats was significantly lower on day P10 ($p=0.045$) and significantly higher on day P16 ($p=0.002$) and P38 ($p=0.018$) than in females. Finally, the mean N/C of the males was significantly higher on day P10 ($p=0.037$) and significantly lower on day P16 ($p<0.001$) than in females. On day P38, this ratio was still lower in males than, but this difference was not significant ($p>0.05$) (Table 2).

DISCUSSION

The CP develops early and possesses a functional BCSF within the first several weeks of embryogenesis [3]. According to literature data there are differences between fetal and adult (mature) CPEC and its postnatal transition is probably complex. The isolation of the developing CNS allows the influence of local signals for the organization of networks in a relatively confined microenvironment [3,21]. The tight junctions between adjacent epithelial cells appear to be quite well developed in immature CP suggesting that in a growing brain the properties of BCSFB are largely similar to those of adults [1,3,21]. The developmental BCSFB restricts the passage of lipid insoluble molecules by the same mechanism as in the adult (tight junctions) [4]. The young rats normally can maintain plasma-to-CSF gradients similar to those in the adult. The onset of CSF K+ homeostasis takes place approximately at birth in rats [22], but in younger postnatal animals CSF K\Na+ and Ca^{2+} ions concentrations show higher values than in the older ones [23]. The barrier mechanisms in the developing brain are different from those in the adult brain, but these differences do not necessarily reflect immaturity of the system [24].

The basic pattern of postnatal dynamics found in our morphometric study (increase from P10, to P16 followed by a decrease on P38) is in accordance with other parameters, like delta-6 desaturase specific activity in CP [25], or specific transport mechanism of albumins through BCSFB [26]. Also, nucleus volume fraction in CPEC of the lateral ventricle in rats increased from P0 to P10 and then decreased slightly on P30, CPEC glycogen decreased from P0 to P10 and remained low, while at the same time cell height decreased from P0 to P10, and then only slightly increased on P30 [16]. In rats postnatal morphological changes in CPEC correlate well with the maturation of the CP capability to transport K and Cl [16,27]. Our results confirm the statement that in rats during postnatal development there was an overall decrease in BCSFB exchange with increasing age [28]. In all studies of rat's CP development different postnatal growth patterns must be considered, because its growth in the third ventricle ended

by the 5th postnatal week, but in the fourth and possibly in the lateral ventricles CP extended to develop through the period from P3 to P25 [29-33].

All cited data suggest that the postnatal developmental changes of CP are critical in the transition of its specific functions in embryonic periods to those necessary in the external environment. However, there is no available data about potential sex differences during CP postnatal development or in adults. In this study we recorded significant sex differences in the parameters of CP cellular size (A_E, B_E, A_{BR}). These differences were present on P10, but were greatest on P16 (early juvenile period) (Tables 1 and 2) and remained to a lesser extent on P38 (late juvenile period in rats). In spite of the observed sex differences, the postnatal development of CPEC has basically the same cytoplasm and nucleus dynamics. Namely, for most of parameters lower values on P10 in males are due to lower starting values, and probably therefore they showed greater increases, and inverted behavior of N/C follows described patterns. Our results which contribute to the knowledge of transition from fetal to adult CP also suggest that the studies of postnatal development of CP epithelial cells must include extended time periods.

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MORFOMETRIJSKA ANALIZA POSTNATALNOG RAZVOJA EPITELA HORIOIDNOG SPLETA MUŽJAKA I ŽENKI PACOVA

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Morfometrijski parametri (prosečna površina, obim, površina najmanjeg opisanog četvorougla, prosečna površina nukleusa, obim nukleusa, zaokrugljenost nukleusa i prosečni nukleocitoplazmatski odnos) epitelnih ćelija *Plexus choroideus*- a lateralnih komora ispitani su na 15 mužjaka i 15 ženki pacova u neonatalnom i juvenilnom periodu (10., 16. i 38. postnatalni dan). Rezultati su statistički analizirani faktorskom ANOVA. Srednje vrednosti površine epitelnih ćelija, površina najmanjeg opisanog četvorougla i obim bili su signifikantno veći kod pacova starih 16 dana, nego kod onih starih 10 i 38 dana. Nasuprot tome, nukleocitoplazmatski odnos je bio niži kod pacova starih 16 dana, nego kod onih starih 10 i 38 dana. Prosečna površina i obim nukleusa pokazali su slične trendove, dok je zaokrugljenost nukleusa porasla od 10. do 38. dana. Signifikantne polne razlike su postojale u vrednostima površine epitelnih ćelija, površina najmanjeg opisanog četvorougla i obima, koje su bile više u mužjaka nego u ženki, kako u grupi staroj 16, tako i u grupi staroj 38 dana. Nukleocitoplazmatski odnos bio je veći u mužjaka nego u ženki starih 10 dana, ali niži u mužjaka starih 16 i 38 dana.

U celini, veličina epitelnih ćelija horioidnog spleta porasla je 16. i potom se smanjila 38. dana, kad je još uvek bila veća nego 10. dana. Veličina nukleusa posle porasta u 16. danu, takođe se smanjila u 38. danu, ali do vrednosti koje su manje nego 10. dana. Opšte smanjenje nukleocitoplazmatskog odnosa koje je pratilo ove promene indirektno ukazuje na opadanje funkcija. U izučavanom periodu, epitelne ćelije horioidnog spleta mužjaka pacova bile su veće, ali su njihovi funkcionalni parametri postali manji nego u ženki, što bi ukazivalo na polne razlike u dinamici rasta horioidnog spleta pacova.