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Differential expression of HIF-1α and its hypoxia-related inducers in the spleens of plateau yaks and plain yellow cattle

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Summary. The present study aims to investigate the distribution and expression characteristics of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 in the spleen of plateau yaks and plain yellow cattle and to speculate the possible regulatory role of HIF-1α and its related hypoxia-inducible factors in the adaptation of the yak spleen to the plateau hypoxic environment. Histological features were observed using H&E and PAS stains. Immunohistochemical staining and optical density analysis were applied to investigate the distribution and differences in the expression of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 in the spleen of yaks and cattle. The results showed that the area of splenic trabeculae and splenic nodules was significantly larger in the yak than in yellow cattle ($P<0.05$). Glycogen was mainly distributed in splenic arterial endothelial cells, vascular smooth muscle cells, splenic blood sinusoidal endothelial cells, and fibroblasts, and the distribution was significantly higher in the spleen of yaks than in cattle ($P<0.05$). HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 were mainly expressed in lymphocytes, arterial endothelial cells, vascular smooth muscle cells, splenic blood sinusoidal endothelial cells, and fibroblast cytoplasm, with higher expression in yak spleen ($P<0.05$). In conclusion, combining the differences in spleen tissue structure, glycogen distribution, and expression distribution of several hypoxia-related factors between yaks and cattle, we suggest that HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 may be important factors in the adaptation of yak spleen to the plateau environment, which provides a theoretical basis for further exploring the adaptation mechanism of plateau hypoxia in yaks.

Key words: Yak, Spleen, HIF-1α, VEGF, VCAM-1, IL-4

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Introduction

Hypoxia and low pressure are typical features of the plateau environment. As the altitude increases, the atmospheric pressure gradually decreases, resulting in a decrease in the atmospheric oxygen content and oxygen partial pressure. Upon entry of plain animals into the plateau, their organisms may experience an overall comprehensive reaction to the environment, such as the occurrence of upper respiratory tract infections. In contrast, yaks that have resided in the Tibetan plateau region for a long time are less sensitive to oxygen and have developed good adaptability (He et al., 2016). The correlation between hypoxia and immune response is significant and its effect on immunity and inflammation depends on the microenvironment and immune processes occurring in a particular ecological niche (Taylor and Colgan, 2017). The spleen, which is the largest peripheral immune organ, is associated with hypoxic adaptation as well as hypoxic stress (Wang et al., 2021a). When exposed to brief hypoxia induced by exercise, apnea, or simulated living at high altitudes, the spleen volume decreases due to the evacuation of stored red blood cells (Purdy et al., 1985; Sonmez et al., 2007; Wang et al., 2021a). Hypoxia, caused by the physiological environment or pathological conditions, generates a molecular response from cells in which gene expression levels are mediated by the hypoxia-inducible factor-1α (HIF-1) family of transcriptional regulators, which induce transcriptional changes in a specific set of

target genes that regulate immune cell proliferation, development, and effector functions to control innate and adaptive immunity (Balamurugan 2016; Taylor and Colgan, 2017).

HIF-1 is a transcription factor composed of two subunits: HIF-1α, which is sensitive to oxygen levels and has a short half-life, and HIF-1β, which is a nuclear translocator (Zhang et al., 2018). Under normal oxygen conditions, HIF-1α is rapidly degraded by von Hippel-Lindau tumor suppressor protein (VHL) via the ubiquitin-proteasome system (Yang et al., 2021). Under hypoxic conditions, hydroxyl reductase is inactivated, so HIF-1α cannot be hydroxylated, preventing proteasomal degradation, gradually increasing the protein content after hypoxic injury, and activating the expression of downstream factors such as erythropoietin (EPO) and glucose transporter protein-1 (GLUT1) to restore tissue homeostasis by stimulating erythropoiesis, anaerobic glycolysis, and other adaptive processes (Majmundar et al., 2010; Xie et al., 2019; Zhao et al., 2021). HIF-1α expression is associated with pro-angiogenic genes, mediates the stroma, and can mediate endothelial and vascular support cells to perform their biological functions (Pedrosa and Lemes, 2020). Vascular endothelial growth factor (VEGF), one of the downstream pro-angiogenic target genes promoted by HIF-1α activation, is widely present in various tissues and organs of the body and plays a crucial role in cellular adaptation to hypoxia (Majmundar et al., 2010). Vascular endothelial growth factor receptor-2 (VEGFR-2) is the primary mediator of VEGF-driven responses in lymphocytes. VEGF can specifically bind to VEGFR-2 to promote endothelial cell proliferation, enhance vascular permeability, and stimulate vascular neovascularization, and remodeling (Melincovici et al., 2018; Xiang et al., 2019; Wang et al., 2021b). Vascular cell adhesion mole-1 (VCAM-1) is a 90 kDa glycoprotein, a member of the immunoglobulin family, which is expressed in the endothelial cells of large and small vessels after stimulation by cytokines and mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils to the vascular endothelium (Agassandian et al., 2015). Upregulation of VCAM-1 significantly increases the expression of HIF-1 regulatory genes such as VEGF, which plays a crucial role in angiogenesis and erythropoiesis in a hypoxic environment (Nakao et al., 2003; Yamashita et al., 2008; Dong et al., 2011; Kim et al., 2017; Wu et al., 2021). VEGF specifically stimulates VCAM-1 expression through phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and activating transcription factor (ATF-2) (Fearnley et al., 2014). It has been shown that pro-inflammatory factors and some inflammatory mediators can upregulate HIF-1α protein expression in either hypoxic or normoxic conditions. Previously, Scharte et al. (2006) demonstrated that IL-4 can increase hypoxia-induced HIF-1α protein levels in human transformed intestinal cells. Meanwhile, IL-4 is a pleiotropic cytokine that promotes pulmonary angiogenesis under hypoxic conditions and induces the expression of HIF-1α, VEGF, and VCAM-1 in the lung via the p42/p44-MEK/ERK pathway (Yamaji-Kegan et al., 2009).

It is well known that yaks are typical indigenous animals adapted to plateau environments, there is currently no research available on the regulation of hypoxic factors, such as HIF-1α, in relation to the adaptation mechanisms of the yak's spleen. In this study, we conducted a comparison of the expression distribution of HIF-1α and its related hypoxic factors in splenic tissues of adult yaks and yellow cattle by H&E staining, PAS staining, immunohistochemistry, and optical density analysis. We also speculate on the functional correlation between HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 and the adaptation to the hypoxic plateau environment in yaks. The study aims to identify biological patterns in indigenous animals living in high-altitude plateaus, which can be used as a reference for human hypoxic adaptation, and to provide basic information for the prevention and treatment of plateau diseases in humans and plateau animals.

Materials and methods

Animals and tissue collection

Spleens were collected from adult yaks (n=6) in Hezuo City (Gansu Province, China, approximately 3500 m above sea level) and from adult yellow cattle (n=6) in Chifeng City (Inner Mongolia Autonomous Region, China, approximately 1000 m above sea level). All experimental animals were handled according to the Animal Ethics Procedures and Guidelines of the People's Republic of China and the study was approved by the Animal Ethics Committee of Northwest University for Nationalities. All yak and yellow cattle were considered clinically healthy based on the results of physical examination and serum biochemical tests. The animals were euthanized by administering pentobarbital sodium (200 mg/kg) via IV injection. Immediately after euthanasia, samples were collected and preserved in 4% paraformaldehyde for tissue fixation.

Reagents

In the study, anti-HIF-1α antibody (Bioss, Beijing, China; rabbit bs-20398R), anti-VEGF antibody (Bioss, Beijing, China; rabbit bs-1665R), anti-VEGFR-2 antibody (Bioss, Beijing, China; rabbit bs-0565R), anti VCAM-1 antibody (Bioss, Beijing, China; rabbit bs-0396R), anti-IL-4 antibody (Bioss, Beijing, China; rabbit bs-0581R), AEC peroxidase substrate kit (Solarbio, Beijing, China; A2010), Periodic Acid Schiff (PAS) staining kit (Solarbio, Beijing, China; G1281), and Histostatim plus kit (Bioss, Beijing, China; PV-0023) were used.

H&E staining

The fixed 1 cm^3 spleen samples of yak and yellow cattle were processed by means of routine histotechniques, embedded in paraffin, and 5-μm-thick sections were taken from paraffin blocks. Following this, the sections were stained with the standard H&E staining procedure and mounted with neutral resin.

Periodic Acid-Schiff (PAS) staining

The standard PAS staining procedure was followed. Nuclei were stained with hematoxylin, and the specimens were mounted with neutral resin.

Immunohistochemistry

Immunohistochemical staining was conducted using the Histostain™-Plus kit to analyze the expression levels of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4. Spleen tissue sections were dewaxed in xylene and subjected to gradient alcohol hydration. After being rinsed in phosphate-buffered saline buffer (PBS), the sections were subjected to antigen retrieval (15 minutes in a microwave oven) in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was inactivated with 3% hydrogen peroxide treatment for 10 min at 37°C. The sections were then incubated with anti-HIF-1α polyclonal antibody (1:200 dilution), anti-VEGF polyclonal antibody (1:200 dilution), anti-VEGFR-2 polyclonal antibody (1:200 dilution), anti-VCAM-1 polyclonal antibody (1:200 dilution), and anti-IL-4 polyclonal antibody (1:200 dilution) at 4°C overnight in a humid chamber.

Following antibody binding, the sections were stained with the AEC substrate development kit, the nuclei stained with hematoxylin, and then the specimens were sealed with a water-soluble blocker. Negative controls were created using bovine serum albumin as the primary antibody while all other steps and conditions were constant.

Statistical analysis

Images of stained tissue sections were observed and captured using a light microscope, and the results of positive HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 expression were quantified by the image analysis software Image Pro Plus 6.0. Statistical analysis was performed using GraphPad Prism 8.0, and $P<0.05$ was considered a significant difference.

Results

H&E staining

In the H&E-stained specimens (Fig. 1a-f), the spleens of adult yaks and yellow cattle were found to be well-structured and in good developmental condition, with no evidence of lesion sites. Splenic parenchyma in

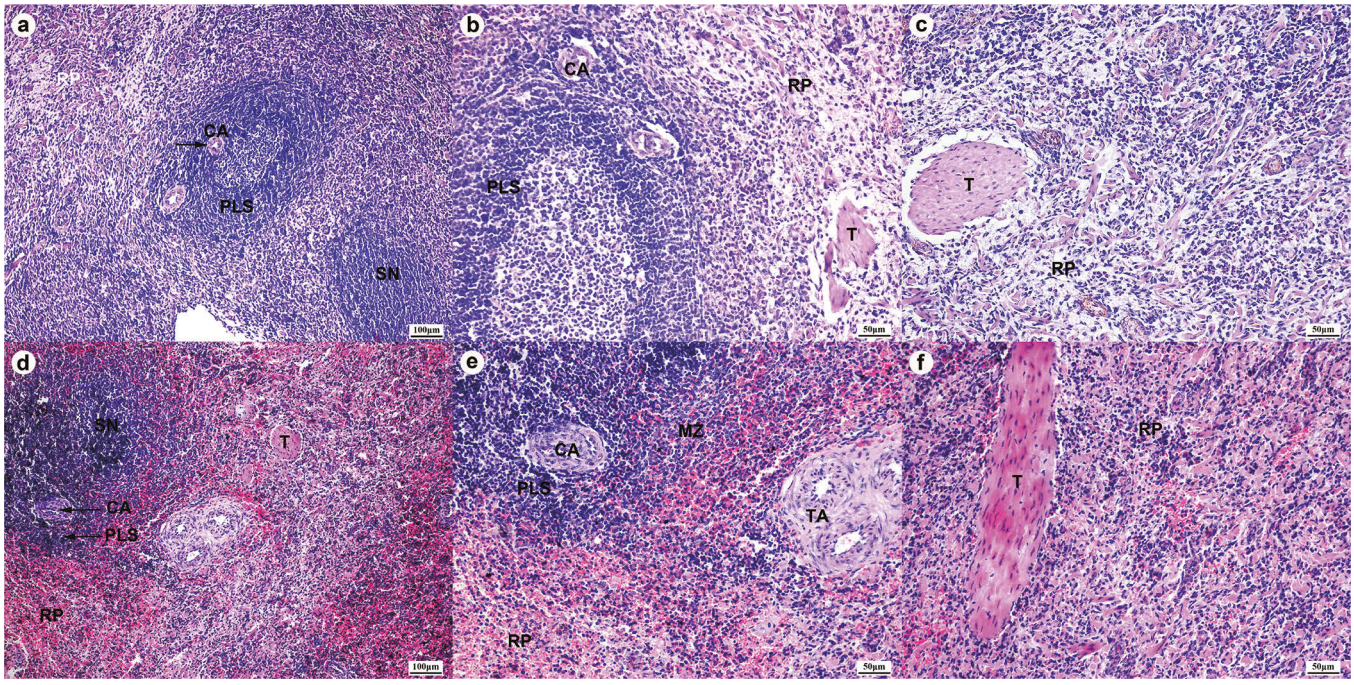

Fig. 1. H&E staining results of yak and yellow cattle spleens. **a-c.** Yak spleen tissue sections. **d-f.** Yellow cattle spleen tissue sections. RP red pulp; CA central artery; PLS periarterial lymph sheath; SN splenic nodule; T trabeculae; MZ marginal zone; TA trabecular artery. a, d, x 100; b, c, e, f, x 200.

both groups were clearly distinguishable, consisting of white pulp, marginal zone, and red pulp. The histological structure of the spleen in yak and yellow cattle was compared, finding that the yak spleen had a significantly larger (P<0.05) splenic trabeculae area than that of yellow cattle (P<0.05). Additionally, the area of white pulp in the yak spleen was significantly smaller than that of yellow cattle (P<0.05) (Table 1).

PAS staining

The results of the staining (Fig. 2 a-f) indicate that the PAS reaction was positive in the spleen cells of both adult yaks and yellow cattle. Additionally, there was significant glycogen accumulation in the endothelial cells, vascular smooth muscle cells, and outer fibroblasts of the vessel wall, as well as the splenic blood sinusoidal

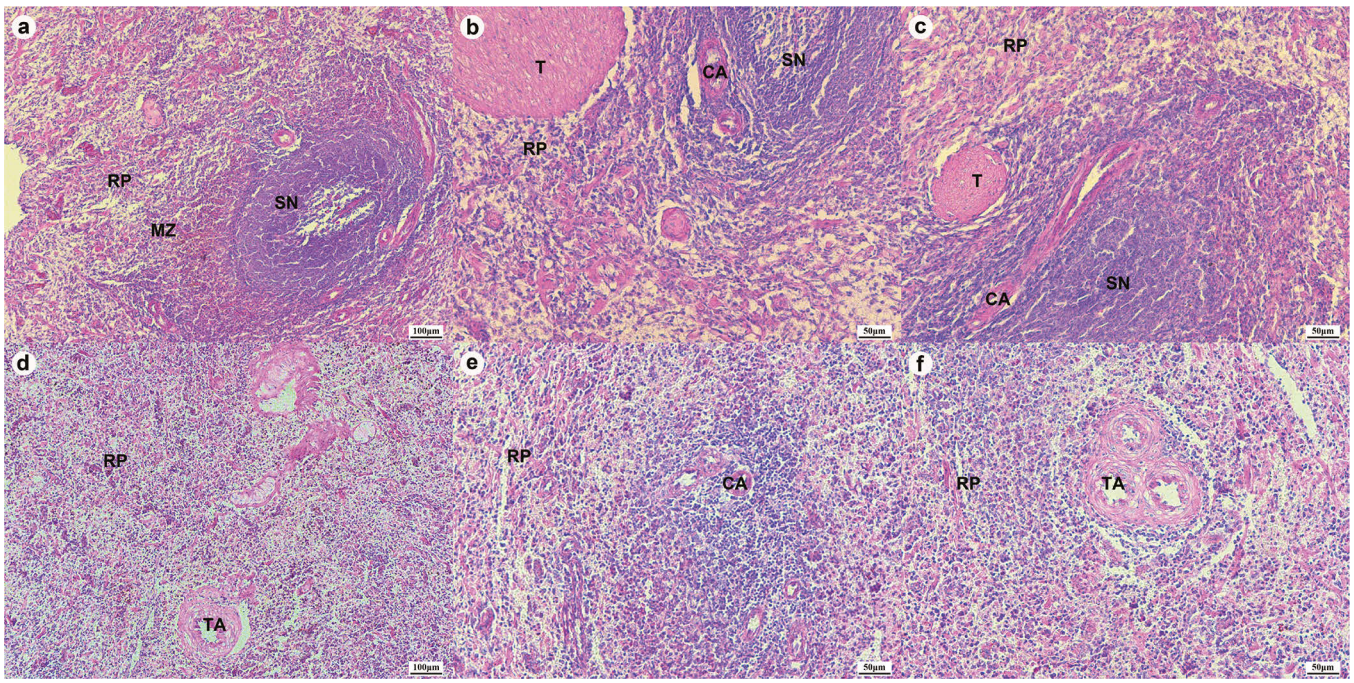

Fig. 2. PAS-stained spleen sections of yak and yellow cattle. **a-c.** Yak spleen tissue sections. **d-f.** Yellow cattle spleen tissue sections. RP red pulp; CA central artery; SN splenic nodule; T trabeculae; MZ marginal zone; TA trabecular artery. a, d, x 100; b, c, e, f, x 200.

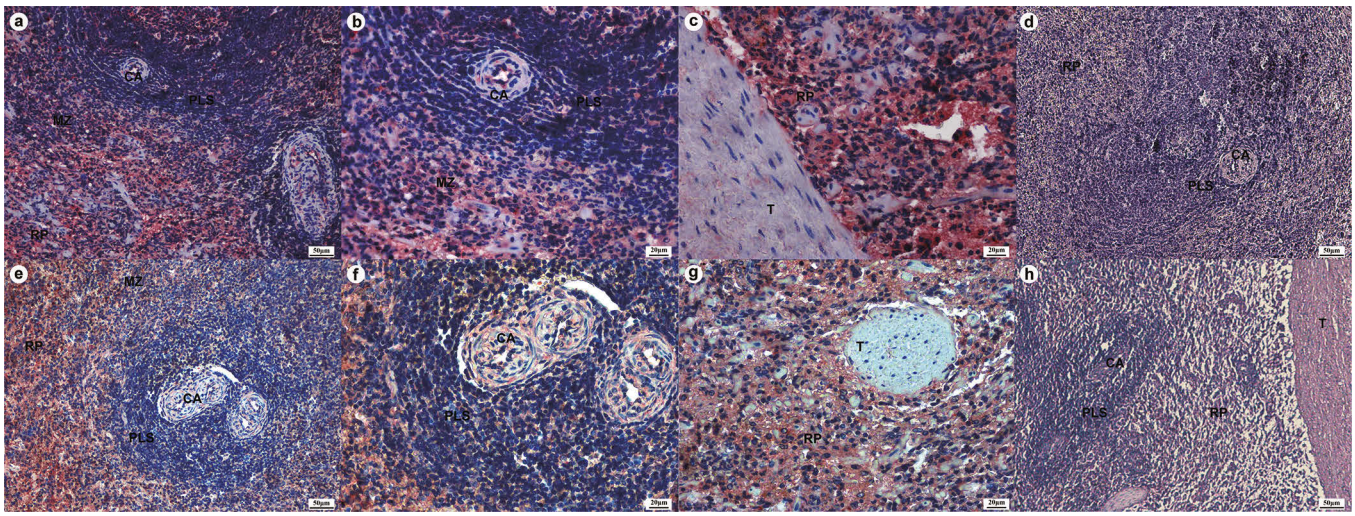

Fig. 3. Immunohistochemical staining results of the HIF-1α protein in different splenic regions of yak and yellow cattle. **a-c.** Localization of HIF-1α in splenic tissue of yak. **e-g.** Localization of HIF-1α in splenic tissue of yellow cattle. **d, h.** Respectively correspond to the negative control of yak and yellow cattle spleen tissue. RP red pulp; CA central artery; PLS periarterial lymph sheath; T trabeculae; MZ marginal zone. a, d, x 200; b, c, e, f, x 400.

Table 1. Comparative results of histological indices of spleen in yak and yellow cattle.

Histological indices of the spleen	Yak	Yellow Cattle
White pulp area (×10^6/μm^2)	0.357±0.007^a	0.457±0.011^b
Trabecular area (×10^6/μm^2)	0.031±0.001^a	0.023±0.001^b
Splenic nodule area (×10^6/μm^2)	0.206±0.002^a	0.163±0.008^b

Data are expressed as mean ± standard deviation. Different letters indicate significant differences (P<0.05).

Table 2. Proportion of glycogen distribution in splenic tissue of yak and yellow cattle.

	Yak	Yellow Cattle
Glycogen distribution proportion /%	26.343±1.482^a	15.574±2.501^b

Data are expressed as mean ± standard deviation. Different letters indicate significant differences (P<0.05).

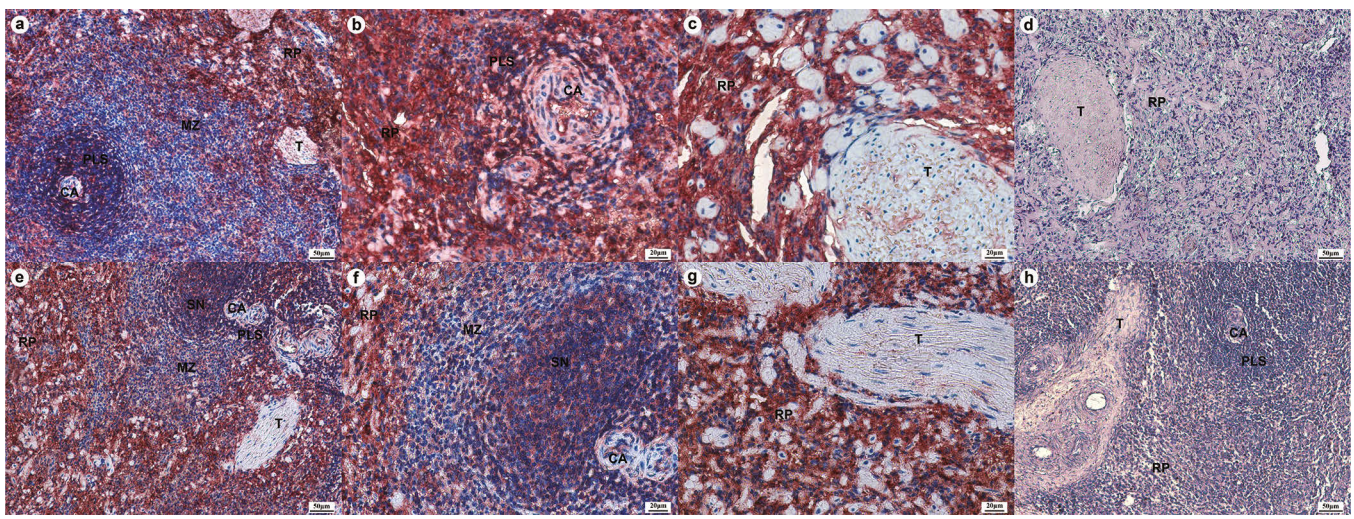

Fig. 4. Immunohistochemical staining results of the VEGF protein in different splenic regions of yak and yellow cattle. **a-c.** Localization of VEGF in splenic tissue of yak. **e-g.** Localization of VEGF in splenic tissue of yellow cattle. **d, h.** Respectively correspond to the negative control of yak and yellow cattle spleen tissue. RP red pulp; CA central artery; PLS periarterial lymph sheath; SN splenic nodule; T trabeculae; MZ marginal zone. a, d, x 200; b, c, e, f, x 400.

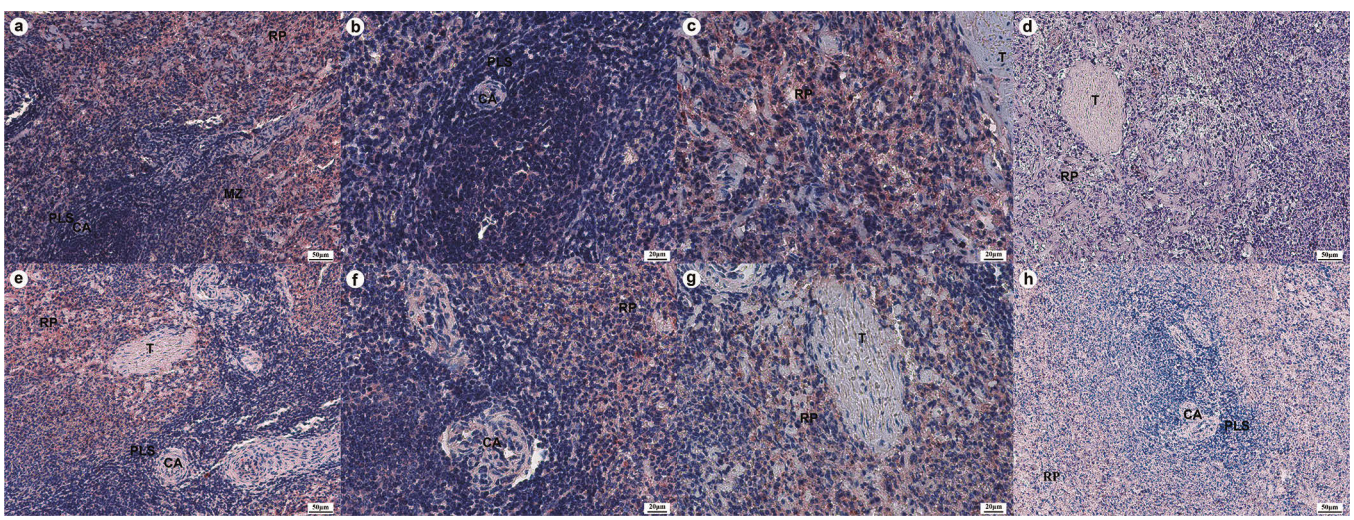

Fig. 5. Immunohistochemical staining results of the VEGF-2 protein in different splenic regions of yak and yellow cattle. **a-c.** Localization of VEGFR-2 in splenic tissue of yak. **e-g.** Localization of VEGFR-2 in splenic tissue of yellow cattle. **d, h.** Respectively correspond to the negative control of yak and yellow cattle spleen tissue. RP red pulp; CA central artery; PLS periarterial lymph sheath; T trabeculae; MZ marginal zone. a, d, x 200; b, c, e, f, x 400.

endothelial cells in the trabeculae, central, and trabecular arteries of both yak and yellow cattle, and the glycogen score was significantly higher ($P<0.05$) in yak spleen than in yellow cattle (Table 2).

Immunohistochemical staining

Immunohistochemical results showed that HIF-1α, VEGF, VEGFR-2, and IL-4 positive reactions were widely distributed in spleens, mainly condensed in the red and white pulp and trabeculae. HIF-1α, VEGF, VEGFR-2, and IL-4 showed strong positive reactions in the cytoplasm of yak lymphocytes, splenic blood sinusoidal endothelial cells, central arterial endothelial cells, vascular smooth muscle cells, and outer fibroblasts of the vessel wall (Figs. 3a-c, 4a-c, 5a-c, 7a-c). Among them, HIF-1α, VEGF, and IL-4 were highly expressed in trabeculae, while VEGFR-2 was less expressed in trabecular smooth muscle fibroblasts (Fig. 5c, f). VCAM-1 was expressed in the cytoplasm of lymphocytes, splenic blood sinusoidal endothelial cells, central arterial endothelial cells, vascular smooth muscle cells, and outer fibroblasts of the vessel wall and trabeculae (Fig. 6a-c).

In yellow cattle, HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 were strongly expressed in different splenic

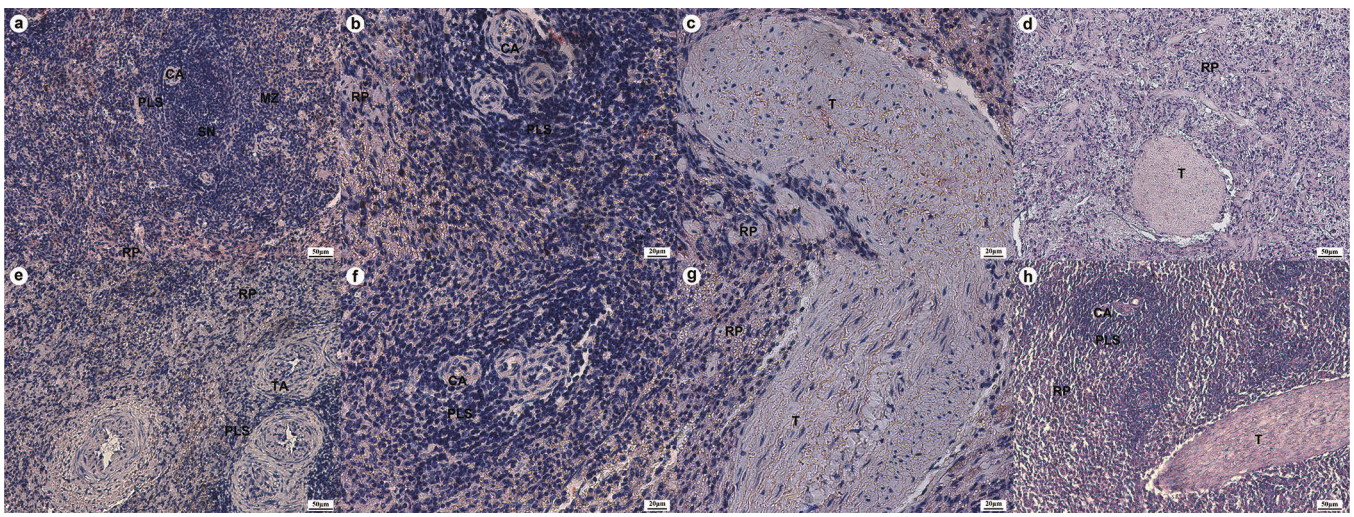

Fig. 6. Immunohistochemical staining results of the VCAM-1 protein in different splenic regions of yak and yellow cattle. **a-c.** Localization of VCAM-1 in splenic tissue of yak. **e-g.** Localization of VCAM-1 in splenic tissue of yellow cattle. **d, h.** Respectively correspond to the negative control of yak and yellow cattle spleen tissue. RP red pulp; CA central artery; PLS periarterial lymph sheath; SN splenic nodule; T trabeculae; MZ marginal zone; TA trabecular artery. a, d, x 200; b, c, e, f, x 400.

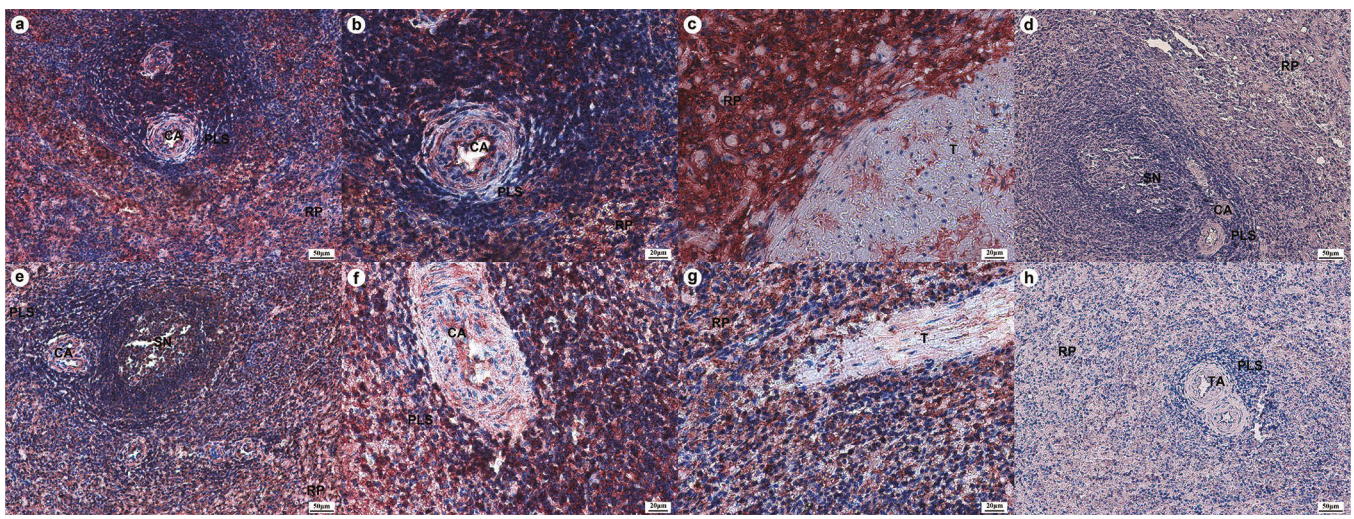

Fig. 7. Immunohistochemical staining results of the IL-4 protein in different splenic regions of yak and yellow cattle. **a-c.** Localization of IL-4 in splenic tissue of yak. **e-g.** Localization of IL-4 in splenic tissue of yellow cattle. **d, h.** Respectively correspond to the negative control of yak and yellow cattle spleen tissue. RP red pulp; CA central artery; PLS periarterial lymph sheath; SN splenic nodule; T trabeculae. a, d, x 200; b, c, e, f, x 400.

cell types. Immunohistochemical results showed different positivity degrees of HIF-1α, VEGF, VEGFR-2, and IL-4 in lymphocytes, splenic blood sinusoidal endothelial cells, central arterial endothelial cells, vascular smooth muscle cells, and outer fibroblasts of the vessel wall (Figs. 3d-e, 4d-e, 5d-e, 7d-e). In the trabeculae, VEGF and IL-4 were strongly expressed, while HIF-1α and VEGFR-2 were weakly expressed (Figs. 3f, 4f, 5f, 7f). VCAM-1 positivity is located in the cytoplasm of lymphocytes, sinusoidal endothelial cells, central arterial endothelial cells, vascular smooth muscle cells, and outer fibroblasts of the vessel wall and trabecular smooth muscle fibroblasts, however, the positive reaction was weak (Fig. 6d-f).

Results of the optical density analysis

Intensities of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 immune reactions were differentially expressed in different regions of adult yak and yellow cattle spleen. The immunostaining intensity of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 were significantly higher (P<0.05) in yak spleen than in yellow cattle spleen (Fig. 8a-j). The expression of HIF-1α and VEGF was the highest in the red pulp of both yak and yellow cattle spleens, followed by the white pulp, with the lowest expression in the trabeculae (Fig. 8b, d). The expression of HIF-1α was similar in yak red pulp as well as white pulp (P>0.05), while the expression of HIF-1α was significantly higher in yellow cattle red pulp than in white pulp and trabeculae (P<0.05) (Fig. 8b). The splenic red pulp, white pulp, and trabecular of the yak displayed fairly similar (P > 0.05) VEGFR-2 expression. The reaction score was the highest in red pulp, followed by white pulp, and the lowest in trabecular cells of yellow cattle spleen, with significant differences among the three (P<0.05) (Fig. 8f). VCAM-1 expression the highest in the red pulp of yak spleen, followed by the white pulp, and lowest in the trabeculae, with no significant differences among the three (P>0.05). The red pulp displayed the highest expression score, followed by the trabeculate, and the score was lowest in the white pulp of yellow cattle spleen, with significant differences among them (P<0.05) (Fig. 8h). IL-4 positive responses were similar in yak and yellow cattle spleen, with the highest expression in the white marrow, followed by the red marrow and the weakest in the trabeculae, with significant differences among regions (P<0.05) (Fig. 8j).

Discussion

The spleen serves as a storage site for erythrocytes and hematopoietic stem cells. When the body experiences exercise, apnea, or sudden exposure to severe hypoxia, it triggers adaptive responses to hypoxia. These responses stimulate erythropoiesis through EPO and/or BMP4-dependent pathways to restore the oxygen supply to hypoxic tissues (Alafi et al., 1956; Stutte et al., 1986; Pernett et al., 2021; Wang et al., 2021; Zhang et al., 2022). This study utilized H&E staining to observe the spleen histology of both yak and yellow cattle. The results showed that the area of the trabeculae and tubercles in yak splenic histology was larger than those of yellow cattle. This could be attributed to the fact that yaks reside in a low-oxygen environment, which promotes the development of certain organs. Additionally, glycogen plays a crucial role in supporting the daily activities of animals, and in situations of hypoxia, cells depend heavily on anaerobic glycolysis to maintain a steady ATP supply (Pescador et al., 2010). Previous studies have shown that exposure of cells to hypoxic environments leads to an increase in glycogen (Mamedova et al., 2003; Vigoda et al., 2003). This study revealed that the glycogen content in the spleen of yaks was significantly higher than in yellow cattle. This is likely due to the prolonged exposure of yaks to a hypoxic plateau environment. It is hypothesized that the increased glycogen content in the spleen provides energy for yaks to sustain their life activities in such an environment. However, further research is required to understand the exact mechanism.

HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 are transcription factors that have been identified as vital in regulating hypoxic signaling. They play a crucial role in hypoxic injury and repair and can adapt to hypoxic environments by stimulating angiogenesis, erythropoiesis, glycolysis, and iron metabolism in various cells and tissues. This accelerates oxygen transport and regulates energy requirements (Shu et al., 2019; Xie et al., 2019). In the present study, the expression and distribution of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 were determined in the spleen of yaks and yellow cattle. The findings revealed that all five factors were expressed in all tissues, and their distribution varied among different tissues.

The results of this study showing that HIF-1α is expressed at different levels in different cell types of yak spleen is consistent with the findings of previous researchers (Wang et al., 2006; Xiong et al., 2015;). In our study, yaks expressed stronger HIF-1α positivity than yellow cattle. The hypothesis is that a hypoxic environment caused by increased altitude, which results in decreased oxygen concentration, may lead to increased expression of HIF-1α in yak spleen. HIF-1α is responsible for regulating glycolysis through the PI3K/Akt pathway and upregulating EPO, which helps in maintaining cellular ATP production and splenic erythropoiesis during hypoxic conditions. This process also facilitates cell survival during hypoxia (Alafi and Cook 1956; Haase 2013; Xie et al., 2019; Kierans et al., 2021; Wang et al., 2021a). Therefore, elevated HIF-1α levels in yak spleen aid in the regulation of cellular energy demand and oxygen supply during hypoxia.

In a previous study (Youssef et al., 2019), VEGF and VEGFR-2 positivity were found to be widely distributed in the spleen, mainly in splenic blood sinusoidal endothelial cells. Our analysis showed that a variety of

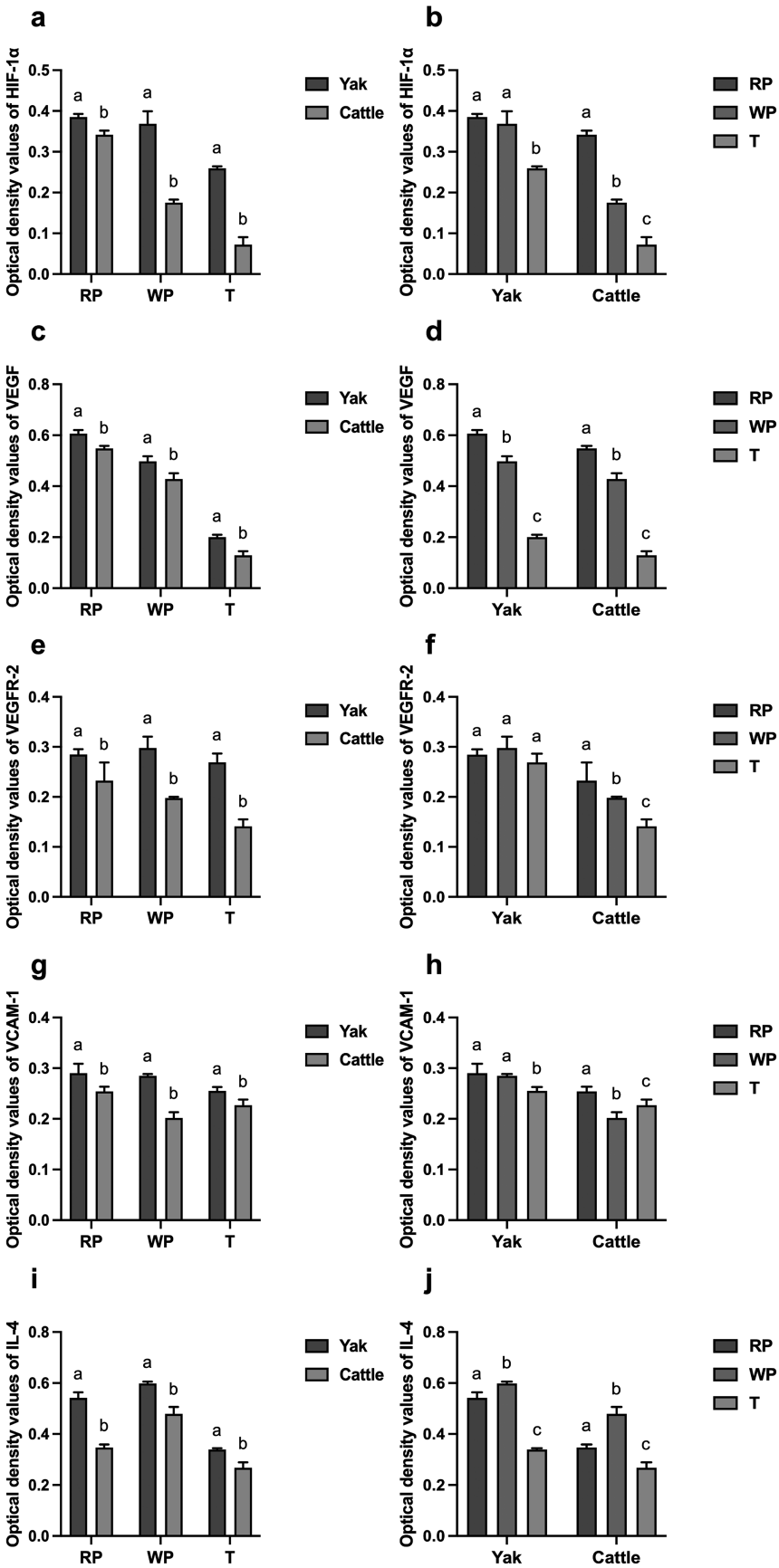

Fig. 8. Mean optical density scores of HIF-1α, VEGF, VEGFR-2, VCAM-1and IL-4 immunostaining in the spleen of yak and yellow cattle. **a, c, e, g, i.** Mean optical density values of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 in different regions of adult yak and yellow cattle spleens; **b, d, f, h, j.** Mean optical density values of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 in the same regions of adult yak and yellow cattle spleens; RP: red pulp; WP: white pulp; T: trabeculae. Data are expressed as mean ± standard deviation. Different letters indicate significant differences (P<0.05), and the letters indicate insignificant differences (P>0.05).

cell types, including lymphocytes, splenic blood sinusoidal endothelial cells, central arterial endothelial cells, vascular smooth muscle cells, outer fibroblasts of the vessel walls, and trabecular fibroblasts caused VEGF and VEGFR-2 immune reactivity. The results of the study are consistent with the results of Youssef et al. (2019). Optical density analysis revealed stronger expression levels of VEGF and VEGFR-2 in the trabeculae of yak spleens. It is hypothesized that trabeculae play an important role in hypoxic adaptation in yaks by regulating the blood content of the spleen and the blood supply to the spleen through diastole and stretch. VEGF is the most crucial angiogenic factor activated by HIF-1α. The binding of VEGF to its receptor VEGFR-2 facilitates blood flow by promoting the formation of blood vessels in the hypoxic region of the yak. This, in turn, reduces hypoxia-induced tissue damage (Lin et al., 2004; Yang et al., 2023). Research has also demonstrated that HIF-1α is co-expressed with VEGF and VEGFR-2, indicating that hypoxia stimulates high levels of HIF-1α expression. Subsequently, HIF-1α can induce the expression of VEGF and VEGFR-2, thereby promoting angiogenesis to adapt to the hypoxic environment.

VCAM-1 is an angiogenesis stimulating factor, and its expression is regulated by hypoxia. It can stimulate the expression of HIF-1 in a hypoxic environment and promote neovascularization to adapt to the hypoxic environment (Dong et al., 2011; Fukushi et al., 2000; Beck-Schimmer et al., 2001). VCAM-1 is expressed in various types of cells such as vascular smooth muscle cells, vascular endothelial cells, and human umbilical cord endothelial cells (Barks et al., 1997; Blease et al., 1998; Ishiyama et al., 1998; Lee et al., 2001; Xia et al., 2001). Our study aligns with these findings as we observed positive VCAM-1 responses mainly in vascular endothelial cells, smooth muscle cells, and lymphocytes. Hypoxia is known to stimulate the expression of VCAM-1 in vascular endothelial cells, smooth muscle cells, and lymphocytes. It also increases the expression of VEGF, which is a regulatory gene of HIF-1. Optical density analysis showed that VCAM-1 expression was higher in the spleen of yaks than in that of yellow cattle. Therefore, it is presumed that hypoxia stimulates the expression of VCAM-1 in vascular endothelial cells, smooth muscle cells, and lymphocytes, secretes soluble VCAM-1, and increases the expression of VEGF, a regulatory gene of HIF-1, to promote cell adhesion and differentiation and angiogenesis, which play a facilitating role in the adaptation of yak spleen to hypoxia.

IL-4, a pleiotropic cytokine produced mainly by activated T lymphocytes, mast cells, and basophils, exhibits angiogenic activity under *in vivo* and *in vitro* conditions (Fukushi et al., 2000; Kelly-Welch et al., 2003). Immunohistochemical staining showed that IL-4 was mainly distributed in the cytoplasm of splenic lymphocytes, endothelial cells, smooth muscle cells, and fibroblasts and showed strong expression in the white marrow. The expression of IL-4 is regulated by hypoxia and it induces the expression of VEGF, VCAM-1, and HIF-1α in the lung. This response promotes angiogenesis in the hypoxic microenvironment. Therefore, it has been suggested that IL-4 is expressed in response to hypoxic damage in the yak spleen, in turn this upregulates the expression of HIF-1α, VEGF, and VCAM-1. This upregulation promotes cell proliferation, vascular remodeling, and energy supply in the spleen.

Conclusion

The study observed similar expression and distribution characteristics of HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4 in the cytoplasm of lymphocytes, central arterial endothelial cells, vascular smooth muscle cells and outer fibroblasts of the vessel wall, splenic blood sinusoidal endothelial cells and trabecular smooth muscle fibers in the spleen of yaks. It is hypothesized that the yak spleen adapts to hypoxic conditions through the regulation of pro-angiogenesis, erythropoiesis, glycolysis, and oxygen transport via HIF-1α, VEGF, VEGFR-2, VCAM-1, and IL-4. However, further investigation is needed to fully understand the specific regulatory mechanisms involved.

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