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Expression of (ZO-1 & PLVAP) Antibody in the Sulcus Medianus Organum of Rat

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Abstract--- *The circumventricular organs (CVOs) are peculiar structures that border the brain ventricles, they are highly vascularized and lined by a specialized ependymal cells called tanycytes. There are seven CVOs that are generally characterized by deficient blood brain barrier and can be classified into two main groups (sensory& secretory CVOs). The sulcus medianus organum (SMO) is a previously described CVO, is located at the floor of the fourth ventricle at the rostral part of median sulcus, little information is available about this region of the brain. This research performed an immunohistochemical study considering the capillaries and the ependymal lining of this region to explore observations suggesting this region as a CVO. Twenty adult male rats (Rattus Norvegius Albinus) aged 3-6 months were used to study the immunohistochemistry of the SMO & the median eminence (ME) which was used as a control region since it is a well-known CVO lined by specialized ependymal cells (tanycytes) and richly vascularized with fenestrated capillaries. The immunohistochemical study involved the use of (ZO-1 antibody) to detect the tight junctions and the (PLVAP antibody) to explore the presence of fenestrated capillaries. The immune histochemical labeling findings showed a ZO-1 antibody reactivity that forms a continuous green line at the apical aspects of the ependymal lining of both SMO & ME. The PLVAP antibody labeling showed a negative reactivity at the region of the SMO in comparison to the ME which showed a positive reactivity. Conclusively, the SMO region contain an ependymal lining cells that could be regarded as a tanycytes-like cells and the blood vessels in this region showed features indicating the presence of blood brain barrier. However, the presence of fenestrated capillaries cannot be excluded totally, and the use of vascular permeability study or electron microscopy to explore the ultra-structural properties of the ependymal lining and the blood vessels at that region was recommended.*

Keywords--- *Circumventricular Organs(CVO), Sulcus Medianus Organum (SMO), Median Eminence (ME), ZO-1 Antibody (Zonula Occludens-1 Antibody), PLVAP Antibody (Plasmalema Vesicle Associated Antibody), Tanycytes.*

I. INTRODUCTION

The circumventricular organs (CVOs) are midine structures, bordering the third and fourth encephalic ventricles, characterized by deficient blood-brain barrier (Kaur & Ling, 2017), they are neurohemal organs (Joly et al., 2007) and Although there is no uniformity of structural construction, several features differentiate CVOs from other regions of the brain, all have extensive blood supply and possess special vascular arrangements, with many capillary loops getting near to the ventricular surface (Duvernoy & Risold, 2007).

The ventricular surface of CVOs have ependymal specializations, that lie adjacent to the capillary loops making

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them able to receive chemical information from cerebrospinal fluid, blood and other specialized brain nuclei (Jose Ruberte, 2017).

The ependymal cells of the CVOs are different from normal ependyma by the presence of occluding junctions between adjacent cells that limits the passage of molecules between cerebrospinal fluid and the CVO interstitium and vice versa, thus, it is thought that the ependymal cells of the CVOs take the main responsibility in formation of the blood–brain barrier instead of the capillary endothelial cells (Paxinos, 2015).

The median sulcus of the fourth ventricle contains a formerly undescribed CVO, it extends from the caudal end of the mid brain aqueduct to the inferior part of floor of fourth ventricle, the nonciliated cells predominante in this region with apical surface of each cell has characteristic microvillous projections with pinocytotic vesicles, the cell body is elongated with basal projection that give it a general morphology that is similar to tanycytes found in other CVOs (Collins, 1989).

This study aims to explore the presence of zonula occludens tight junctions between the ependynal cells linning the SMO & explorating the existence of fenestrated capillaries in that region in comparison to the median eminence which is a well known highly vascularized CVO with fenestrated capillaries.

II. MATERIALS AND METHODS

2.1. Animals & Housing

A sample of 20 adult male rats (Rattus Norvegicus Albinus) aged 3-6 months were taken from the animal house of Al-Nahrain University/College of Medicine and the National Center for Drug Control and Research during the years 2016-2017 on basis of being apparently active and healthy, with 300 ± 50 g body weight.

2.2. Collection of Samples

Animals were euthanized with chloroform impregnated cotton wool in an air tight chamber for 5 minutes, then pinned on a dissection board in the prone position and the skull was opened dorsally by a strong pair of scissors starting from the foramen magnum to the nasal bones.

In order to expose the entire contents of the skull, the parietal and temporal bones had to be cut, the cranial nerves were severed and the brain was delivered in one piece. Next, the brain was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 36 hours and dissected into two parts under dissecting lens, one part contained the median eminence (ME) and the other part included the cerebellum, 4th ventricle and sulcus medianus organum (SMO), ME specimen extends from the optic chiasm rostrally to the rostral part of the brain stem caudally, SMO specimen extends from the rostral limit of the cerebellum to beginning of the spinal cord caudally.

2.3. Preparation of Paraffin Sections

Paraffin sections were prepared according to (Bancroft and Gamble, 2008).

2.3.1. Fixation

Tissue samples were fixed for 36 hour at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) solution which was prepared according to (Bancroft and Gamble, 2008) by mixing the following:

Paraformaldehyde (Fisher scientific®)	4 g
Distilled water	95 ml
Phosphate buffer saline (PBS) concentrate (Birmingham®)	5 ml

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The solution heated to 60-65ºC while stirring and a few drops of 1N NaOH was added until solution became clear, left to cool and filtered.

2.3.2. Dehydration

Tissue was transferred into increasing concentrations of ethyl alcohol (70 % ethanol for 24 hours, 90 % ethanol for 6 hours, 100 % ethanol for 2 hours).

2.3.3. Clearing

Specimens were cleared with two ten minutes changes of xylene to ensure good tissue transparency.

2.3.4. Paraffin Infiltration & Embedding

The cleared specimens were infiltrated two times (2 hour for each one) with melted paraffin that was kept liquefied at (56-58º C) in hot oven. Then, tissue blocks were made using stainless steel molds (paraffin base molds) and plastic cassettes.

An electric wax dispenser was used for embedding the specimen. When the blocks completely solidified, they were separated from their molds and kept in refrigerator at 4 ºC until sectioning.

2.3.5. Sectioning

Tissue blocks were mounted on the holder of the electric microtome. Sections of 5 µm thickness from the regions of ME and SMO were cut and floated on 45º C water bath for 5-10 seconds before attaching the sections to slides (1-2 sections per -slide). Two sets of slides were made: one for staining with Hematoxylin& Eosin by using ordinary glass slides and the other for immunohistochemistry by using positive charge slides. These slides were kept in a 37º C oven to ensure good adhesion of sections.

2.4. Immunohistochemical Staining

Paraffin slides were transferred into a 60º C hot air oven for about 30 minutes to remove excess paraffin, left to cool down for 15 minutes and then dewaxed in two washes of xylene, each for 10 minutes.

The xylene was removed by 5 minutes washes subsequently in a decreasing concentrations of ethanol (100%, 95%, 80 & 75%) and then rehydration in phosphate buffered saline (PBS) for 10 minutes.

The hydrated slides then processed for antigen retrieval by putting them in a coplin jar containing sodium citrate buffer (antigen retrieval solution) which was prepared according to IHC protocol provided by biorbyt company by taking 2.94 gm of (Trisodium citrate) mixed with 1000ml of distilled water and the pH was adjusted to 5 with 1NHCl, 0.5 ml of tween 20 was added and the solution stored at 4º C.

The coplin jar transferred to an autoclave (90-98° C, 1.2 Bar) for 15 minutes and left to cool down to room temperature for 20 minutes.

The sections then washed in phosphate buffered saline (PBS) three times (5 minutes each) and blocked in 3% BSA (Bovine Serum Albumin) in phosphate buffered saline (PBS) for1 hour at 37º C.

The blocked tissue sections were “encircled” using wax pen and incubated with the primary unconjugated antibodies (ZO-1 & PLVAP antibody) in a humid chamber overnight at (4°C). The primary and secondary antibodies were diluted at (1:100) in antibody diluents except the PLVAP antibody diluted in a ratio of (1:400).

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The next step, sections labeled with (ZO-1 antibodies) rinsed by phosphate buffered saline four times (10 minutes each) and incubated in the dark humid chamber at (37°C) for 1 hour with Goat anti-Rabbit (FITC) & those labeled with PLVAP antibody incubated in the dark humid chamber at (37°C) for 1 hour with (Texas red) secondary antibody.

After incubation for 1hour, sections rinsed carefully by phosphate buffered saline using micropipette for 3 times (10 minutes each) and then a small drops of DAPI chromogen were added to the tissue sections & incubated for ten minutes at (37°C) to stain nuclei and lastly a small drops of antifade mounting medium were added to the sections and cover slip were put.

2.5. Examination of Slides

The labeled Slides were examined after 2-3 days (stored in a dark chamber at 4°C) under fluorescent microscope (AXIO-ZEISS®) with (10X, 20X, 40X) objective lenses. Three UV filters were used: FITC filter (519 nm), Texas Red filter (619 nm), and DAPI filter (435 nm) for bright green, red, and blue fluorescence, respectively (according to AXIO-ZEISS® and NIKON®). Digital camera (AXIO-ZEISS®) mounted to the fluorescent microscope was used to take the pictures.

III. RESULTS

3.1. ZO-1 Antibody Labeling

3.1.1. Median Eminence

The immunoreactivity of the ZO-1 antibody showed a more strong signal especially on the apical aspect of the ependymal cells covering the median eminence with more continuity than that of the ependyma covering the lateral wall third ventricle, the cells covering the medial eminence appeared as a monolayer flattened cells with rounded nuclei (Fig. 3.1)

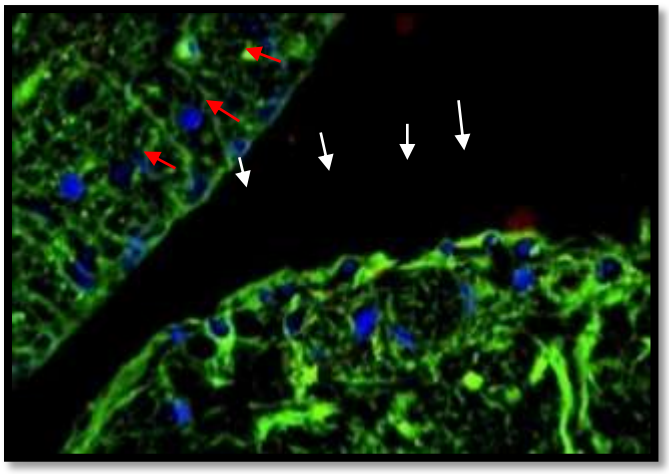

Figure 3.1: Coronal Section at the Region of the third Ventricle and the Median Eminence, the Ependymal Cells (Indicated by White Arrows) Covering the Median Eminence show a More Strong ZO-1 Immuno-Reactivity than that of the Ependymal Cells (indicated by red arrows) Covering the Lateral Wall of the Third Ventricle. The Cells Appear as a Monolayer with Flattened Nuclei

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The brain tissue parenchyma next to the lateral wall of the third ventricle showed a bright ZO-1 reactivity expressed as a continuous lines surrounding the transverse sectioned blood vessels, however the median eminence parenchyma showed a similar pattern of reactivity but the lines around the transversely sectioned blood vessels expressed a less continuity (Fig. 3.2).

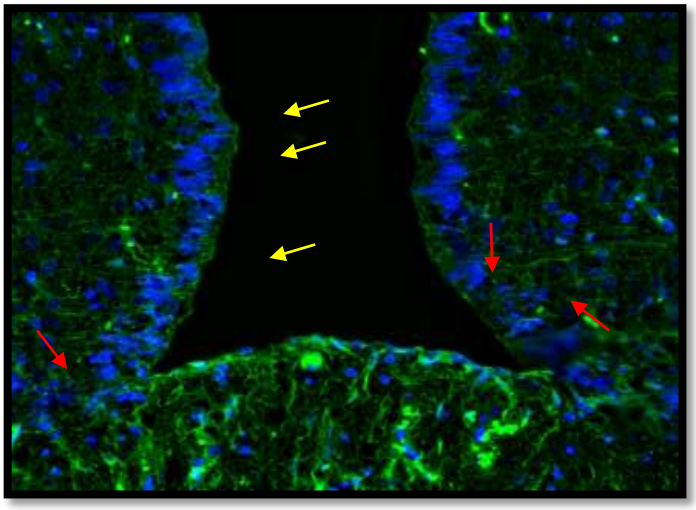

Figure 3.2: Coronal Section at the Region of the Third Ventricle & the Median Eminence, the Brain Tissue Parenchyma on the Lateral Side of the Third Ventricle Shows a ZO-1 Reactivity Expressed as Rounded Continuous Lines (Indicated by the Red Arrows) While the ZO-1 Reactivity in Region of the Median Eminence Shows a Rounded Lines but with Less Continuity (Indicated by the Yellow Arrows)

3.1.2. Sulcus Medianus Organum

The ZO-1 Antibody immune histochemical labelling in the region of fourth ventricle & median sulcus showed a positive reactivity in the ependymal lining of the floor of the fourth ventricle as shown in (Fig. 3.3).

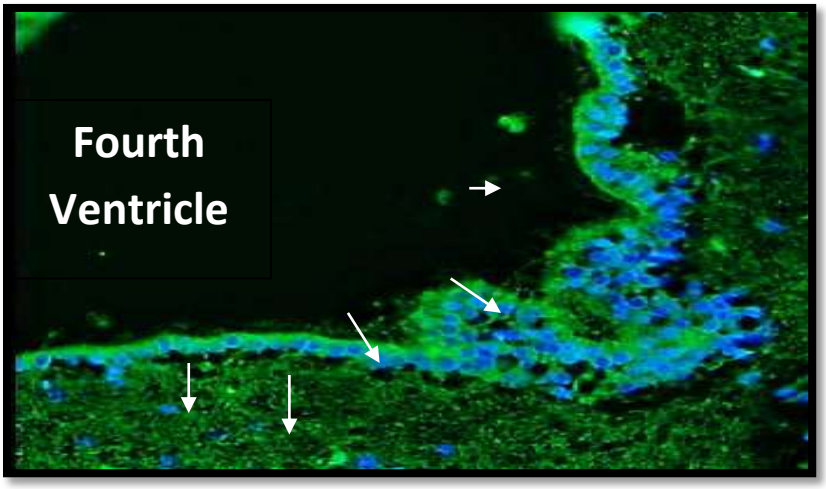

Figure 3.3: Coronal Section at the Regions of the Fourth Ventricle and the Median Sulcus Showing the Expression of the ZO-1 Antibody in the Floor of Ependymal Lining (Arrows) of the Fourth Ventricle

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The brain tissue parenchyma inferior to the ependymal cells covering the floor of the fourth ventricle showed a green fluorescence ZO-1 reactivity expressed as rounded continuous lines surrounding the transverse sectioned blood vessels (Fig. 3.4).

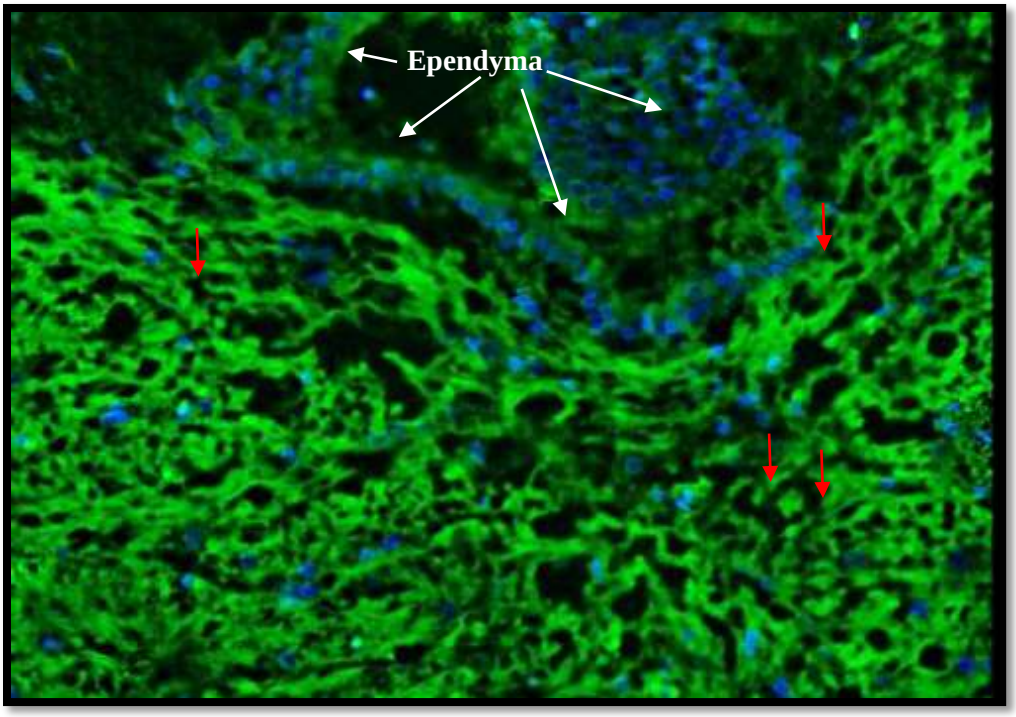

Figure 3.4: Coronal Section at the Region of the Fourth Ventricle & the Median Sulcus Showing the Cavity of the Fourth Ventricle, the Ependymal Lining the Floor

The brain parenchymal tissue below the ependyma at the region of the furrow of the median sulcus showing a ZO-1 antibody reactivity expressed as a continous rounded green lines (red arrows)

3.2. PLVAP Antibody Labeling

3.2.1. Median Eminence

The region of the median eminence at the ventral aspect of the third ventricle showed positive immunohistochemical reactivity to PLVAP antibody compared to the parenchymal brain tissue subjacent to ependyma of the lateral walls of the third ventricle, The pattern of the positive immunohistochemical reactivity in the median eminence showed rounded linear configuration, these reactivities showed variable intensity of the red fluorescent reactivity indicating the detection of the labeling of this plasma lemma vesicle associated protein to endothelial cells, this labeling was not detectable in the brain parenchyma forming the lateral walls of the third ventricle (Fig. 3.5).

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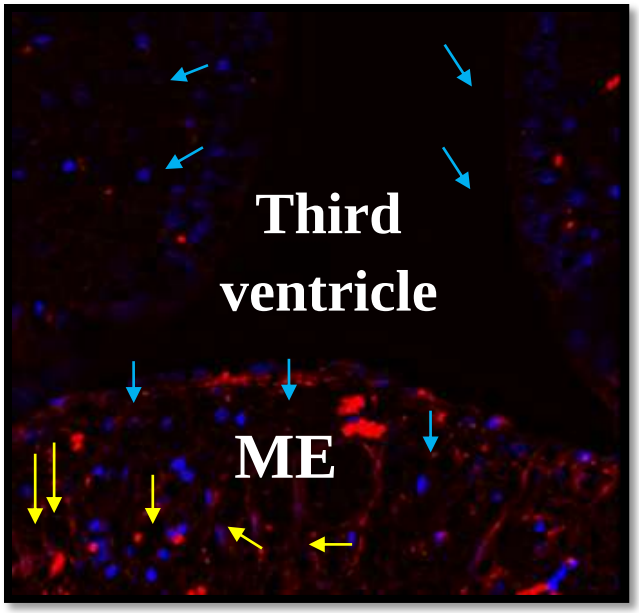

Figure 3.5: Coronal Section at the Region of the Third Ventricle and the Median Eminence (ME) Showing the PLVAP Antibody Immunore Activity (as Indicated by the Yellow Arrows) Expressed as Rounded Linear Lines of Variable Intensity (Indicated by the Yellow Arrows) that is not Detected in the Parenchymal Brain Tissue Forming the Lateral Walls of the Third Ventricle, the Ependymal Cells Covering the Lateral Wall of the Third Ventricle and the Median Eminence are Indicated by Blue Arrows

3.2.2. Sulcus Medianus Organum

The Immunohistochemical expression in the region of the fourth ventricle and the brain tissue parenchyma inferior to the median sulcus didn't showed detectable positive reactivity signal to the PLVAP antibody (Fig.3.6)

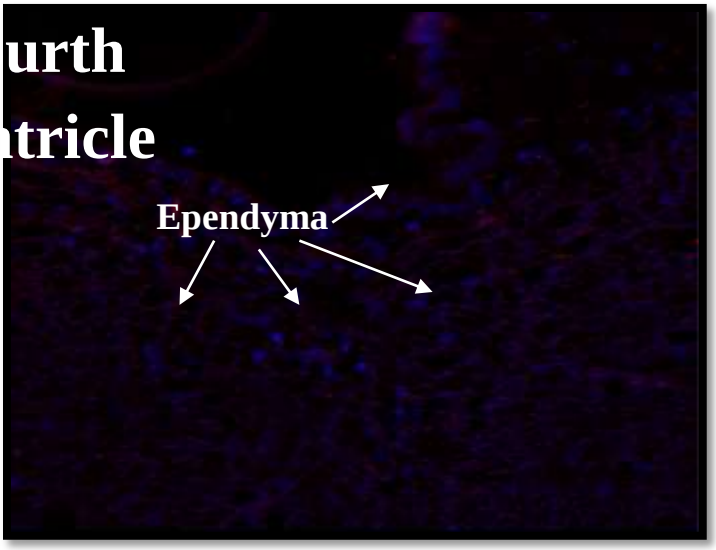

Figure 3.6: Coronal Section at the Region of the Fourth Ventricle and the Median Sulcus, the Parenchymal Brain Tissue Inferior to the ependyma didn't Showed Positive Reactivity to PLVAP Antibody

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IV. DISCUSSION

The immunoreactivity of the ZO-1 antibody seen in the results of this study showed intense continuous reactivity on the apices of the ependymal cells covering the median eminence and the rostral region of median sulcus (the region of the SMO) in comparison to the ependyma covering the adjacent walls of the ventricles. This pattern was in agreement with description reported in previous articles (Petrov et al.,1994). The immunoreactivity of the ZO-1 marker of this study goes with the suggestions of (Ahmed, 2017) that the ependymal cells exhibiting tight junction complexes are a unique cell population which could be considered as a strong evidence for the presence of tanycyte or tanycyte-like cells covering a circumventricular organ. Those authors established this finding as an additive confirmation to the recognition of the SMO.

The ZO-1 immunohistochemical reactivity obtained in this study is supported by the finding of (tight junction) that the median eminence contains tanycytes which cover the floor of the third ventricle, these tanycytes express occludin, ZO-1, and claudin 1 and 5, but not claudin 3. These molecules are organized as a continuous belt around the cell bodies of the tanycytes (Mullier, 2010), these authors suggested that the technique of permeability studies using peripheral or central injections of Evans blue dye confirm the existence of tanycytes barrier function.

The results of this study showed ZO-1 reactivity expressed in the brain tissue parenchyma next to the lateral wall of the third ventricle and the parenchyma subjacent to median sulcus of 4th ventricle floor , with a continuous linear ZO-1 reactivity around the lumen of the transverse sectioned blood vessels. This pattern also was seen at the parenchyma of median eminence, apart from discontinuous linear reactivity around the sectioned blood vessels. These histochemical finding could be related to the detection of tight junctional complexes of the blood brain barriers in these regions. The certainty that the median eminence contains fenestrated capillaries is appropriate interpretation of the discontinuous ZO-1 immunohistochemical expression of the vascular lumen in this region.

Tight junctions are physical barrier in the BBB between endothelial cells and the blood circulation that prevent the free movement of substances and protect neurons from toxic substances (Saunders et al., 2008).

The negative polyclonal PLVAP immunohistochemical reactivity found in this study could be considered as criteria excluding the presence of endothelial diaphragms maintaining permeability of fenestrated blood vessels at the region of SMO. However, the presence of fenestrated capillaries in the region of SMO needs to be further investigated using more comprehensive research techniques.

In agreement with the above conclusion for the need of more elaborated investigation for the presence of fenestrated capillaries in the region of SMO, the pattern of distribution of the PLVAP protein was reported to be restricted to the membrane of a subset of endothelial cells in the normal microvasculature (Tse, 2010). The highest detection of PLVAP was found in the lungs, kidneys, spleen, endocrine glands and digestive tract (Stan, 1999), PLVAP was not detected in the endothelial cells of large vessels, with the exception of the endocardial lining of the heart chambers (Stan, 2005, Deharvengt, 2012).

The detection of PLVAP in the brain tissue has been documented to be upregulated during ischemic brain disease in association with damage to the blood-brain barrier resulting in an increased permeability of the microvasculature (Shue, 2008) as PLVAP was induced during hypoxia (Carson-Walter, 2005).

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The results obtained in this study regarding the absence of PLVAP immunohistochemical reactivity in the region of the SMO compared to the reactivity at the ME make a controversy about the broad-spectrum expression made by the report of Umans, (2017) that plvap expression in brain endothelial cells is absent from the adult brain, except for the vasculature of circumventricular organs. As this report followed the conclusions made by Hallmann et al., (1995) in a study to describe the novel Mouse Endothelial Cell Surface Marker (MECA-32) which is a monoclonal antibody with high specificity for mouse endothelium in both embryonic and mature tissues, the absence of PLVAP immunohistochemical reactivity found in this study necessitates the recommendation for further investigation for this topic using the suitable techniques and markers. The explanation of the negative PLVAP reactivity at the SMO in consideration to the proposal of Hallmann et al., (1995) may be attributed to miscellaneous possibilities including; the sensitivity and specificity of the marker used, the absence of fenestrated capillaries at SMO, or may the need for certain modifications of the labeling technique of the marker.

In support to the above conclusion, it was documented that large number of problems could be challenged during the physiological and molecular biology studies done for barrier mechanisms in the brain, accordingly there will be some animal of choice on which it can be most conveniently studied. Achievement of conclusive results depends upon the convenience of the species and its relevance to the problem being studied (Saunders, 2018).

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