ORIGINAL ARTICLE

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF HEPATIC NUCLEAR FACTOR 4 ALPHA EXPRESSION IN THE CHOROID PLEXUS OF THE LATERAL AND 4TH VENTRICLES OF ADULT MALE RAT BRAIN

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ABSTRACT

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

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

Results: 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 equalled 0.264 ± 0.083 pixel/micron^2 while the fourth ventricle CP has mean positivity 0.297 ± 0.043 pixel/micron^2. The immunohistochemical expression of marker in the fourth ventricle CP was significantly ($p \leq 0.05$) higher than those in the lateral ventricle ($P \leq 0.05$).

Conclusions: 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.

KEY WORDS: Choroid Plexus, Lateral Ventricles, Fourth Ventricle, HNF4α

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ABBREVIATION

Choroid plexus: cp,
Cerebrospinal fluid: csf,
hepatocyte nuclear factor 4 alpha: 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 forming the blood-cerebrospinal fluid (CSF) barrier. Together with adherent 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].

THE AIM

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

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 LABELLING

The Super Sensitive IHC for Detection Kit HNF4α antibody was found in CP by following all subsequent steps, which were carried out at room temperature in a humidified chamber. Super Sensitive IHC Detection Kit was used. Sectioning at 5 µm and deparafinization were used. We incubated tissue in appropriate pre-treatment or digestive enzyme for primary antibody and PBS/TBS was washed 3 times for 2 minutes. Then incubate was put in Hydrogen Peroxide Blocking Reagent for 10 minutes, PBS/TBS washed 3 times for 2 minutes. After we applied Blocking Reagent and incubated for 5 minutes, PBS/TBS washed 3 times for 2 minutes. We applied primary antibody and incubated it according to manufacturer's recommended protocol (overnight) incubation, PBS/TBS washed 3 times for 2 minutes. There were applying of HRP Polymer and incubating for 10 minutes, PBS/TBS washed 3 times for 2 minutes. Then adding of 20µl of DAB Chromogen to 1 ml of DAB Substrate, mixing by swirling and applying to tissue. Then incubated for about 3-5 minutes, PBS/TBS washed 3 times for 2 minutes. Finally counter stain and cover slip using a permanent mounting media were done [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, forty 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 was 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 means in this study.

RESULTS

IMMUNOHISTOCHEMICAL LABELLING OF THE CHOROID PLEXUS HEPATOCYTE NUCLEAR FACTOR 4 ALPHA (HNF4A)

Light microscopic examination of sections labelled with anti-HNF4α showed high reactivity in choroidal epithelium compared to 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 demonstrated in (Figure 3).

APERIO IMAGESCOPE SOFTWARE AND STATISTICAL ANALYSES ASSESSMENT OF ANTI-HNF4A REACTIVITY

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

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 labelled with the specified marker and categorized them into three groups: strongly positive, positive, and weakly positive, while negative areas were those with no 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 labelled cells were marked up with Aperio ImageScope software as brown, light brown and yellow coloured, indicating strongly positive, positive and weakly positive, respectively (Figure 4). In this study, it was not

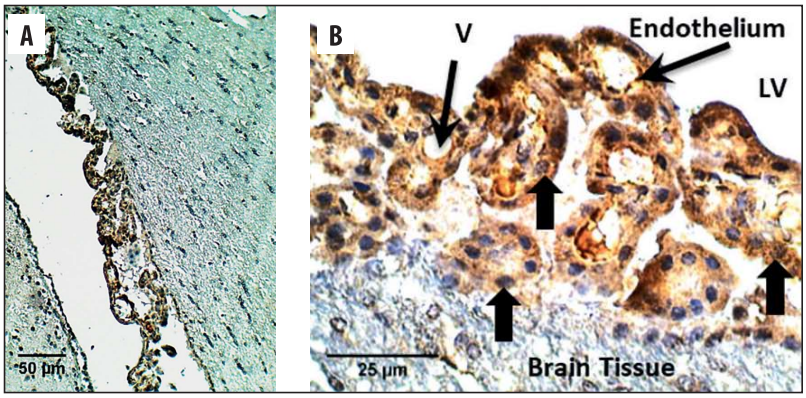

Fig. 1. (A) Coronal section of rat brain showing lateral ventricle (LV) choroid plexus (block arrows) labelled with HNFα marker. Highlighting bigger reactivity in the choroid plexus compared to the surrounding brain structures. The ependymal cells have weaker labelling than that of the choroid plexus but still higher than the brain tissue. (B) Higher magnification of lateral ventricle (LV) choroid plexus (block arrows) in rat brain showing brown color labelling with HNFα marker. Many choroidal blood vessels (V) are seen. Large choroidal epithelium nuclei stained whit hematoxylin counterstain whereas blood cells (BC) inside vessels do not stain. (A) 100X, (B) 400X.

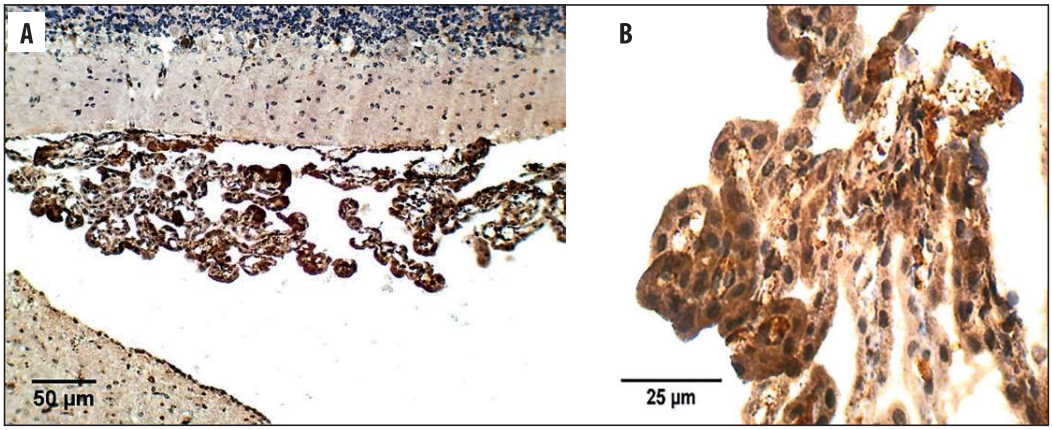

Fig. 2. (A) Coronal section through the fourth ventricle (4th V) of rat brain where the choroid plexus (block arrows) is labeled with anti-HNF4α (brown color) compared to other parts of the brain. (B) The single-layer ependymal lining of the fourth ventricle has weaker labeling with this marker compared to that of the choroid plexus. Fourth ventricle (4th V) choroid plexus (block arrows) showing brown color reactivity with anti- HNF4α. The endothelium of blood vessels is also labeled with HNF4α marker whereas blood cells (BC) inside these vessels do not show such reactivity. Choroidal epithelium nuclei are of a blue color as they stain with hematoxylin counterstain. (A) 100X (B) 400X.

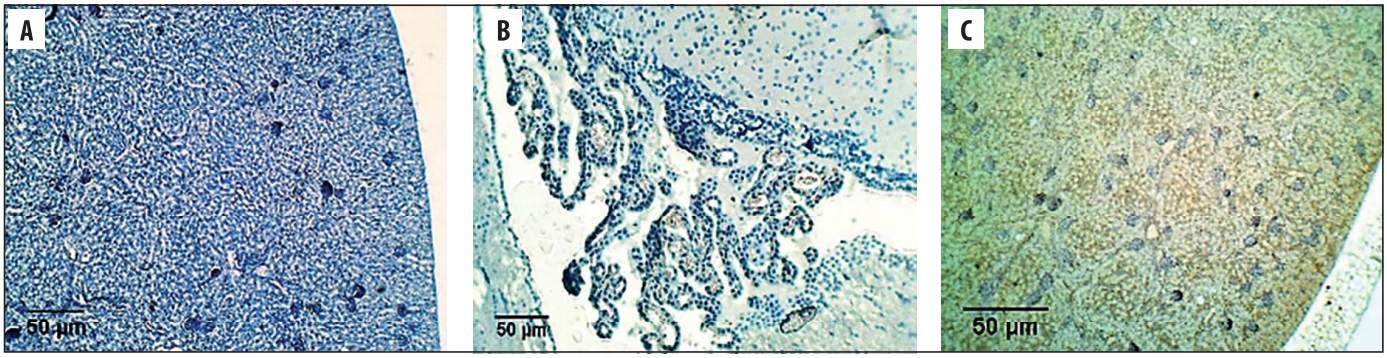

Fig. 3. Controls used for immunohistochemical labeling. Kidney external positive controls for HNF4α (A) marker, revealing brown labelling of renal tubules with nuclei counterstained with hematoxylin. Kidney external negative controls for HNF4α (B) marker, show no brown labelling. Nuclei are counterstained with hematoxylin. Choroid plexus internal negative controls for HNF4α (C) marker, highlight no immune histochemical labeling while the nuclei are counterstained with hematoxylin. 100X.

possible to localize precisely the HNF4α receptors in cells, which were previously localized in the basolateral side of choroidal epithelium plasma membrane, though it was clear to identify HNF4α labelling 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 transthyretin. 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 indexes in the fourth ventricle CP compared to that of the lateral ventricle (Tables I-II), indicat-

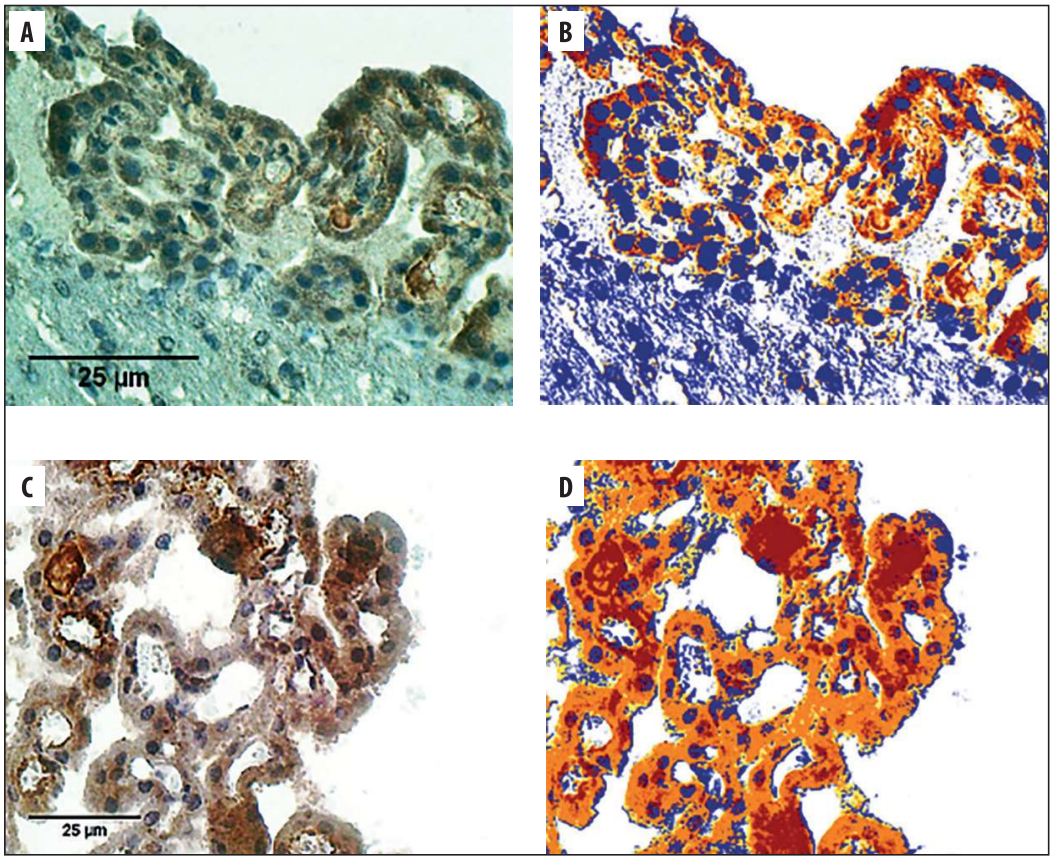

Fig. 4. Immune histochemical labeling of the choroid plexuses with HNF4α in the lateral ventricle (A) and fourth ventricles (C) with their respective makeover images (B) and (D) obtained via Aperio ImageScope software. Reaction force is detected visually where the red color represents areas of strong positivity, orange color represents positivity, yellow color represents weak positivity, and blue color indicates negative or no reaction. 400X.

Table I. Descriptive statistics of HNF4α marker labelling 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

ing 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 transthyretin 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 labelled 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].

Table II. 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	

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 analysed 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].

CONCLUSIONS

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 new knowledge 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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The Authors declare no conflict of interest.

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