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## **Analysis of age-related differences in hypoxia-related factors in yak brain tissue**

Lan Zhang et al., Analysis of age-related differences in hypoxia-related factors in yak brain tissue

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## **Abstract**

The brain is an important part of the mammalian nervous system, is highly sensitive to hypoxia, and plays an important role in the adaptation of the body to hypoxic environments. This study was conducted to study the distribution and expression of hypoxia-related factors

(hypoxia-inducible factor 1 $\alpha$ , HIF-1 $\alpha$ ; erythropoietin, EPO; vascular endothelial growth factor, VEGF; vascular cell adhesion molecule, VCAM) in the cerebellum, cerebrum, medulla oblongata, and corpora quadrigemina in yaks of different ages (4d, 6-months-old and adult). Paraffin sections were obtained from the cerebellum, cerebrum, medulla oblongata, and corpora quadrigemina of healthy yak for 4-day-old, 6-months-old and adult yaks. Histological characteristics were assessed by haematoxylin staining. Immunohistochemical staining was performed to detect the distribution and expression of HIF-1 $\alpha$ , EPO, VEGF and VCAM proteins. Immunohistochemical results showed that HIF-1 $\alpha$ , EPO, VEGF, and VCAM were expressed in the pyramidal cell layer of the yak cerebrum, and distributed in the cerebellum granule cell layer, Purkinje cell layer and medulla layer, and were mainly positive in Purkinje cells and medulla. It is expressed in the cell bodies of the medulla oblongata and the quadrimotoneurons. The expression level in the medulla oblongata was higher, indicating may play a crucial role in functional cohesion. The expression of HIF-1 $\alpha$  in 4 d cerebellar tissues was higher than that in other age groups, and the expression of HIF-1 $\alpha$  in the medulla oblongata increased with age. In addition, the expression levels of EPO and VEGF in the 6-month-old group were slightly higher than those in the other age groups. It is speculated that EPO and VEGF have obvious protective effects on brain tissue in the 6-month-old age group; VCAM showed no significant differences in the cerebrum, cerebellum, medulla oblongata, or corpora quadrigemina of the yaks. This study provides basic data for further exploration of the adaptive mechanism of plateau yak brain tissue.

**Key words:** Yaks, brain tissue, development, EPO, VEGF, VCAM

## INTRODUCTION

*Bos grunniens*, a unique species adapted to high-altitude and cold climates, live in plateau areas with an altitude of 3,000 ~ 6,000 m. Through long-term natural selection, *Bos grunniens* has formed a unique adaptive structure and physiological mechanism in the low-oxygen environment of the plateau. In recent years, domestic and foreign studies on the adaptation of yaks to hypoxia have mostly focused on the urinary, cardiovascular, respiratory, and digestive systems<sup>1-4</sup> while there are few histological studies on the aging changes of the

yak nervous system. Yak brain tissue is sensitive to the oxygen environment. Although, although it only accounts for 2% of the body weight, but the oxygen demand of the brain accounts for 20% of the oxygen demand of the body. Yak brain tissue can function normally under extremely harsh conditions, which must be closely connected to its special physiological mechanisms.

Hypoxia-inducible factor (HIF) is a key transcriptional regulator of the adaptive response to the hypoxic microenvironment, which can regulate a variety of hypoxia adaptive genes in response to a decrease in oxygen concentration in cells. It regulates cell proliferation and apoptosis through interactions with downstream target genes, transcription factors, and intracellular activators in the cytoplasm, enabling cells to adapt to hypoxia and survive. It also and plays an important role in cell genesis and development under hypoxic conditions. HIF is a basic heterodimer protein, and its complex consists of an O<sub>2</sub>-dependent alpha subunit (HIF- $\alpha$ ) and a subunit (HIF- $\beta$ ). Among them, HIF- $\beta$  belongs to the aromatic hydrocarbon receptor protein. There are three independent subunits of HIF- $\alpha$ , HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ , which are similar in structure but different in function. HIF-1 $\alpha$  is an oxygen-sensitive subunit and plays an important role in mediating hypoxia signal transduction in multiple signal pathways. When cells are cultured under low oxygen conditions, transcription factors in the cytoplasm are activated to enter the nucleus, bind specifically to target DNA sequences, and regulate the expression of target genes. When cardiovascular cells were cultured in a simulated hypoxic environment in vitro, HIF-1 $\alpha$  overexpression increased the expression of angiogenic genes and VEGF, which play a protective role in the heart. Erythropoietin (EPO) is a glycoprotein peptide. High-altitude and low-oxygen environments can stimulate EPO expression in plasma, thereby promoting erythropoiesis to improve hypoxia-induced physiological responses, Additionally, erythropoiesis can inhibit EPO expression in a feedback manner to control red blood cell volume and accurately adapt to the needs of the body<sup>5</sup>. Generally, EPO expression is low in brain tissues of normal animals. However, when the central nervous system suffers from ischaemia and hypoxia, EPO expression in the brain increases significantly, thus promoting the recovery of neurological function after brain injury<sup>6</sup>. More scholars have given attention to the neuroprotective effect of EPO, but little is known about the exact mechanism

of EPO's neuroprotective function, and there studies to confirm the role of EPO in brain tissue are still lacking. Vascular endothelial growth factor (VEGF) is part of the important factors that regulate blood vessel growth in animal tissues. Previous studies have shown that VEGF is a regulatory protein related to hypoxia adaptation at high altitudes, and has a neuroprotective effect on ischaemic hypoxic brain tissues<sup>7</sup>. Vascular cell adhesion molecule (VCAM) is an important adhesion molecule in the body and is closely related to vascular endothelial function. It circulates widely on the surface of vascular endothelial cells and macrophages<sup>8</sup>. The unusual structure of the cerebrovascular endothelial cells differs from that of other tissues. This results in high levels of adhesion molecules. Studies have found that, under normal conditions, low VCAM content can promote cell proliferation and participate in the repair process of vascular endothelial cell damage. Contemporary studies have shown that VCAM is involved in brain tissue damage in animal models<sup>9</sup>. Hypoxia is a pathogenic factors that can cause altitude sickness and acute brain injury. Brain injury caused by hypoxia is serious and will mainly have adverse effects on brain cognitive function<sup>10</sup>. Therefore, the distribution and expression of EPO, VEGF, and VCAM in the brain tissues of yaks of different ages play an important role in the mechanism of action of yak brain tissues in high-altitude and low-oxygen environments. It is of great research value to reveal the mechanisms of the central nervous system in yaks inhabiting high-altitude hypoxia environments.

## **MATERIALS AND METHODS**

### **Animal ethics and Materials**

Brain tissues (cerebellum, cerebrum, medulla oblongata and corpora quadrigemina) of yaks of different ages (4d, 6 months and adult) in this experiment were collected from healthy yaks in a slaughterhouse in Xining, Qinghai Province. All experimental and surgical procedures are approved by the Committee for the Care and Use of Animals for Biological Research of Qinghai Province, PRC. The brain samples were attached with 4% paraformaldehyde, followed by immunohistochemical staining. The study was approved by the State Forestry Administration, and all procedures were performed in compliance with guidelines for the care and use of laboratory animals adopted by the Ministry of Science and

Technology of the People's Republic of China.

### **H&E staining detection**

Fully fixed samples were embedded in paraffin and the tissue blocks then were sliced into 5  $\mu\text{m}$  sections for subsequent processes. Haematoxylin and eosin (H&E) staining was used to observe the histological features of the samples.

### **Immunohistochemical staining**

Immunohistochemical staining was performed based on Histostain<sup>TM</sup>-Plus Kits (Bioss, China, SP-0023). Briefly, tissue sections were deparaffinised in xylene and dehydrated in different concentration gradient of alcohol. After being rinsed in phosphate buffered saline buffer (PBS), sections were autoclaved (15 min in a microwave oven) in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. The sections were then incubated with anti-EPO polyclonal antibody, anti-VEGF polyclonal antibody and anti-VCAM polyclonal antibody (Bioss, China, 1:200 dilution) at 4°C overnight in a humid chamber. Antibody binding was coloured with DAB Substrate kit (Solarbio, China, DA1010) and tissue sections were countersigned with haematoxylin. All washing steps in-between were made in PBS. To assess the specificity of the immunolabelling, negative control slides were created using the bovine serum albumin as the primary antibody while all other steps and conditions remained the same. Finally, the sheet is sealed with neutral gum. Results The positive expression intensity was a comprehensive criterion. Dark brown-yellow was strongly positive, light brown-yellow was positive, and close to background color or colorless was negative. The images were made by Olympus DP71 microphotography system.

### **Statistical analysis**

Images of the stained tissue sections were noted and captured by a light microscope (Olympus CX31, Tokyo, Japan). Image-Pro Plus (Version 6.0, Media Cybernetics, Inc., Bethesda, MD, USA) was utilized to quantify the positive results of expression of HIF-1 $\alpha$ , EPO, VEGF and VCAM. The measurement parameters included some of the area and sum of

the integrated optical density (IOD). The IOD of HIF1 $\alpha$ , BNIP3 and beclin-1 was calculated by immunohistochemical staining of 5 fields (original magnification of 400 times) randomly selected in the section. Five sections were randomly selected for each brain tissue. GraphPad Prism software was utilized to analyse the statistical significance for all statistical analyses. The data were analyzed by one-way ANOVA. P value of less than 0.05 between the groups was considered statistically significant.

## **RESULTS**

### **H&E staining detection**

HE staining was used to observe the histomorphology of yak brain tissues (cerebellum, cerebrum, medulla oblongata and corpora quadrigemina) (Fig.1). The results showed that the cerebellar cortex was composed of three layers: a molecular layer, Purkinje cell layer, and granular cell layer. The molecular layer is dominated by a small number of glial cells. The granular layer is composed of numerous small granular cells. The Purkinje cell layer consists of larger Purkinje cells. The cerebral cortex was divided into six layers. The neurons in the cortex include granular cells, pyramidal cells and spindle cells. White matter and myelin sheaths are present in the medulla oblongata and mainly contain horns and glial cells. The quadrilateral body is primarily comprised of glial cells and neurons. In addition, the brain thickened with age, indicating that the cerebral cortex function in yaks gradually strengthened with age.

### **Immunohistochemical observation of HIF-1 $\alpha$ , EPO, VEGF, and VCAM in yak brain tissue**

Immunohistochemical staining of yak brain tissues (cerebellum, cerebrum, medulla oblongata, and tetrad) at different ages (4d, 6 months, and adult).

### **Distribution and expression of HIF-1 $\alpha$ in brain tissue**

The results of HIF-1 $\alpha$  staining at different ages showed that cytoplasmic staining was strongest in cerebellar Purkinje cells, and a small amount of HIF-1 $\alpha$  was expressed in granular

cells. In the cerebral cortex, the cytoplasm of pyramidal cells, mainly distributed in the second and third layers had a small amount of expression. HIF-1 $\alpha$  is positively expressed in glial cells and neurons of the quadrimata gray matter. It was expressed in small amounts in the horn cells of the medulla oblongata (Fig.2). In addition, the expression of HIF-1 $\alpha$  in 4-day-old cerebellar tissues was higher than that in other age groups ( $P<0.01$ ). There were no significant age differences between the brains ( $P>0.05$ ). The expression levels in the medulla oblongata increased with age ( $P<0.01$ ). The expression level in the tetrastroma decreased with increasing age ( $P<0.01$ ) (Fig.3).

### **Distribution and expression of EPO in brain tissue**

Immunohistochemical staining was performed on paraffin sections of yak brain tissue, and EPO staining in different age groups showed that EPO was localised to the cytoplasm of the cells. Positive expression was found in basket cells, Purkinje cell cytoplasm, and granulos cell cytoplasm of the cortical molecular layer in the cerebellum, of which Purkinje cells had the strongest positive staining. It is expressed in the cytoplasm of glial and pyramidal cells in the medulla of the cerebrum. In the medulla oblongata, it is strongly expressed in the horn, glial and neuronal cells. There was strong positive expression in the corpora quadrigemina of the quadrimatous gray matter (Fig.4). In contrast, EPO expression in the medulla oblongata was greater than that in other tissues( $P<0.01$ ). In addition, the results of EPO in different age groups showed that the expression level of EPO in the brain was the highest in 4D mice, and the expression level of EPO in other tissues at 6 months was higher than that in other age groups (Fig.5)( $P<0.01$ ).

### **Distribution and expression of VEGF in brain tissue**

VEGF expression in the brain tissues from different age groups showed that VEGF was present in the cytoplasm of the cells. Cytoplasmic staining of Purkinje cells in the cerebellum was the strongest, and a small amount of positive staining was observed in the granuloma cells and basket cells. VEGF is expressed in the cytoplasm of glial and pyramidal cells in the medulla of the cerebrum. In the medulla oblongata, strong positive expression was observed



in horn cells, glial cells, and neurons cells. There was a strong positive expression in the quadrimatous gray matter of the corpora quadrigemina (Fig.6). The results showed that the expression of VEGF in the medulla oblongata was higher than that in other tissues( $P<0.01$ ). The results of different age groups showed that the expression level of VEGF was highest in 6-month-old cerebellar tissues, whereas, expression level was the lowest in the 6-month-old cerebral tissue( $P<0.01$ ). In 4-day-old medulla oblongata tissue, the expression level was the highest in the adult corpora quadrigemina (Fig.7)( $P<0.05$ ).

### **Distribution and expression of VCAM in brain tissue.**

The results of VCAM analysis at different ages showed that VCAM was located in the cytoplasm. Cytoplasmic staining of Purkinje cells in the cerebellum was the strongest, and there was a small amount of positive staining in granulosa cells; a small amount was expressed in the cytoplasm of pyramidal cells in the medulla of the cerebrum. It is expressed in small amounts in the horn cells of the medulla oblongata. Positive expression was observed in glial cells and neurons of the quadrimatous gray matter (Fig.8). The results showed that the expression of VCAM in the tetrad was greater than that in other tissues( $P<0.01$ ). In addition, the VCAM group showed no age-related differences (Fig.9). Figure 10 shows the negative control.

### **Discussion**

Yaks, as a unique species adapted to the plateau, have formed a unique adaptive structure and physiological mechanism to the low- oxygen environment on the plateau. Brain tissue plays an irreplaceable role in the regulation of respiration, and circulation, and other physiological functions. Brain tissue plays an irreplaceable role in regulating physiological functions such as respiratory circulation. HIF-1 $\alpha$  is an important component of the brain's adaptive response to hypoxia and is involved in the regulation of developmental cellular processes and cellular oxygen homeostasis. Bergeron et al. showed that hypoxia can increase HIF-1 $\alpha$  level in the adult rat brain and plays an important role in adapting to a hypoxic environment. The results of this experiment showed that the expression of HIF-1 $\alpha$  protein in

the medulla oblongata was higher than that in other tissues, mainly in horn cells, glial cells and neurons. In addition, age-related results showed that the expression of HIF-1 $\alpha$  protein in brain tissue at 4 days was higher than that in other age groups. There were no significant age differences between the brains ( $P > 0.05$ ). The expression levels in the medulla oblongata increased with age ( $P < 0.01$ ). The expression of HIF-1 $\alpha$  decreased with the increase of age ( $P < 0.01$ ), indicating that HIF-1 $\alpha$  plays a regulatory role in the medulla oblongata.

Combined with the results of this study, EPO and VEGF are expressed in the yak cerebellum, cerebrum, medulla oblongata, and corpora quadrigemina. The expressions of EPO and VEGF in the medulla oblongata tissues was higher than that in other tissues and was, mainly expressed in the horn cells, glial cells, and neurons of the medulla oblongata, suggesting that the medulla oblongata may play a crucial role in the process of functional cohesion. The main function of the medulla oblongata is to regulate the visceral activities of the body and is the basic centre necessary to maintain life. Medulla oblongata neurons have high oxidative capacity, and hypoxia reduces aerobic metabolism, resulting in the loss of cell function and ultimately cell death. However, hypoxia can also cause protective changes in two main ways: 1) systemic expression such as that of example EPO promoting erythrocyte hyperplasia and thus improving oxygen supply; and 2) procaine expression, such as VEGF, which increases vascular permeability and stimulates angiogenesis. Research shows that at the nervous system level, low oxygen promotes EPO and the expression of VEGF<sup>11,12</sup>, as well as their activation in neurons, glial cells, endothelial cells, stem cells, and neural precursor cells via a specific receptor<sup>4,13</sup>. They also play a role in recovery, including the maintenance of neural function, nerve regeneration after injury, neurogenesis, and vascular remodelling. Studies have shown that EPO is produced when the medulla oblongata neurons are hypoxic, is located in other brain tissue regions such as the medulla oblongata and quadrilateral, and is up-regulated under hypoxia<sup>14</sup>, which is consistent with the results of this study. Studies have shown that the neural protective mechanism induced by the hypoxic environment of EPO includes direct regulation of Akt phosphorylation to prevent DNA fragmentation, thus reducing mitochondrial membrane depolarisation and cytochrome c release<sup>15,16</sup> enhancing erythropoiesis, accelerating the blood- carrying oxygen transport ability, and protecting brain

tissue from damage<sup>17,18</sup>. In addition, VEGF, as a mitogen for endothelial cells, plays an important role in regulating neurogenesis and promoting angiogenesis. VEGF is also related to the destruction of the blood-brain barrier, can be found in brain tissue after injury, and has a neuroprotective effect. In models of hypoxia<sup>19</sup>, ischemia<sup>20</sup> and hypoxia/reoxygenation injury<sup>21</sup>, VEGF was expressed in neurons<sup>22</sup>, which was consistent with the results of this study. Increasing evidence has shown that<sup>23,24</sup> EPO and VEGF have shielding effect on the central nervous system under hypoxic conditions. VCAM is an important irreducible cell adhesion molecule. In this study, VCAM was mainly expressed in the glial cells, which is consistent with the findings of Miyamoto et al. VCAM is expressed in oligodendrocytes, participates in the initiation of myelination, regulates myelination initiation, and was a positively regulates oligodendrocyte differentiation and initiation of myelination<sup>25</sup>. Combined with the results of this study, the expression distribution of EPO, VEGF, and VCAM in 4-day-old, 6-month-old, and adult yaks is consistent, further indicating that there is a connection between the three functions. The medulla oblongata may be more sensitive to ischaemia and hypoxia than the other brain tissue regions.

The expressions of HIF-1 $\alpha$ , EPO, VEGF, and VCAM in the cerebellum, cerebrum, medulla oblongata, and tetrad of yaks of different ages were detected. The results showed that there was no difference in the expression of VCAM in the brain tissues of yaks, whereas the expression of EPO and VEGF in the cerebellum, medulla oblongata, and quadrangle showed a trend of initially increasing and then decreasing with age. The expression levels of EPO and VEGF in the brain tissues of yaks were slightly higher in the 6-month-old group, and then decreased and increased with age. This helps to increase the protective effect on yak brain tissue after birth, it dicating that yaks, with aging, have adapted well to the environment of high cold and low oxygen through their own adjustment ability and adaptability to the environment, which is similar to the results of Li et al.<sup>26-29</sup>. Studies have shown that during the growth and development of yaks from birth to six months of age, brain tissue development is relatively slow owing to the influence of low-oxygen environments and cold weather<sup>30</sup>. EPO and VEGF are considered important potential regulatory proteins in yaks that adapt to hypoxic environments and play important roles in brain tissue development. It is speculated

that EPO and VEGF have obvious protective effects on brain tissue in the 6-month-old age group, but their functional association with yak brain tissue has not yet been confirmed. Therefore, it is important to reveal the operating mechanism of the yak central nervous system of yaks in high-altitude and low-oxygen environments.

Our results show that HIF-1 $\alpha$ , EPO, VEGF, and VCAM have a protective effect on brain tissue and show correlation with the age of yaks in a hypoxic environment. However, the specific mechanism by which this occurs still needs further validation. This study is a preliminary experiment, and provides basic data for a comparative study of the adaptation mechanisms of yak brain tissues in different age groups under high-altitude hypoxia.

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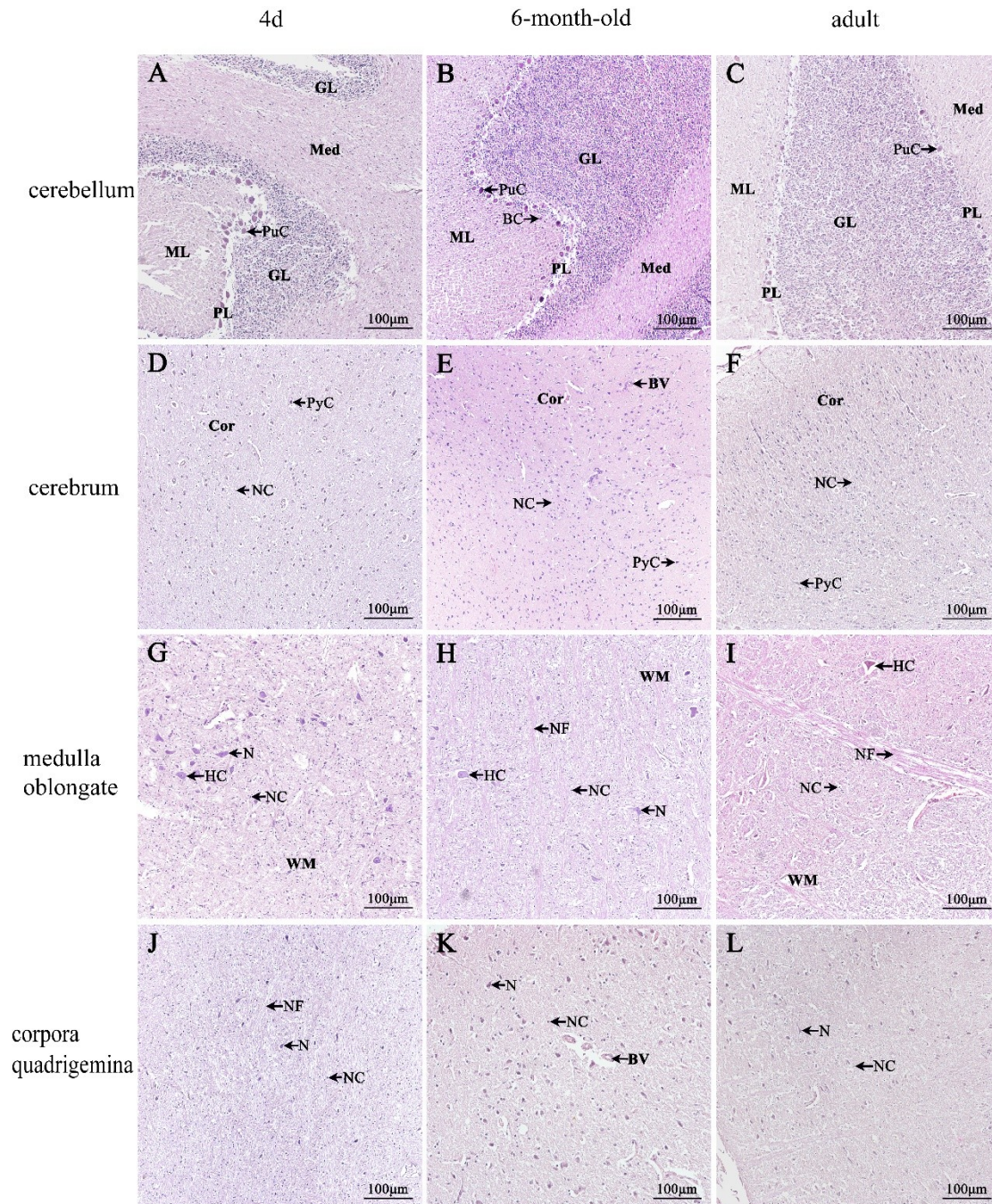
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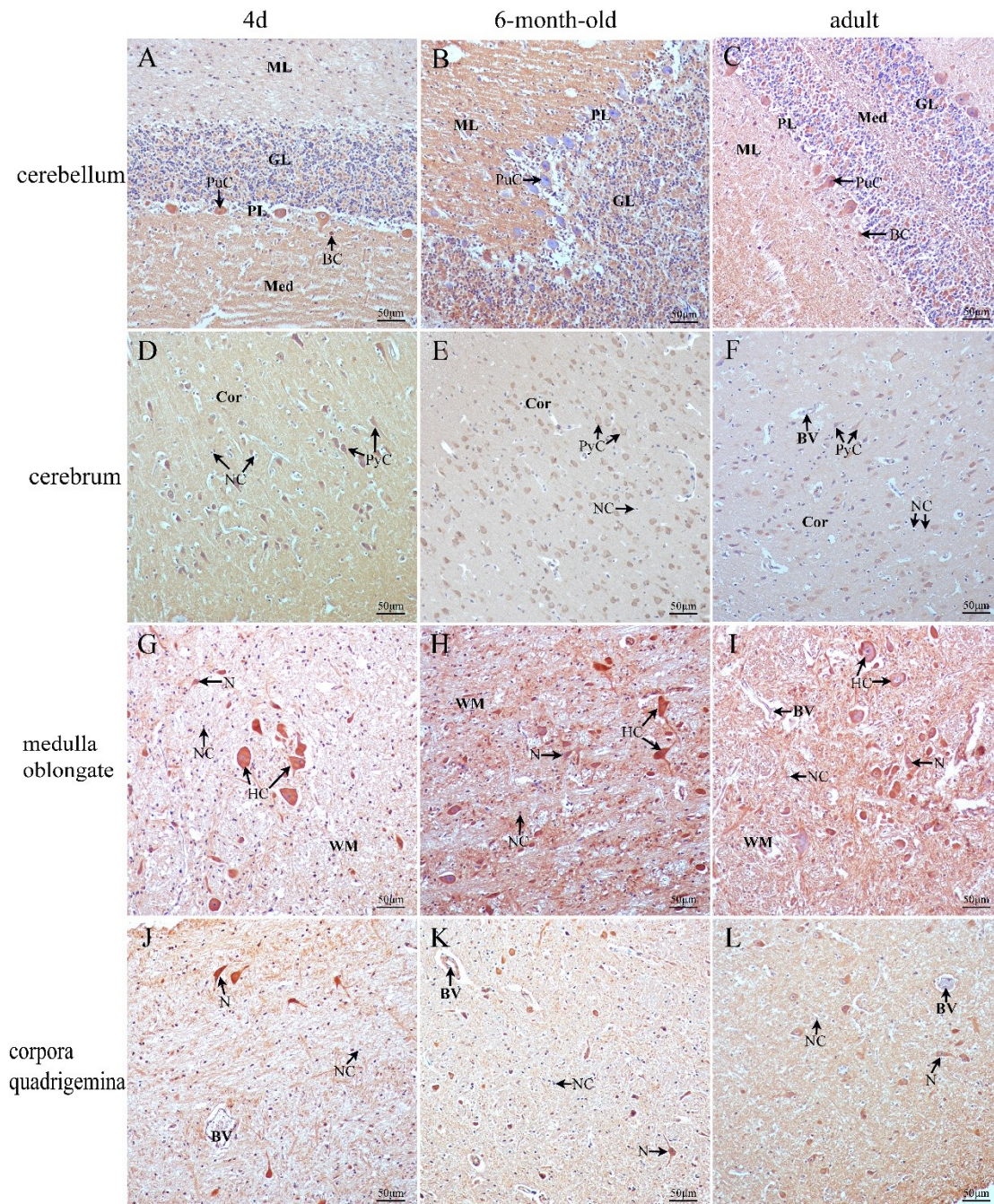
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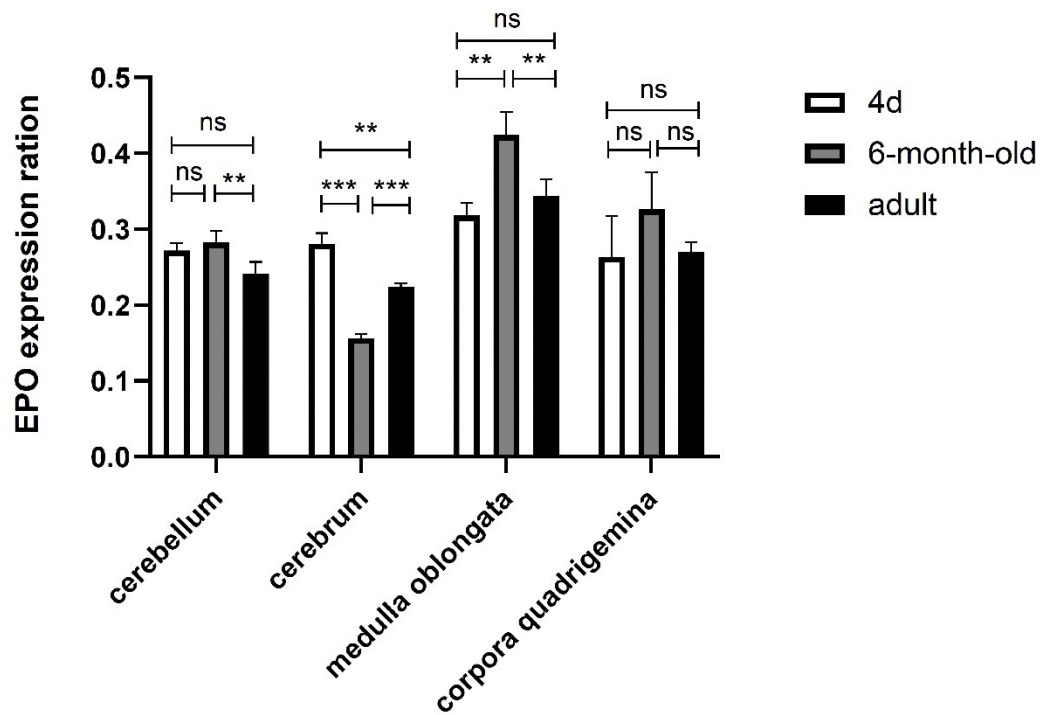


**Figure 1.** Study on brain tissue of yak in different age groups. A-L were 4d, 6-months-old and adult cerebellum, cerebrum, medulla oblongata, and tetrad respectively. ML — molecular layer; GL — granular layer; PL — Purkinje cell layer; Med — medulla; WM — white matter; BV — blood vessel; PuC — Purkinje cell; BC — basket cell; Cor — cortex; PyC — pyramidal cell; HC — horn cell; N — neuron; NC — neuroglia cell; NF — nerve fiber.



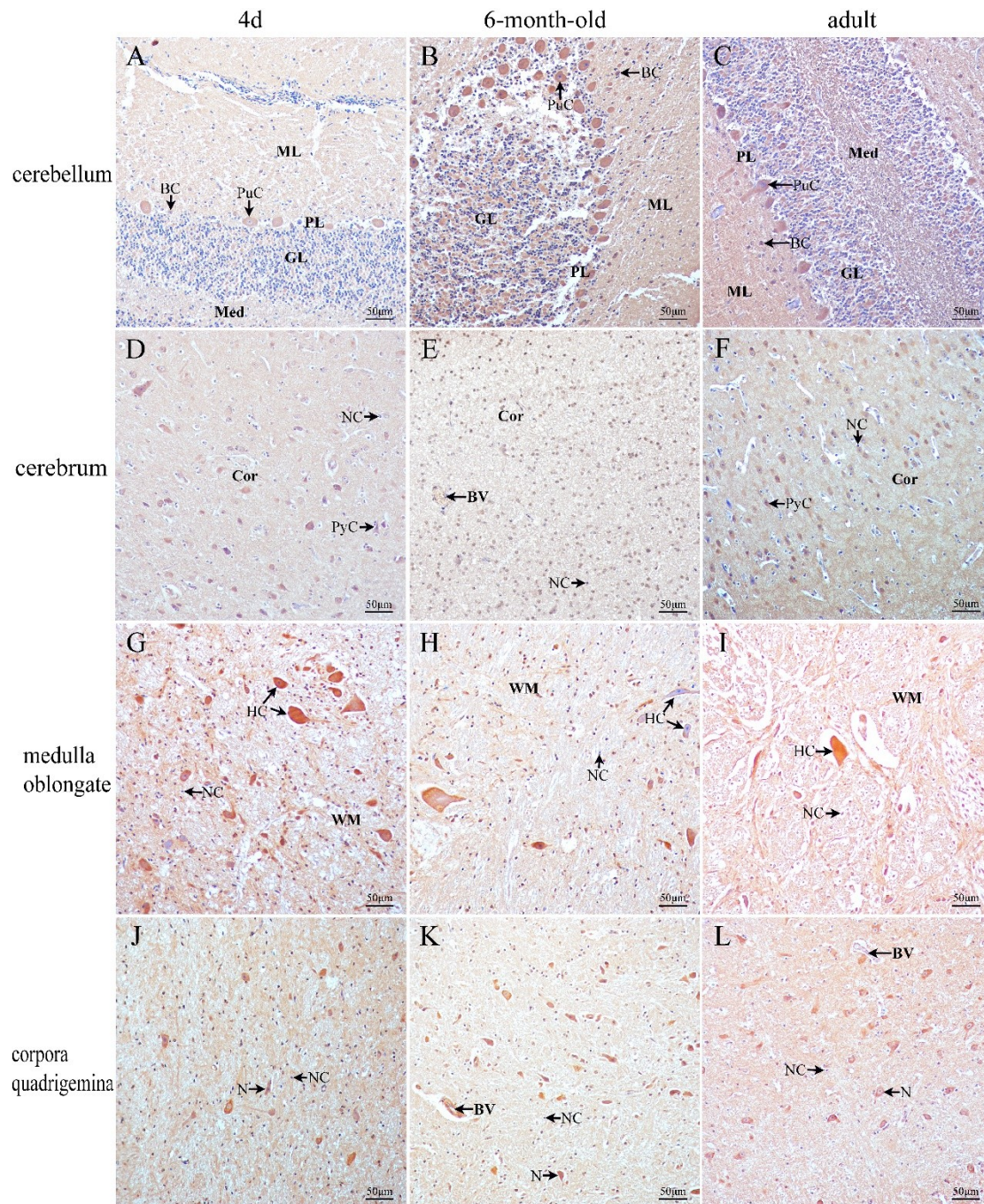


**Figure 2.** Distribution and expression of EPO in brain tissue; A-L were 4d, 6-months-old and adult cerebellum, cerebrum, medulla oblongata, and tetrad respectively. ML — molecular layer; GL — granular layer; PL — Purkinje cell layer; Med — medulla; WM — white matter; BV — blood vessel; PuC — Purkinje cell; BC — basket cell; Cor — cortex; PyC — pyramidal cell; HC — horn cell; N — neuron; NC — neuroglia cell.

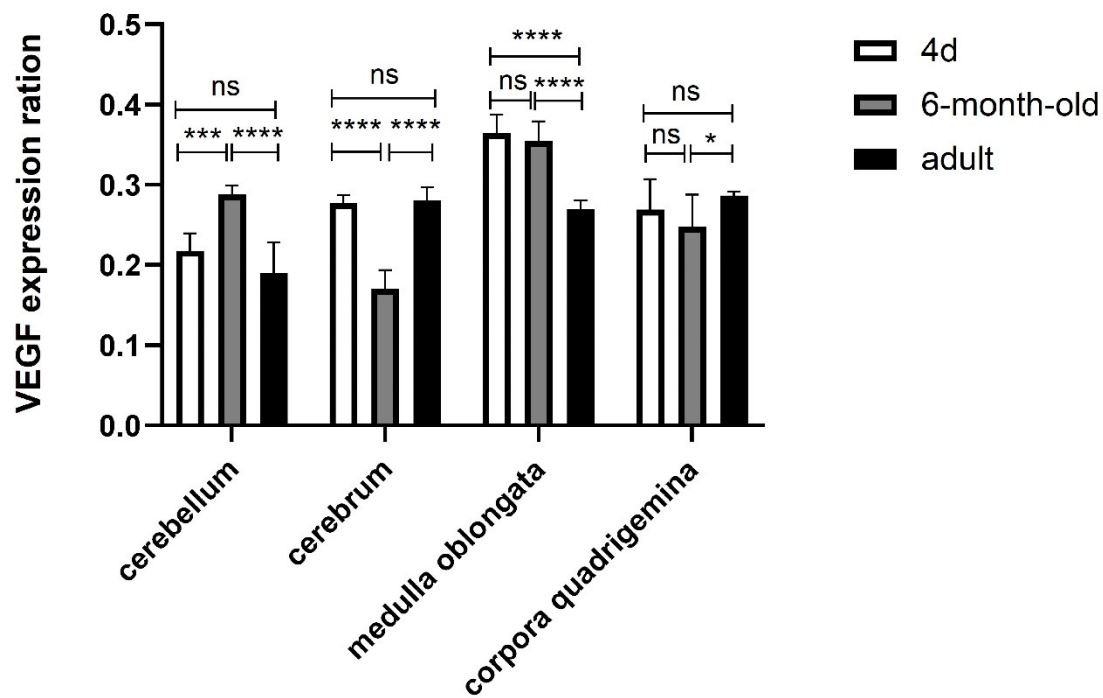


**Figure 3.** Optical density analysis of EPO in brain tissue, \*\*indicated significant difference ( $P < 0.01$ ), \*\*\*indicated extremely significant difference ( $P < 0.001$ ), ns indicated no difference ( $P > 0.05$ ).



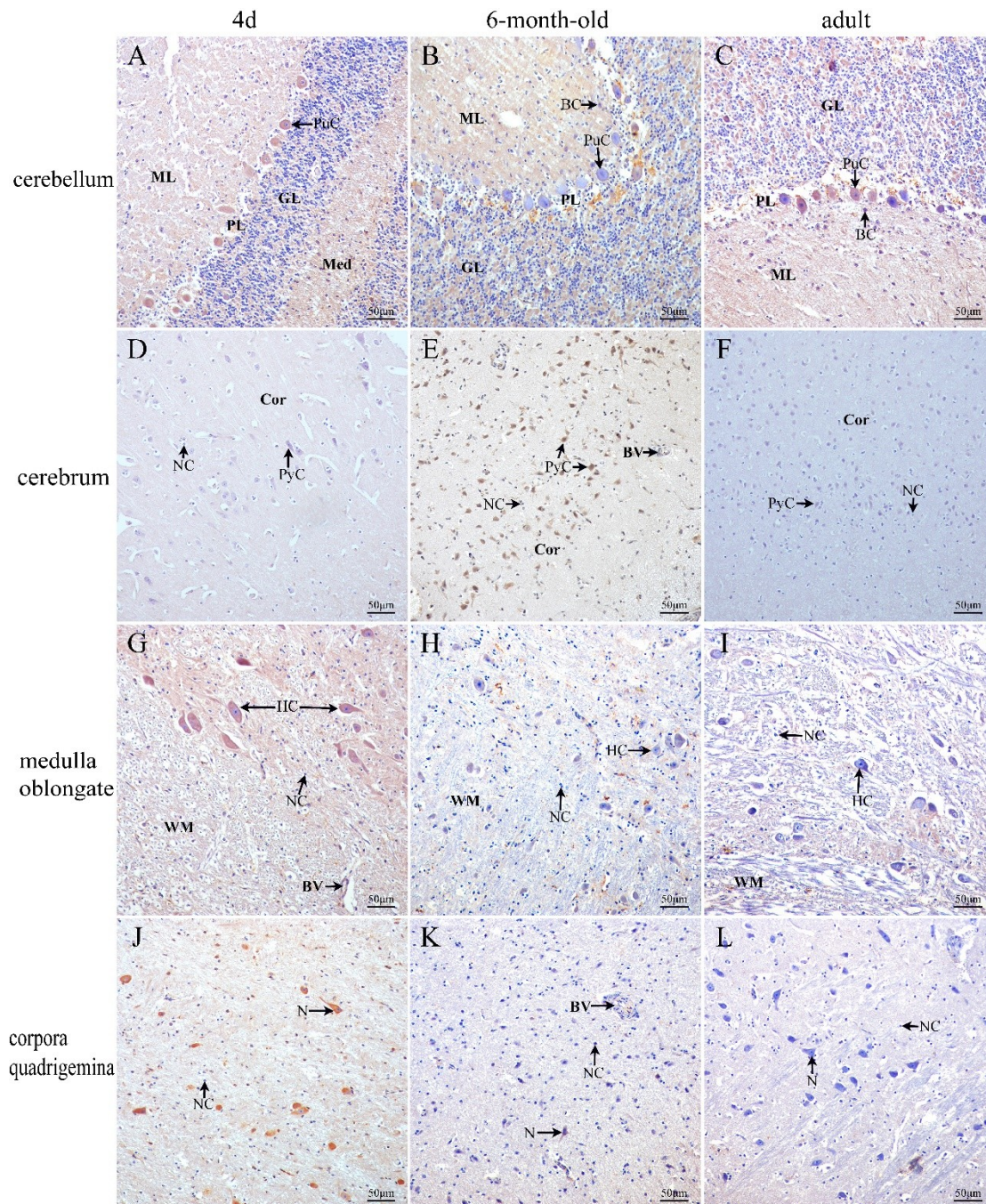


**Figure 4.** Distribution and expression of VEGF in brain tissue; A-L were 4d, 6-months-old and adult cerebellum, cerebrum, medulla oblongata, and tetrad respectively. ML — molecular layer; GL — granular layer; PL — Purkinje cell layer; Med — medulla; WM — white matter; BV — blood vessel; PuC — Purkinje cell; BC — basket cell; Cor — cortex; PyC — pyramidal cell; HC — horn cell; N — neuron; NC — neuroglia cell.

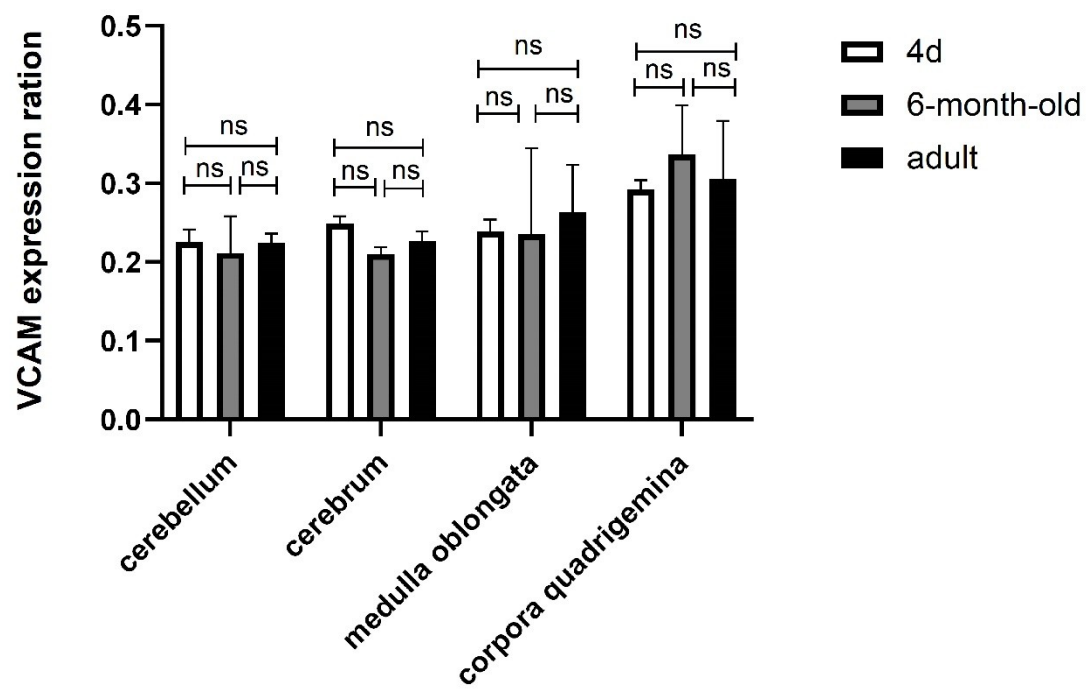


**Figure 5.** Optical density analysis of VEGF in brain tissue, \*indicated significant difference ( $P < 0.05$ ), \*\*indicated significant difference ( $P < 0.01$ ), \*\*\*indicated extremely significant difference ( $P < 0.001$ ), ns indicated no difference ( $P > 0.05$ ).



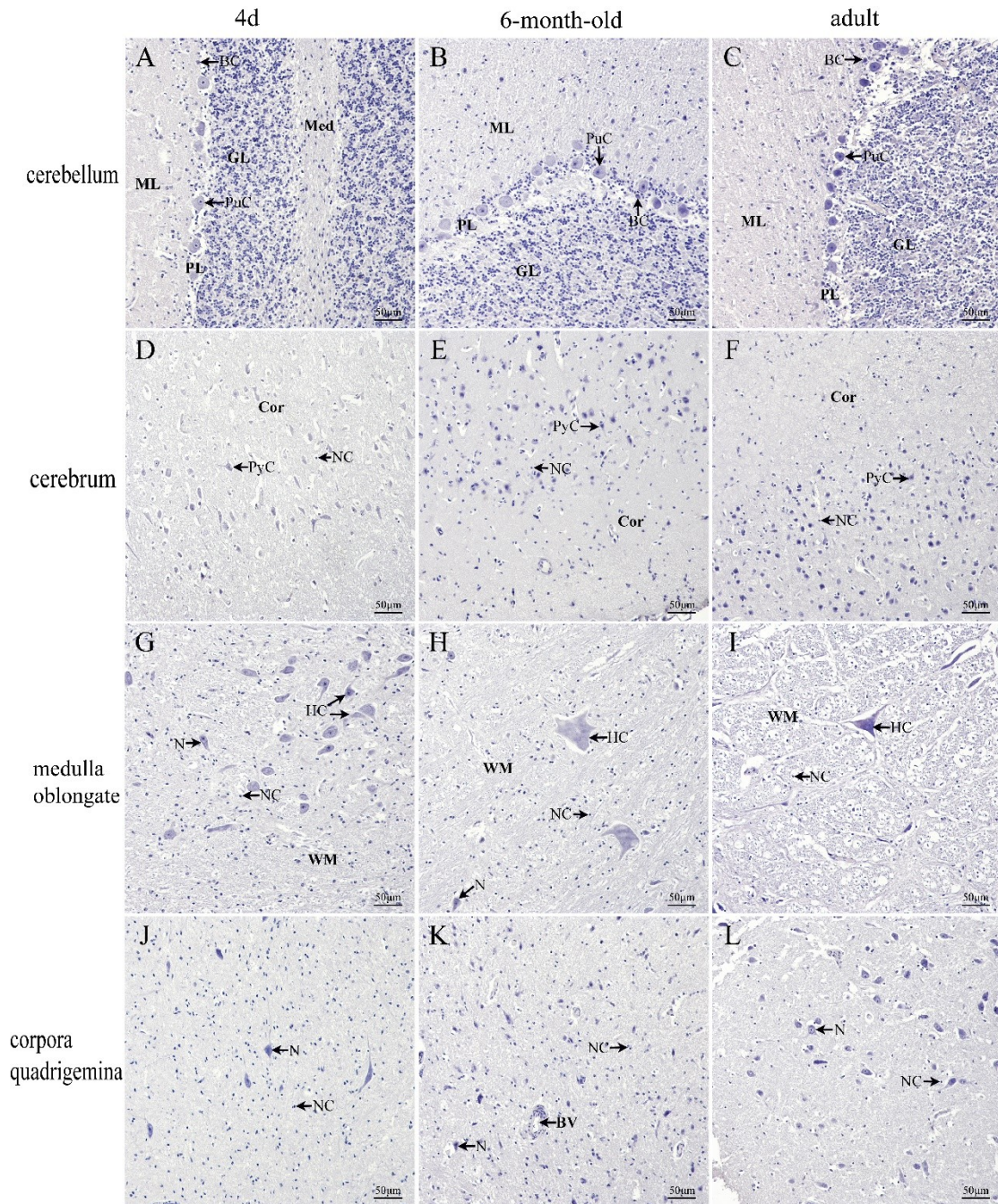


**Figure 6.** Distribution and expression of VCAM in brain tissue; A-L were 4d, 6-months-old and adult cerebellum, cerebrum, medulla oblongata, and tetrad respectively. ML — molecular layer; GL — granular layer; PL — Purkinje cell layer; Med — medulla; WM — white matter; BV — blood vessel; PuC — Purkinje cell; BC — basket cell; Cor — cortex; PyC — pyramidal cell; HC — horn cell; N — neuron; NC — neuroglia cell.



**Figure 7.** Optical density analysis of VCAM in brain tissue; ns indicated no difference ( $P > 0.05$ ).





**Figure 8.** Negative control; A-L were 4d, 6-months-old and adult cerebellum, cerebrum, medulla oblongata, and tetrad respectively. ML — molecular layer; GL — granular layer; PL — Purkinje cell layer; Med — medulla; WM — white matter; BV — blood vessel; PuC — Purkinje cell; BC — basket cell; Cor — cortex; PyC — pyramidal cell; HC — horn cell; N — neuron; NC — neuroglia cell.