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The Role of MMP-9 in Regulating Degradation of the Splenic Microvascular Basement Membrane Induced by High Altitude Hypoxia

Rol de la MMP-9 en la Regulación de la Degradación de la Membrana Basal Microvascular Esplénica Inducida por Hipoxia de Altura

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SUMMARY: To investigate changes of MMP-9 in the rat spleen and hypoxia-induced microvascular basement membrane under high altitude hypoxia. Thirty male specific pathogen-free Sprague Dawley rats were randomly divided into control and hypoxia groups, with 15 rats in each group. The rats in the control group were placed in Dingxi City, Gansu Province (2080 m above sea level) for 30 days. Rats in the hypoxia group were raised in a hypoxic environment in Maduo County, Qinghai Province (4300 m above sea level), for 30 days to establish a hypoxic rat model. Routine blood tests, MMP-9 mRNA, MMP-9 protein, and the spleen microvascular basement membrane were detected. (1) Compared with the control group, the red blood cell count, hemoglobin, and hematocrit levels of the rats in the hypoxia group were all increased; thus, a hypoxia model was successfully established. (2) Compared with the control group, the expression of MMP-9 mRNA and protein was significantly higher in the spleen of rats in the hypoxic group, and the difference was statistically significant ($P < 0.05$). (3) Compared with the control group, the blood vessel basement membrane in the spleen of the hypoxia group was degraded. Under natural low air pressure and high altitude conditions, the expression of MMP-9 in rat spleen tissue increases and participates in the degradation of the microvascular basement membrane.

KEY WORDS: Hypoxia; Spleen; Basement membrane; MMP-9.

INTRODUCTION

Low air pressure, high temperature, strong radiation, and low oxygen content are all characteristics of the Qinghai-Tibet Plateau. Most living organisms on Earth depend on oxygen for biological activities, and the greatest impact on an organism occurs when oxygen concentration decreases (Zhang *et al*., 2020; Liu *et al*., 2021b). Recent studies have suggested that a sudden arrival from plain to plateau environments can lead to different degrees of acute mountain sickness symptoms and associated hypoxic injury (van Hulten *et al*., 2021; Li *et al*., 2022). Long-term exposure to low oxygen on the plateau has damaged resident health, resulting in chronic altitude sickness, exacerbating original systemic diseases, and affecting the quality of life. In a plateau environment, due to the rise of oxidizing free radicals in the body caused by low oxygen and strong radiation, the antioxidant capacity of red blood cells is weakened, active vasodilator substances are reduced, vascular contraction is strengthened, and the body is prone to vascular diseases (Pak *et al*., 2022; Shah *et al*., 2022). The body can activate target genes related to hypoxia, such as VEGF and MMP-9, causing microvascular alterations in a number of organs (Zhu *et al*., 2020). Basement membrane disintegration and microangiogenesisare caused by MMP-9 upregulation in the hypoxic milieu of tumor tissue, which encourages tumor cell invasion and distant metastasis (Najafi *et al*., 2019). However, the involvement of MMP-9 in altering tissue microvascular basement membranes under chronic hypoxic conditions remains unclear. We speculate that microvascular

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injury and its basement membrane degradation are highly likely to occur in the organism during chronic hypoxia, which has not yet been fully elucidated due to the complexity of the pathological mechanisms.

Normal physiological processes such as embryonic development, tissue remodeling, wound healing, and vascular proliferation are regulated by MMP-9, which particularly hydrolyzes type IV collagen in the extracellular matrix and vascular basement membrane (Chen *et al*., 2013; Lazaro *et al*., 2016; Rahat *et al*., 2016). Furthermore, MMP-9 is involved in developing various systemic diseases, such as cerebral infarction caused by cerebral ischemia and hypoxia and the formation of pulmonary hypertension induced by hypoxia (Yan *et al*., 2016). However, there are few studies on microvascular injury induced by chronic plateau hypoxia.

To this end, we established a plateau hypoxia rat model, explored changes in MMP-9 expression levels in spleen tissues, and observed the degradation of microvessels in pathological tissues. With these results, we were able to elucidate the mechanism of microvascular alterations in the spleen in a plateau environment and provide information to broaden potential treatment methods of plateau related diseases.

MATERIAL AND METHOD

Animals. Specific pathogen-free (SPF) male Sprague Dawley (SD) weighing 200 ±10 g were purchased from the Xi'an Jiaotong University Animal Center, China (Grant No. SCXK (Shan) 2018-005). The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Qinghai University (P-SL-202102) and was conducted in strict accordance with the recommendations of the Chinese Ministry of Health's guidelines for animal management. Throughout the trial, all rats were housed at a constant temperature of 18 ± 2 °C and relative humidity of 40–60 %. The rats received a regular pellet diet and unlimited access to water.

Reagents and instrumentation. The following regents and instruments were used: Anti-MMP-9 (antibody catalog #ab38898), RNA Extraction Kit QIAGEN miRNeasy Mini Kit (catalog #217004); Reverse Transcription Kit Takara PrimeScript RT reagent kit (catalog #RR036A), Takara TB Green Premix Ex Taq (catalog #RR820A); MMP-9 inhibitor HY135232 (MCE) MMP-9 primer and GAPDH primer (Nanjing, China); veterinary automatic hematology analyzer (Mindray Biomedical Electronics Co., Ltd. BC-5000Vet, Shenzhen, China); real-time fluorescence PCR instrument (ABI, USA); transmission electron microscope (Olympus, Germany).

Establishment of the hypoxic animal model. Thirty rats were assigned into two groups at random and kept under these conditions for 30 days: the hypoxic group was placed in Maduo County, Qinghai Province (4300 m); while the control group was placed in Dingxi City, Gansu Province (2080 m). All SD rats were housed in IVC cages with good ventilation, regular disinfection, and free access to water and food.

Collection of blood and spleen samples. All surgical procedures were performed under urethane (1.0 g/kg) and every effort was made to minimize suffering. Blood (2 mL) was taken from the abdominal aorta and tested on a veterinary automatic hematology analyzer.

Small spleen sections were promptly separated and stored in liquid nitrogen for fast storage at –80 °C for RT-PCR and western blotting. The remaining pieces were prepared for transmission electron microscopy by cutting into 2×2×2 mm^3 blocks of spleen tissue and fixing in a 2.5 % glutaraldehyde solution.

RT-PCR for MMP-9 mRNA. We weighed 50 mg of spleen tissue and ground it into a homogenate, extracted the total RNA, and determined the RNA concentration. Then we performed sequential de-genomic DNA, reverse transcription into cDNA, primer dilution, and fluorescence quantification, then protected the configured reaction tubes from light, performed on the machine, and recorded the results. The MMP-9 and GAPDH primer sequences are shown in Table I. The amplification conditions were: 95 °C initialization for 10 min; denaturation at 94 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min for a total of 40 cycles. The MMP-9 primer and GAPDH primer sequences are shown in Table I.

Table I. Primer sequences.

Name	Primer sequence	Product (bp)
MMP-9	Forward: 5'-GCATCTGTATGGTCGTGGCT-3'	112
	Reverse: 5'-TGCAGTGGGACACATAGTGG-3'	
GADPH	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3'	201
	Reverse: 5'-GAACTTGCCGTGGGTAGAGT-3'	

Western blotting for MMP-9 protein. Using 40 mg of spleen tissue, we added 400 mL protein lysis solution and homogenized the tissues. We conducted lysis at 4 °C for 30 min and centrifuged at 12000 rpm for 20 min. The supernatant was then collected, and tissue protein determination and protein sample preparation were performed. After gel preparation and sample loading, electrophoresis was started according to the "red positive and black negative" connection of positive and negative electrodes. Briefly, first with a low voltage of 80 V, when the indicator entered the top of the separation gel, the voltage was increased to 120 V. The sandwich structure was made, PVDF membrane was activated by methanol, and the sequence of "black gel and white film" was followed. The membrane was transferred in the order of "black glue and white film"; 5 % skimmed milk was closed, and the primary antibody and secondary antibody were incubated after continuous shaking for 1 h on the shaker. Finally the color development was exposed three times, and the average value was taken. We analyzed the protein electrophoresis images and recorded the expression results.

Transmission electron microscopy. Using the 2×2×2 mm^3 spleen tissue block, we conducted transmission electron microscopy using the following steps:

Fixation: 2.5 % glutaraldehyde solution was fixed for 2 d, rinsed three times with 0.1 M phosphoric acid for 20 min each time. Osmium acid (50 %) and sodium dimethylarsenate (50 %) were fixed for 2 h, then rinsed three times with 0.1 M phosphoric acid for 20 min each time. Dehydration: spleen tissues aspirated by pipette were placed in 30 %, 50 %, and 70 % ethanol for 10 min each, stained with sodium acetate overnight, and finally treated with 90 % ethanol and anhydrous ethanol for 10 min each; this was repeated three times. Impregnation: Alcohol was replaced with anhydrous propylene oxide for 5–10 min, then the epoxy resin was impregnated overnight. Embedding: pure embedding solution was used to embed the spleen tissue in the embedding molds for 12 h. The temperature was set at 37 °C. Curing: tissues were placed in an oven at 60 °C for 72 h. Slicing: tissue was removed and first cut into semi-thin sections with a thickness of 2 μm, stained for 3 min, and then cut into ultrathin sections of 50 nm after microscopic observation of the vascular structure. The sections were doubly stained with sodium acetate and lead citrate, then examined and photographed using transmission electron microscopy.

Statistical methods. SPSS 28.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for analysis. Data were expressed as mean ± standard deviation ($x \pm s$), and independent sample t-tests were used for comparison between two groups ($\alpha = 0.05$).

RESULTS

Characteristics of the hypoxia rat model: Blood routine tests included white blood cells (WBC), red blood cells (RBC), hemoglobin (HB), hematocrit (HCT), and platelets (PLT). The results of the two groups are shown in Table II.

RT-PCR results. The relative expression level of MMP-9 mRNA in spleen tissue of the hypoxic group was 3.61 ± 0.39 higher than that of the control group, which was 0.94 ± 0.11. The difference was statistically significant (t = –25.615, P <0.05), as shown in Figure 1.

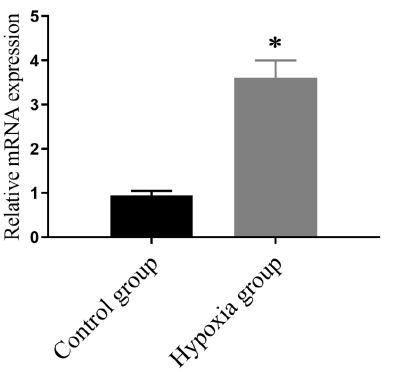

Fig. 1. MMP-9 mRNA expression level. Notes:*P <0.05 vs. control group.

Western blotting results. The relative expression level of MMP-9 protein in spleen tissue of the hypoxic group was 1.77 ± 0.39 higher than that of the control group, which was 0.65 ± 0.17. The difference was statistically significant (t = –10.244, P <0.05), as shown in Figure 2.

Table II. Blood routine results of rats ($\bar{x} \pm s$).

Group	n	WBC (×10⁹/L)	RBC (×10¹²/L)	HB (g/L)	HCT (%)	PLT(×10¹²/L)
Control group	15	6.00±1.15	6.10±0.23	162.21±17.10	42.06±5.46	832.13±43.93
Hypoxia group	15	5.35±0.76	11.56±1.51*	236.22±23.18*	68.29±7.54*	603.53±73.00*
t	-	1.847	-13.828	-9.951	-10.914	10.391
P	-	0.075	0.000	0.000	0.000	0.000

Notes:*P <0.05 vs. control group.

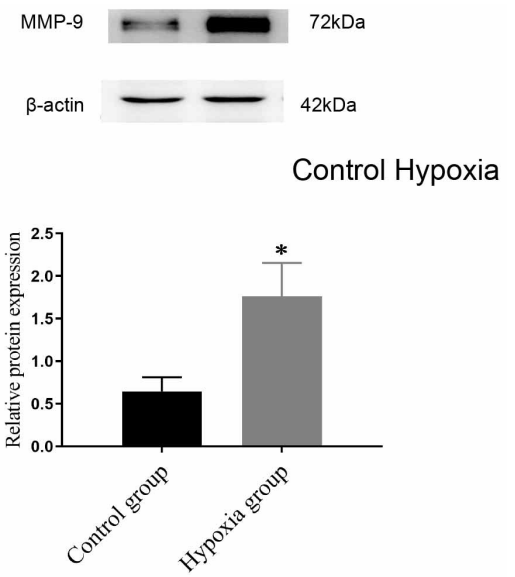

Fig. 2. MMP-9 protein expression level. *P <0.05 vs. control group.

Transmission electron microscopy. In the control group, the vascular basement membrane was thick and continuous. In the hypoxic group, the splenic vascular basement membrane was unevenly thinned, and in some places, even fractured and discontinuous. The basement membrane degradation was more pronounced in the hypoxia group, as shown in Figure 3.

DISCUSSION

The natural environment of the plateau, owing to low oxygen levels, can cause insufficient oxygen supply in blood circulation by increasing the amount of oxygen carried and the number of red blood cells. According to previous research, a large number of red blood cells crowd together, the deformation capacity is reduced, blood flow is slow, microcirculatory disorders occur, and high coagulation of blood can further aggravate the state of tissue ischemia and hypoxia (Kobayashi *et al*., 2022). Our study found an increase in erythrocyte count, hemoglobin, and hematocrit in rats under hypoxic conditions in the highlands, consistent with related studies (Su *et al*., 2015; Shandaulov *et al*., 2020).

Hypoxia tends to cause abnormal pathological processes, such as that in morphology and function of tissues, and is the main influencing factor in the occurrence of vascular diseases such as myocardial infarction, cerebral infarction, and pulmonary hypertension (Chen *et al*., 2020). Hypoxia exposure can cause various vascular pathological changes, ultimately leading to the degradation of the vascular basement membrane (Jiang *et al*., 2019). We found that MMP-9 expression was elevated in the spleen of rats in the hypoxic group compared to the control group, which is consistent with previous findings in bone marrow tissue (Zhu *et al*., 2020). Further, the degradation of the splenic vascular basement membrane increased in the hypoxic group rats, and the splenic vascular basement membrane was uneven with discontinuous thickness thinning. We analyzed the reasons for this, on the one hand, mainly because the typical feature of the plateau area is hypoxia, and in the process of chronic hypoxia, a series of pathological changes occur in multiple systems and organs of the organism (Ma *et al*., 2019; Liu *et al*., 2021a). On the other hand, type IV collagen is the main component of the vascular basement membrane, and type IV collagen is hydrolyzed by the primary biological function of MMP-9, which is also the primary mechanism of vascular basement membrane disintegration (Wu *et al*., 2022).

Oxidative stress marker levels are elevated in the highland natural environment, which contribute in different ways to the onset of the inflammatory response, and the subsequent inflammatory state can activate the regulated expression of multiple pathway-related proteins, including the MMP-9 protein (Ling *et al*., 2022; Zhou *et al*., 2022), which further modulates ischemic-hypoxic injury in the organism. In this experiment, splenic MMP-9 expression in the hypoxic group was substantially higher than that of the control group, further confirming that splenic basement membrane degradation was associated with MMP-9.

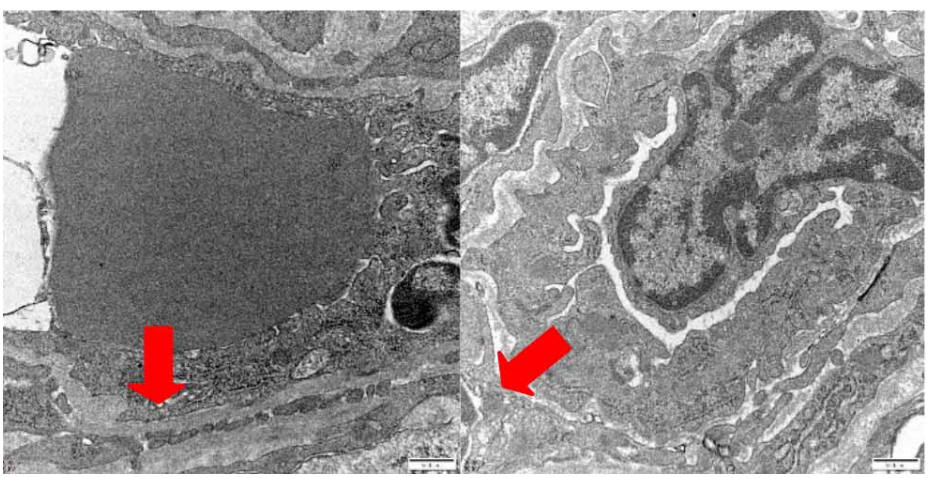

Fig. 3. Splenic microvascular basement membrane changes. Scale = 500 nm; the thickness is 50 nm; magnification (×30000).

Previous studies have indicated (Zhu *et al*., 2022) that the mechanism of its action may be that under hypoxic conditions, the body undergoes oxidative stress, which promotes the production of inflammatory factors and activates multiple pathways, such as IL-6-JAK2-STAT3-MMP-9, causing the secretion of MMP-9 by vascular endothelial cells in the body tissue, leading to microvascular proliferation (Moshapa *et al*., 2019; Zhu *et al*., 2020). Therefore, the high expression of MMP-9 in rat spleen tissue under chronic plateau hypoxia conditions may be closely related to microvascular basement membrane degradation.

In conclusion, the present study showed that hypoxia in the plateau could lead to microvascular basement membrane degradation and elevated MMP-9 expression in the spleen of rats, and the high expression of MMP-9 was involved in microvascular basement membrane degradation. The specific mechanism of hypoxia-induced MMP-9 high expression is to be further investigated.

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RESUMEN: El objetivo de este trabajo fue investigar los cambios de la MMP-9 en el bazo de la rata y la membrana basal microvascular inducida bajo hipoxia a gran altura. Treinta ratas macho Sprague Dawley, libres de patógenos específicos, se dividieron aleatoriamente en dos grupos de 15 ratas cada uno, un grupo control y un grupo hipoxia. Durante 30 días las ratas del grupo control estuvieron en la ciudad de Dingxi, provincia de Gansu (2080 m sobre el nivel del mar). Las ratas del grupo de hipoxia se criaron en un entorno hipóxico en el condado de Maduo, provincia de Qinghai (4300 m sobre el nivel del mar), durante 30 días para establecer un modelo de rata hipóxica. Se realizaron análisis de sangre de rutina, ARNm de MMP-9, proteína MMP-9 y de la membrana basal microvascular del bazo. En comparación con el grupo control, el recuento de glóbulos rojos, la hemoglobina y los niveles de hematocrito de las ratas del grupo de hipoxia aumentaron; por lo tanto, se estableció con éxito un modelo de hipoxia. En comparación con el grupo control, la expresión de ARNm y proteína de MMP-9 fue significativamente mayor en el bazo de las ratas del grupo hipóxico, siendo la diferencia estadísticamente significativa (P <0,05). En comparación con el grupo control, la membrana basal de los vasos sanguíneos estaba degradada en el bazo del grupo hipoxia. En condiciones naturales de baja presión atmosférica y gran altitud, la expresión de MMP-9 en el tejido del bazo de la rata aumenta y participa en la degradación de la membrana basal microvascular.

PALABRAS CLAVE: Hipoxia; Bazo; Membrana basal; MMP-9

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