Immunohistochemical Characterization of Hepatic Nuclear Factor 4 Alpha Expression in the Choroid Plexus of the lateral and 4th ventricles of adult Male Rat Brain

Rasha A. Salman[1], Taghreed Abdulrsool Ali[2], Duaa AL Musawi[3]

[1]Assistant lecturer, Dept. of Biology, Madenat Alelem University College, Baghdad, Iraq, [2]Assistant lecturer, Dept. of Dentist, AlTurath University College,[3]Lecturer Baghdad, Iraq, Dept. of Biology, Madenat Alelem University College

Abstract

The choroid plexus (CP) is present in brain ventricles. It is responsible for cerebrospinal fluid (CSF) secretion and various vital functions. Special proteins present in choroidal epithelium play important roles in CSF production and energy metabolism.

This study aims to compare between the lateral and fourth ventricles CPs using hepatocyte nuclear factor 4 alpha (HNF4α), metabolism marker, to evaluate the functional activity of this tissue in the two regions.

Ten adult male albino rats were used to study the histological features of the CPs and to study the functional activity by quantitative immunohistochemical labeling with HNF4α marker.

The CP of the fourth ventricle had more functional activity than the CP of the lateral ventricle. A quantitative assessment of HNF4α using Aperio ImageScope Software Analysis showed that the lateral ventricle CP mean positivity 0.264 ± 0.083 pixel/micron² while the fourth ventricle CP have mean positivity 0.297 ± 0.043 pixel/micron². The immunohistochemical expression of marker in the fourth ventricle CP were significantly, $P \leq 0.05$ higher than those in the lateral ventricle at $P \leq 0.05$. Immunohistochemical detection of metabolism marker went along with findings of other histological and biochemical studies to define the CP as a highly dynamic structure with regional variations forming a continuum of one entity tissue capable of functional adaptation according to body needs.

Keywords: *Choroid Plexus, Lateral Ventricles, Fourth Ventricle, HNF4α*

Introduction

The choroid plexuses (CPs) are leaf-like highly vascular structures [(1)]. Four CPs floating inside the ventricular cavities of the brain: one in each of the two lateral ventricles, one in the third, and one in the fourth ventricle [(2)].

The CPs are extensions of the ependymal lining of the ventricular walls and consist of a fenestrated vasculature core surrounded by a single layer of polarized cuboidal epithelium with an interstitial stromal layer of connective tissue rich in fibroblasts and cells of the immune system in between [(3)]. Adjacent CP epithelial cells are joined together by tight junctions to form the blood-cerebrospinal fluid (CSF) barrier. Together with adherens junctions, the tight junctions also ensure the apico-basal polarity of membrane proteins (for example, transporters) that

are critical for normal epithelial cell function [4].

The main known function of the CP epithelium is to produce CSF via passive filtration of peripheral blood across the choroidal capillary endothelium in the vasculature core followed by regulated active secretion across the single-layered epithelium [5].

Hepatocyte nuclear factor 4 alpha (HNF4α) may play a role in the transcriptional control of drug transporters. It is a member of the nuclear receptor superfamily that regulates a broad range of xenobiotic–metabolizing enzymes and thus regulating the metabolism in the CP [6]. The HNF4α gene can also be found in the liver, pancreas, intestine, brain and recently in epithelial cell of CPs [7].

Materials and Methods

Animals and tissue preparation:-

A sample of 10 adult male rats (*Rattus norvegicus albinus*). The animals aged 3-6 months, with 300 ± 50 g body weight, and were fed with standard pellet diet. Animals were euthanized with chloroform soaked cotton in an air tight chamber for 5 minutes, then the brains were removed from the skulls and fixed for 18 hours in 4% paraformaldehyde at room temperature (22°C).

The brains were cut in coronal planes rostral to the optic chiasma and caudal to the midbrain in order to obtain lateral and third ventricles specimens, while fourth ventricle samples were made by trimming the remaining caudal part of the brain (cerebellum and brainstem). The specimens were then left in the fixative for another 18 hours and finally transferred into commercial 70% methanol where they were kept until further processing. Paraffin blocks were made and 5 µm thickness sections were cut for immunohistochemical labelling [8].

Immunohistochemistry Labeling:-

The Super Sensitive IHC for Detection Kit HNF4α antibody was found in CP by following all subsequent steps, which carried out at room temperature in a humidified chamber. Super Sensitive IHC Detection Kit was used. Sectioning at 5 µm were used and deparafinization, Incubate tissue in appropriate pretreatment or digestive enzyme for primary antibody and PBS/TBS wash 3 times for 2 minutes. Then incubate slide in Hydrogen Peroxide Blocking Reagent for 10 minutes, PBS/TBS wash 3 times for 2 minutes. Apply Blocking Reagent and incubate for 5 minutes, PBS/TBS wash 3 times for 2 minutes. Apply primary antibody and incubate according to manufacturer's recommended protocol (overnight) incubation, PBS/TBS wash 3 times for 2 minutes. Apply HRP Polymer and incubate for 10 minutes, PBS/TBS wash 3 times for 2 minutes. Add 20µl of DAB Chromogen to 1 ml of DAB Substrate, mix by swirling and apply to tissue. Incubate for about 3-5 minutes, PBS/TBS wash 3 times for 2 minutes. Finally counter stain and cover slip using a permanent mounting media [9].

Controls

For positive controls, adult male rat kidney sections were labelled for HNF4α in the same procedure, while for negative controls adult male rat brain and kidney sections were labelled in the same procedure except that primary antibodies of HNF4α were replaced by PBS.

Immunohistochemical Reaction Assessment

For HNF4α marker, fourty field images of immunohistochemically labelled slides were captured from the lateral ventricle CP, and a similar number of fields were captured from the 4th ventricle CP. A LEICA DM 750 light microscope equipped with Digital Microscopic Camera 5 Mega pixel digital camera were used to capture the fields. Images were processed with Aperio ImageScope v.11 program for total positivity. Microsoft office Excel® 2013 program was used to describe the collected data by calculating the Descriptive Statistics and t-Test were used to compare between means in this study.

Results

Immunohistochemical Labeling of the Choroid Plexus

Hepatocyte Nuclear Factor 4 Alpha (HNF4α)

Light microscopic examination of sections labeled with anti-HNF4α showed high reactivity in choroidal epithelium compared with other cells of brain tissue. There was no detectable difference between reactivity of lateral and fourth ventricles CPs. Ependyma showed weaker reactivity to HNF4α marker than the choroidal epithelium (Figures 1-2). Endothelium of choroidal vessels were highly reactive to HNF4α marker whereas blood cells inside these vessels were non-reactive with this marker (Figure 2).

Controls

External positive and negative controls, and internal negative controls are seen in (Figure 3).

Aperio ImageScope Software and Statistical Analyses

Assessment of Anti-HNF4α Reactivity

Statistical analysis of anti-HNF4α reactivity in the lateral and fourth ventricles CPs gave mean values of 0.264 ± 0.083 pixel/micron² and 0.297 ± 0.043 pixel/micron², respectively, with a wider range of reaction intensity in the lateral ventricle CP than that in the fourth ventricle CP (Figure 4). Two-sample assuming equal variances t-Test revealed a statistically significant difference between these values ($p<0.05$) (Tables 1-2).

Table 1 Descriptive statistics of HNF4α marker labeling in the lateral and fourth ventricles CPs.

Descriptive Statistics	Lateral ventricle CP HNF4α	Fourth ventricle CP HNF4α
Mean	0.264	0.297
Standard Error	0.013	0.006
Median	0.258	0.292
Mode	#N/A	#N/A
Standard Deviation	0.083	0.043
Range	0.428	0.185
Minimum	0.102	0.208
Maximum	0.531	0.394
Count	40	40

Table 2 Comparison of the total positivity of HNF4α marker in the lateral and fourth ventricle CP.

Variable	Lateral ventricle CP HNF4α	Fourth ventricle CP HNF4α
Mean	0.264	0.297
Variance	0.007	0.001
Observations	40	40
Df	78	
t Stat	-2.211	
P(T<=t) two-tail	0.029	
t Critical two-tail	1.990	

Discussion

Previous studies on the CPs of the lateral, third and fourth ventricles considered them as one entity but some authors reported differences in activities of certain metabolic enzymes of the various CPs [10]. The immunohistochemical reactivity of HNF4α in the CPs of both lateral and fourth ventricles were estimated with Aperio ImageScope software that could detect the cells labeled with the specified marker and categorized them into three groups: strongly positive, positive, and weakly positive, while negative areas were those without any reactivity. That was applicable for choroidal epithelium, ventricular ependyma, and endothelial cells of choroidal vessels, but not blood cells within (Figures 1(B)-3).

Positively labeled cells were marked up with Aperio ImageScope software as brown, light brown and yellow colored, indicating strongly positive, positive and weakly positive, respectively (Figure 4). In this study, it was not possible to localize, with precision, HNF4α receptors in cells which were previously localized in the basolateral side of choroidal epithelium plasma membrane, though it was clear to identify HNF4α labeling as a granular stain occur in the cytoplasm. This might be due to the presence of aggregations of transporter proteins across the B-CSF-B like ABCC, ABCB1, ABCB4 and transthyretein. In addition, HNF4α was observed in the endothelium preventing backflow of metabolites to the blood as ABCC proteins play a protective role in choroidal epithelium and mediate basolateral efflux of conjugates resulting from CSF drugs metabolism into the blood while ABCB1 proteins are distributed in the apical side of endothelium [11, 12].

Analysis of HNF4α reactivity in the CPs showed statistically significant higher readings in the fourth ventricle CP compared to that of the lateral ventricle (Tables 1-2), indicating higher activity in the choroidal epithelium of the fourth ventricle. However, cells of the CP of the lateral ventricle showed wider range of HNF4α expression, possibly reflecting a diverse state of activity in that CP since it is spread over wide regions in the brain's ventricles when compared to the smaller size CP impacted in the fourth ventricle.

Expression of HNF4α regulates many proteins and metabolizing enzymes like the ATP binding cassette ABCB4 and ABCC1 in human and rat [12], and transthyretine which is one of the proteins present in the cytoplasm of CP cells [13] at the BCSFB. Demonstration of intracellular reaction of HNF4α by

binding, for example, with transthyretin in choroidal cells cytoplasm reflects its role in regulation of this protein activity. The presence of well-developed endoplasmic reticulum and Golgi apparatus in CPs makes their ability to secret this protein high [14]. Transthyretin is secreted specifically by the CP and not in other parts of brain and it binds with HNF4α to control drug transportation [15]. All the above mentioned proteins can be labeled with anti- HNF4α to give a cytoplasmic reaction which may highlight an assumption of drugs metabolizing and transporting enzymes to be more concentrated in the fourth ventricle CP than that of the lateral ventricle, with clinical and pharmacological implications [16].

In this study, expression of HNF4α was significantly higher in the fourth ventricle CP compared to that of the lateral ventricle suggesting that protein regulation and metabolic activity are more in fourth ventricle CP, which is in contrast to that reported by Al-Kafagi et al. [16] who suggested the regulation of drug transporters is more in lateral ventricle CP. This disagreement might be due to the lack of use of controls in their work, or it might be caused by the different experimental setting when their conclusions were drawn on a different species. In addition, this study contradicts other findings on certain drugs metabolism where the CPs of lateral and fourth ventricles were found to be of similar activity [17], however, it is understood that the different methodology applied might explain this discrepancy.

Ependymal cells lining the lateral and fourth ventricles showed reactivity to HNF4α marker (Figures 1(B) and 2(B)), albeit at lesser extent on qualitative assessment. The mere observation of the ependymal cells expressing less HNF4α marker than the choroidal epithelium, but higher than the adjacent white matter of the brain, needs to be analyzed quantitatively in a further extension from this study.

In addition to the ependymal lining, HNF4α labelling was also seen in endothelial lining of choroidal vessels (Figures 2(A)), however, assuming equal vascular density of both the lateral and fourth ventricles CPs, this labelling would not bias the results in this study, but further quantitative analysis of the vascular profile of the CPs is indicated.

In this study, the expression of HNF4α in choroidal cells of the fourth ventricle was higher than that of the lateral ventricle. Therefore, it might be expected to have abundant amounts of secreted proteins in the cytoplasm of choroidal cells, suggesting that the endoplasmic reticulum content of the fourth ventricle CP is higher compared with that of the lateral ventricle and consequently the metabolic rate is higher in the fourth ventricle CP, which agrees with previous studies [10].

Conclusion

While carrying the same name as a CP, that part in the fourth ventricle proved distinct functional characteristics from that in the lateral ventricle despite the structural similarities of their cells. In terms of transport system, this study showed preponderance in favour of the fourth ventricle CP, as well as in terms of metabolic activity no matter whether this is related to internal protein synthesis and fluid secretion, or is related to external substance metabolism.

These findings might add to previous works that showed higher functional activity in the CP of the fourth ventricle compared to that of the lateral ventricle, however short of addressing the two regions as distinct entities. Rather, they form a continuum of tissue capable of functional adaptation according to the body needs.

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