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Role of TLR4/NF-κB pathway in the damage of acute hypobaric hypoxia to small intestinal mucosa in rats

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Abstract. The purpose of this study was to investigate the effect of acute hypobaric hypoxia (HH) exposure on small intestinal mucosa in rats and its underling mechanism. The pathological changes of rat small intestine mucosa were detected by HE staining. The expressions of TLR4 and NF-κB in the small intestine were detected by immunohistochemistry (IHC). The levels of Zonulin, TNF-α, IL-1β, and IL-6 in the serum were detected by ELISA. The proportion of natural killer (NK) cells and dendritic cell population in the small intestine was analyzed by flow cytometry. The mRNA and protein levels of TLR4, NF-κB, occludin, HIF-1α, and iNOS were detected by RT-qPCR and Western blot, respectively. Compared with the Control group, the HH groups had different degrees of injury in intestinal mucosa. Meanwhile, in the HH groups, it was also found the increased levels of Zonulin, TNF-α, IL-1β and IL-6 in the serum, the increased $CD4^+/CD8^+$T cells ratio and small intestine NK cells population, the increased mRNA and protein expression levels of small intestine TLR4, NF-κB, HIF-1α and iNOS, and the decreased mRNA and protein expression levels of occludin. Acute HH may damage the intestinal mucosa of rats by inducing TLR4/NF-κB pathway overexpression.

Key words: Acute hypobaric hypoxia — TLR4/NF-κB pathway — HIF-1α — Small intestinal mucosa — Injury

Introduction

High altitude creates a special environment because the atmospheric pressure is lower than at sea level. In North America, South America, East Africa and Asia, more than 1.4 million residents live permanently at high altitudes (> 2500 m). Exposure to high-altitude environment will give rise to severe damage to different organs and tissues, such as brain edema, pulmonary edema, multiple organ dysfunction, intestinal injury (Bailey et al. 2009; Hanaoka et al. 2009). Gastrointestinal disorders such as anorexia, upper abdominal discomfort, indigestion, nausea, and infectious diarrhea are also common among high-altitude climbers. Compared with non-high-altitude areas, residents and immigrants in high altitude areas have been reported to have a higher incidence of digestive system diseases (Recavarren-Arce et al. 2005; Zhou et al. 2009).

High-altitude environment is characterized by low oxygen partial pressure, low pressure, strong radiation, and cold, with hypobaric hypoxia (HH) as one of the most typical ones. HH environment is a challenge for people living in or visiting high-altitude areas, causing progressive hypoxemia, inflammation and increased tissue oxidative stress in the body (Strapazzon et al. 2016). Decreased arterial oxygen saturation and hypoxia in tissues or cells caused by HH will further result in damage to the normal physiological state and function of heart, liver, lung, kidney and brain and other important organs (Mühling et al. 2006), which will eventually pose functional damage to the central nervous system, circulation and respiratory system (Woods et al. 2017). Hypoxia, as a main factor causing multiple organ dysfunction syndrome at high altitude, can also lead to severe primary intestinal barrier dysfunction, promote bacterial and endotoxin translocation, and results in systemic inflammatory response (Colgan and Taylor 2010; Zhou et al. 2011). However, the pathological mechanism of intestinal functional injury engendered by high-altitude HH environment is still unclear.

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Toll like receptors (TLRs) signaling pathway has been documented to play a crucial mediating role in the pathogenesis of intestinal mucositis (Liu et al. 2017). The fundamental immune mechanism driven by TLRs signals is indispensable to protect the integrity of host intestinal barrier and maintain symbiotic composition and tolerance (Chen et al. 2018). TLRs, a family of transmembrane proteins, recognize conserved molecular sequences from microorganisms. With the binding of TLRs and their ligands, two pathways are activated. TLR1, 2, 4, 5, 6, 7, 8, 9 signals pass through MyD88 adaptor protein, while TLR3 signals pass through another MyD88-independent pathway to eventually activate nuclear factor kappa B (NF-κB). It has been reported that inhibition of TLR4/NF-κB pathway activity in rats alleviates colitis induced by trinitrobenzene sulfonic acid (Chamanara et al. 2019), and inhibits the small intestinal epithelial cell apoptosis and inflammatory response induced by lipopolysaccharide (Xie et al. 2019). NF-κB and hypoxia inducible factor 1α (HIF-1α) are thought to be major players in inflammatory and innate immune responses, with pro-inflammatory or anti-inflammatory activity in different cells (Rius et al. 2008; Scholz and Taylor 2013). HIF-1α is widely expressed in all innate and adaptive immune populations, including macrophages, neutrophils, dendritic cells, and lymphocytes. NF-κB and HIF-1α are major regulators of inflammatory gene expression and are involved in a variety of related medical pathological processes (D'Ignazio et al. 2016). According to *in vitro* and *in vivo* studies, NF-κB and HIF signaling are strongly interdependent with NF-κB in the inflammatory response. NF-κB expression promotes HIF-1α expression and is a prerequisite for HIF-1α expression (Rius et al. 2008; Taylor and Cummins 2009). In addition, many pro-inflammatory genes including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) contain functional response elements of NF-κB and HIF in their promoters, and downregulation of NF-κB pathway activity inhibits hypoxia-induced HIF-1α expression (Fitzpatrick et al. 2011). Although the TLR4/NF-κB pathway and HIF-1α play key roles in inflammation, reports on the role of TLR4/NF-κB in HH are very limited.

In this study, the rat model of HH was constructed under a simulated HH environment. Then small intestinal injury, serum and intestinal inflammatory signaling levels were detected, so as to investigate the role of TLR4/NF-κB pathway in the pathogenesis of intestinal mucosal injury induced by HH in rats.

Materials and Methods

Experimental animals and grouping

A total of 32 adult male Sprague-Dawley (SD) rats (180–220 g) were purchased from the laboratory animal center of Sun Yat-sen University. The rats were randomly divided into 4 groups of eight rats each, including normal control group (Control), HH 3-day group (HH-3d), HH 5-day group (HH-5d) and HH 7-day group (HH-7d).

Construction of the rat model of hypobaric hypoxia

Rats in the Control group were fed under normal atmospheric pressure. Rats in the HH-3d, HH-5d and HH-7d groups were exposed to the simulated altitude of 7000 m (atmospheric pressure of about 40 kPa, and oxygen concentration of 10%) in a hypobaric chamber (Shanghai Yuyan instruments Co., Ltd, China) for 3, 5 and 7 days, respectively, so as to construct the rat HH model. During animal modeling, rats in each group would be taken out of the chamber for feeding, weighing, cleaning for 30 min, and then returned to the chamber for continuous exposure to hypoxia. After exposure, rats in each group were anesthetized with 10% ethyl carbamate (1.0 g/kg) and then sacrificed. Blood and intestinal tissue samples of rats in each group were rapidly collected. Then 1.5 ml blood sample of each rat was collected and centrifuged at −4°C (2500 r/min) for 15 min, and the serum was stored in a freezer at −80°C for later use. A segment of jejunum from the same region of the small intestine (5 cm near the terminal ileum) was taken from each rat and fixed in 10% buffered formaldehyde saline for subsequent analysis. All the animal experiments in this study were approved by the Lianyungang Hospital of Traditional Chinese Medicine Ethics Committee.

Hematoxylin-eosin staining (HE)

Morphological and histological changes of small intestinal mucosa in rats were observed using HE staining. The intestinal tissue samples were fixed with 4% paraformaldehyde for histopathological examination. After dehydration with gradient ethanol, the tissues were embedded in paraffin and cut into 4-μm sections, then stained with hematoxylin and eosin.

Immunohistochemical analysis (IHC)

The expression levels of TLR4 and NF-κB in small intestine of rats were determined by IHC. Paraffin sections (4 μm) were deparaffinized by xylene, ethanol and PBS. Then the sections were immersed in a sodium citrate buffer solution at pH 6.0 and heated in a pressure cooker. After rinsed with water and Tris-buffered saline, the sections were incubated overnight at 4°C with mouse anti-human TLR4 antibody (1:500, Abcam, UK) and mouse anti-human NF-κB antibody (1:800, Abcam, UK), and then incubated with the corresponding secondary antibody at room temperature for 30 min. The sections were stained with tissue stain and the third generation immunohistochemical detection kit

(Invitrogen, USA). Finally, protein expression was observed under a fluorescence microscope.

Enzyme-linked immunosorbent assay (ELISA)

The levels of Zonulin, TNF-α, IL-1β and IL-6 in rat serum were measured by ELISA kit (Boster, China). Anti-mouse cytokine antibody was used as capture antibody, and biotin labeled anti-mouse cytokine antibody was used to detect cytokine content. Streptavidin-HRP and tetramethylbenzidine sulfonate are adopted as color development indicators. The values were read at 450 nm wavelength, and the concentration of corresponding cytokines was calculated through the standard curves.

Flow cytometry assay

The small intestine tissues of rats in each group were collected to prepare cell suspension, and then resuspended with RPMI-1640 containing 10% fetal bovine serum (FBS). Subsequently, 1×10^6 cells were incubated with APC-anti-CD4 (BD Biosciences, USA), PE-anti-CD8 (BD Biosciences, USA) at 4°C for 30 min. The negative control was stained with isotype-matched monoclonal antibodies. Next, the cells were rinsed and resuspended using phosphate buffered saline (PBS) for fluorescence activated cell sorting (FACS) analysis. Data were acquired with a FACS Calibur flow cytometer and analyzed using Cell Quest software (BD Biosciences, USA). Natural killer (NK) cells were isolated with rat NK cell isolation kit (Miltenyi, Germany) according to the manufacturer's instructions, and the relative proportion of NK cells in each group was calculated under the microscope after trypan blue staining.

RT-qPCR

The tissue was lysed with TRIzol reagent (Invitrogen, USA). Total RNA was extracted, and cDNA was obtained after reverse transcription. Using cDNA as template, GAPDH as internal reference and the instruction of RT-qPCR detection kit as standard, PCR amplification was carried out in ABI PRISM 7900 PCR detection system (Applied Biosystems, USA). RT-qPCR reaction conditions were as follows: 30 s at 95°C, 5 s at 95°C, 30 s at 60°C, 40 cycles. The relative mRNA expression of target genes was quantified using $2^{-\Delta\Delta Ct}$ method. The primer sequences for the target genes are shown in Table 1.

Western blot

Total protein was extracted from tissues using lysis buffer, and protein concentration was quantified using BCA protein detection kit (Solarbio, China). The same amount (40 μg) of protein samples were separated with 10% SDS-PAGE at a constant voltage of 180 V and current of −100 mA at room temperature. Subsequently, and then were transferred to PVDF membrane (Millipore, USA) at a constant voltage of 100 V and current of −350 mA at 4°C. PVDF membrane was blocked with 10% skim milk (PBS, PH 7.2, containing 0.1% Tween-20) for 2 h, and incubated overnight at 4°C with the following primary antibodies: rabbit anti-human TLR4 antibody (Abcam, UK), rabbit anti-human NF-κB antibody (Abcam, UK), occludin antibody (Abcam, UK), HIF-1α antibody (Abcam, UK), iNOS antibody (Abcam, UK) and β-actin antibody (Wuhan Proteintech Group, Inc., China). At room temperature, the membrane rinsed with TBST buffer 3 times for 5 min each, then incubated with corresponding secondary antibodies for 1 hour, and rinsed with TBST buffer again 5 times for 5 min each. Finally, Enhanced chemiluminescence (ECL) kit (Millipore, USA) was employed in detection of protein band signal, with β-actin protein expression as the internal reference. Protein bands were quantified by using the Image J software (Bethesda, USA).

Table 1. RT-PCR primer sequences

Gene	Primer	Sequence
TLR4	F	5'-CTGCCACCATTTACAGTTCGTC-3'
	R	5'-ATCCAGCCACTGAAGTTGTGAG-3'
NF-κB	F	5'-AGCCCTATGCCTTTTCAACAT-3'
	R	5'-CACTCCTGGGTCTGTGTTGTT-3'
occludin	F	5'-CCATCTGACTATGCGGAAAGAG-3'
	R	5'-TACCAGAGGCGGTGACTTAT-3'
HIF-1α	F	5'- AGCAATTCTCCAAGCCCTCC-3'
	R	5'-TTCATCAGTGGTGGCAGTTG-3'
iNOS	F	5'-CACAGAGGGCTCAAAGGAGG -3'
	R	5'-AAAGTGGTAGCCACATCCCG -3'
GAPDH	F	5'-ACAGCAACAGGGTGGTGGAC-3'
	R	5'-TTTGAGGGTGCAGCGAACTT-3'

Statistical analysis

Data were processed using the SPSS 22.0 software. The data were expressed as mean ± standard deviation (SD). Multiple comparisons between groups were conducted by one-way analysis of variance (ANOVA) followed by LSD *post-hoc* test. $p < 0.05$ considers significantly different.

Results

Effect of hypobaric hypoxia on morphology of rat intestinal mucosa

As shown in Figure 1, after HH exposure, the intestinal mucosa of rats in each group was damaged to different

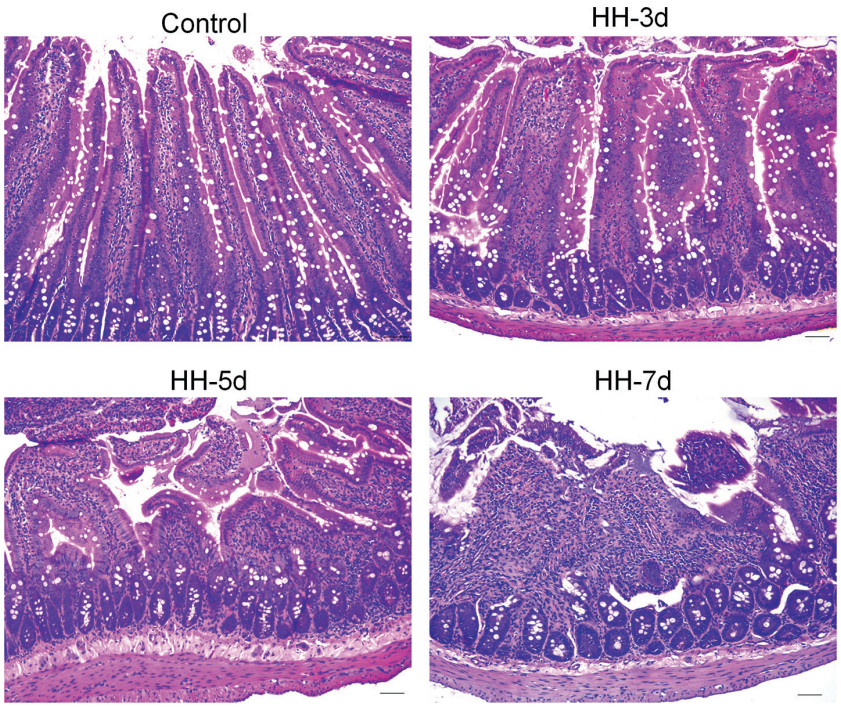

Figure 1. Morphological changes of rat small intestine. Magnification, ×100. HH, hypobaric hypoxia; HH-3d group, rats exposed to HH for 3 days; HH-5d group, rats exposed to HH for 5 days; HH-7d group, rats exposed to HH for 7 days.

degrees. In the HH-3d group, no obvious pathological changes were found in the small *intestinal villi*. However, in the HH-5 group, *intestinal villi* thickened, lodged, integrated or even fell off, and epithelial cells and goblet cells disappeared. In the HH-7 group, the function of *intestinal villi* was lost, epithelial cells and goblet cells migrated into the lumen, the *lamina propria* was exposed, and inflammatory granulocytes were observed. In the Control group of rats, intestinal villi were intact and epithelial cells and goblet cells were evenly arranged. Compared with the Control group, the longer HH exposure time, the shorter the height of *intestinal villi*, the shallower the recess depth, the thinner the mucosal wall thickness, and the smaller the *villi* surface area.

Effect of hypobaric hypoxia on TLR4 and NF-κB in rat small intestine

TLR4 and NF-κB are key factors in inflammation and immune response (Luo et al. 2005; Nikoui et al. 2015). The current study first detected the expression changes of TLR4 and NF-κB in rat small intestine. The results of Figure 2 showed that HH exposure increased the expression of TLR4 and NF-κB in rat small intestine epithelial cells in the HH-3d, HH-5d, HH-7d groups in a time-dependent manner. This result indicated that HH exposure may induce inflammatory response in rat small intestine, and the longer the HH exposure time, the stronger the inflammatory response.

Effect of hypobaric hypoxia on TNF-α, IL-1β, IL-6 and Zonulin levels in rat serum

As shown in Figure 3, in the serum of the HH-3d, HH-5d and HH-7d groups, HH exposure significantly increased the levels of inflammatory factors TNF-α, IL-1β, and IL-6, all of which were time-dependent except for IL-1β. These results indicated that HH exposure induced inflammatory response in rats. Zonulin is one of the important structures of intestinal barrier and a marker of intestinal permeability. Zonulin level in serum has been confirmed to be related to systemic inflammatory level (Qi et al. 2017). The current results showed that HH exposure significantly increased Zonulin level in serum of rats in the HH-3d, HH-5d and HH-7d groups, suggesting that HH exposure caused inflammatory response and increased intestinal permeability in rats. The result was consistent with the changes of inflammatory factor levels in serum.

Effect of hypobaric hypoxia on CD4+/CD8+T cell ratio and natural killer cells in rat small intestine

CD4+ and CD8+T cells are the central factors of the immune response and immune regulation. CD4+/CD8+ maintains a certain proportion and in a relatively balanced and stable state, forming a T lymphocyte network system through mutual induction and restriction. Imbalance of CD4+/CD8+ ratio will lead to abnormal immunoregulation function and a series of pathological changes. It has been reported that in

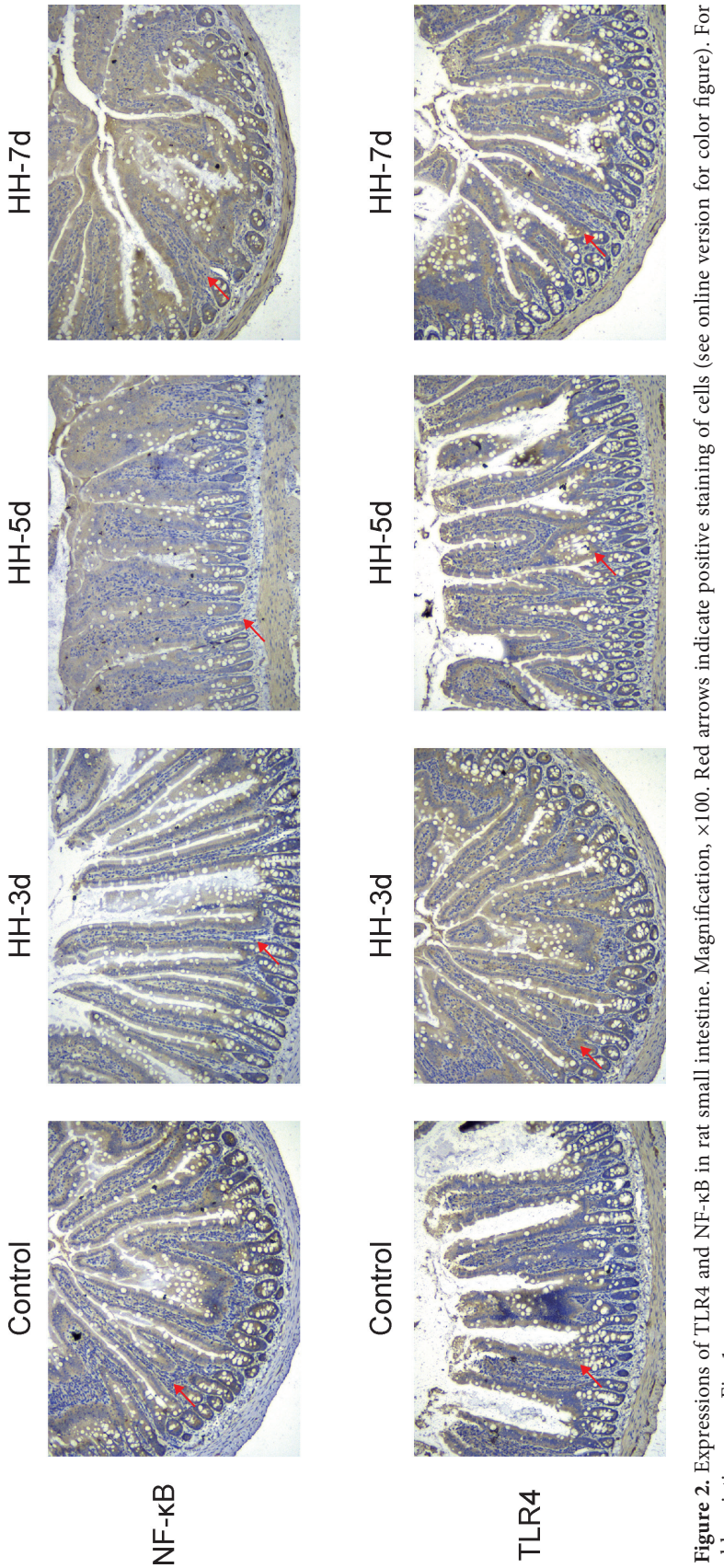

Figure 2. Expressions of TLR4 and NF-κB in rat small intestine. Magnification, ×100. Red arrows indicate positive staining of cells (see online version for color figure). For abbreviations, see Fig. 1.

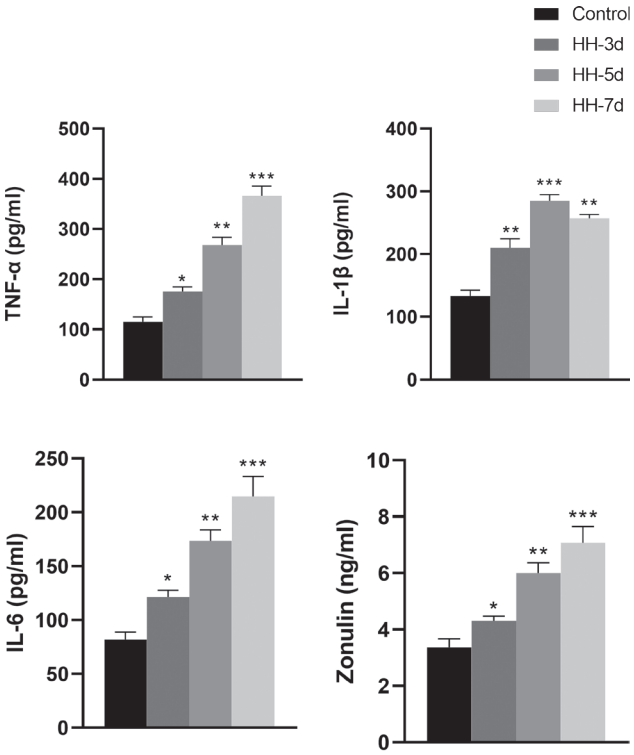

Figure 3. Expression of TNF-α, IL-1 β, IL-6, and Zonulin in rat serum. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Control. For abbreviations, see Fig. 1.

patients with hepatitis C, an increase of CD4+/CD8+ ratio is positively correlated with inflammation (Chiou et al. 2013). The current study detected the relative levels of CD4+ and CD8+T cells in rat small intestine (Fig. 4A). The results showed that HH exposure significantly increased the CD4+/CD8+ ratio in small intestine of rats in the HH-3d, HH-5d, HH-7d groups in a time-dependent manner (Fig. 4B). NK cell is a kind of lymphocyte with key function in innate immunity, which can monitor and resist tumor occurrence and virus infection, and is related to various inflammatory intestinal mucosal diseases (Wingender and Kronenberg 2008). In this study, HH exposure significantly increased the proportion of NK cells in the small intestine of rats in the HH-3d, HH-5d, HH-7d groups in a time-dependent manner (Fig. 4C). These results suggested that HH exposure induced an inflammatory response in rat small intestine.

Effect of hypobaric hypoxia on mRNA and protein expression of TLR4, NF-κB, HIF-1α, iNOS and occludin in rat small intestine

As shown in Figure 5, HH exposure significantly enhanced the mRNA (Fig. 5A) and protein (Fig. 5B) expression of TLR4, NF-κB, HIF-1α, and iNOS in the small intestine of rats in the HH-3d, HH-5d and HH-7d groups in a time-dependent manner. HIF-1α and iNOS are important regulatory proteins in intracellular hypoxia-sensing signals and participate in the regulation of inflammatory signals (Jeffrey Man et al. 2014). The current results suggested that HH exposure caused anoxic environment in intestinal cells, which activated the inflammatory signal pathway in the small intestine of rats. Meanwhile, with the increase of HH exposure time, mRNA (Fig. 5A) and protein (Fig. 5B) expression of occludin were gradually and significantly decreased. Occludin is a tight junction protein. According

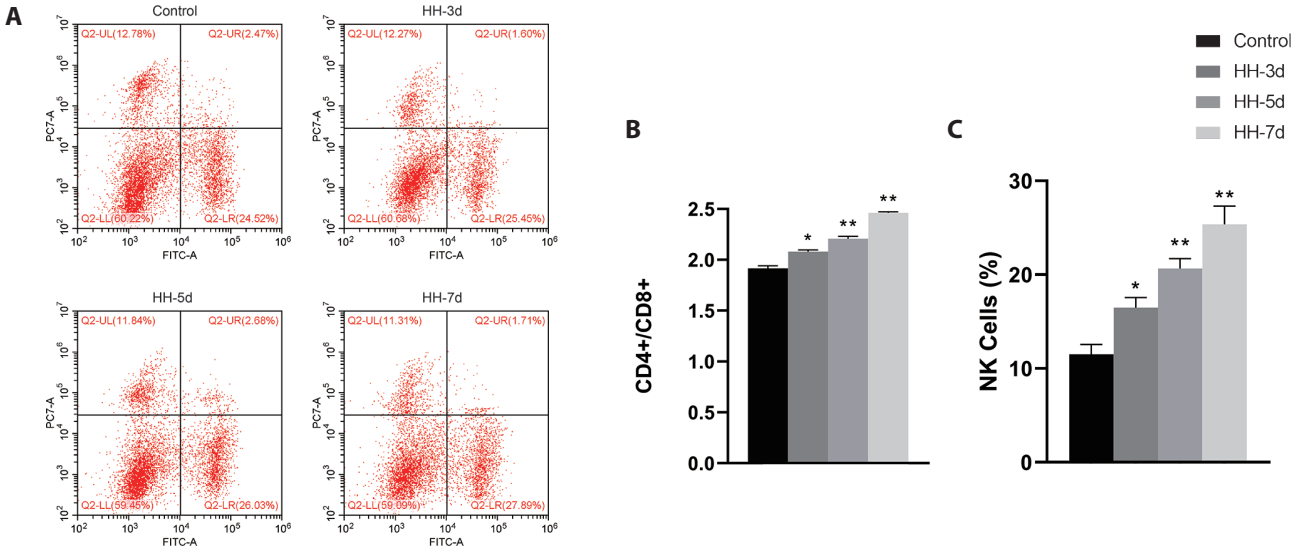

Figure 4. Ratio of $CD4^+/CD8^+$ T cells and proportion of NK cells in rat small intestine. **A.** Flow cytometry assay. **B.** $CD4^+/CD8^+$T cell ratio. **C.** Proportion of NK cells. * $p < 0.05$, ** $p < 0.01$ *vs.* Control. For abbreviations, see Fig. 1.

to researches, the expression of occludin is reduced in intestinal epithelial cells of patients with inflammatory intestinal diseases (Kuo et al. 2019). The results of this study showed that HH reduced the expression of occludin by inducing intestinal inflammation in rats, thus increasing intestinal permeability and further promoting the occurrence of intestinal inflammatory response.

Discussion

Special geological and climatic environment may result in the decline of human body resistance. For example, humans or animals exposed to high altitude are more susceptible to intestinal diseases. According to research, hypoxia environment at high altitude can induce cardiovascular system damage (Blue 2010), intestinal mucosal barrier damage and changes in microbial population (Adak et al. 2013; Zhang et al. 2015). However, the pathogenesis of intestinal injury caused by high altitude remains unclear. Therefore, it is of great practical significance to explore the pathogenesis and find effective measures to prevent and treat intestinal diseases caused by high-altitude environment exposure. In this study, a rat HH model was established by using a hypobaric chamber to simulate a plateau environment (> 7000 m). The pathological changes of small intestinal mucosa were detected 3, 5, 7 days after HH exposure, respectively. It was found that obvious pathological changes occurred in the small intestine of rats in the HH groups, and with the increase of HH exposure time, the pathological changes of small intestinal mucosa became more severe. In the meantime, HH exposure significantly induced intestinal inflammatory response, including activation of inflammatory signals and increase of inflammatory factor level. The results suggested that HH exposure may result in intestinal injury by inducing intestinal inflammation.

TLRs are key factors in inflammation and immune response (Xia et al. 2012; Nikoui et al. 2015). TLR4 has been shown to be act as a main mediator of response to lipopolysaccharide *in vivo* and *in vitro*, which can promote intestinal injury induced by dextran sodium sulfate in mice (He et al. 2016; Xiong et al. 2017). TLR4 increases the expression of pro-inflammatory cytokines by binding to endogenous ligands and activating NF-κB. NF-κB is an important transcription regulator, which plays an important role in regulating inflammation, immune response, cell proliferation, transformation, apoptosis, tumorigenesis and other cellular processes (Li and He, 2004; Vriend and Reiter 2014). Furthermore, dysregulation of NF-κB transcription activity leads to chronic inflammation and cell death (Luo et al. 2005). It has been found that by down-regulating TLR4 and NF-κB p65 expression, the excessive activation of TLR4/NF-κB signaling pathway in mouse colon induced by dex-

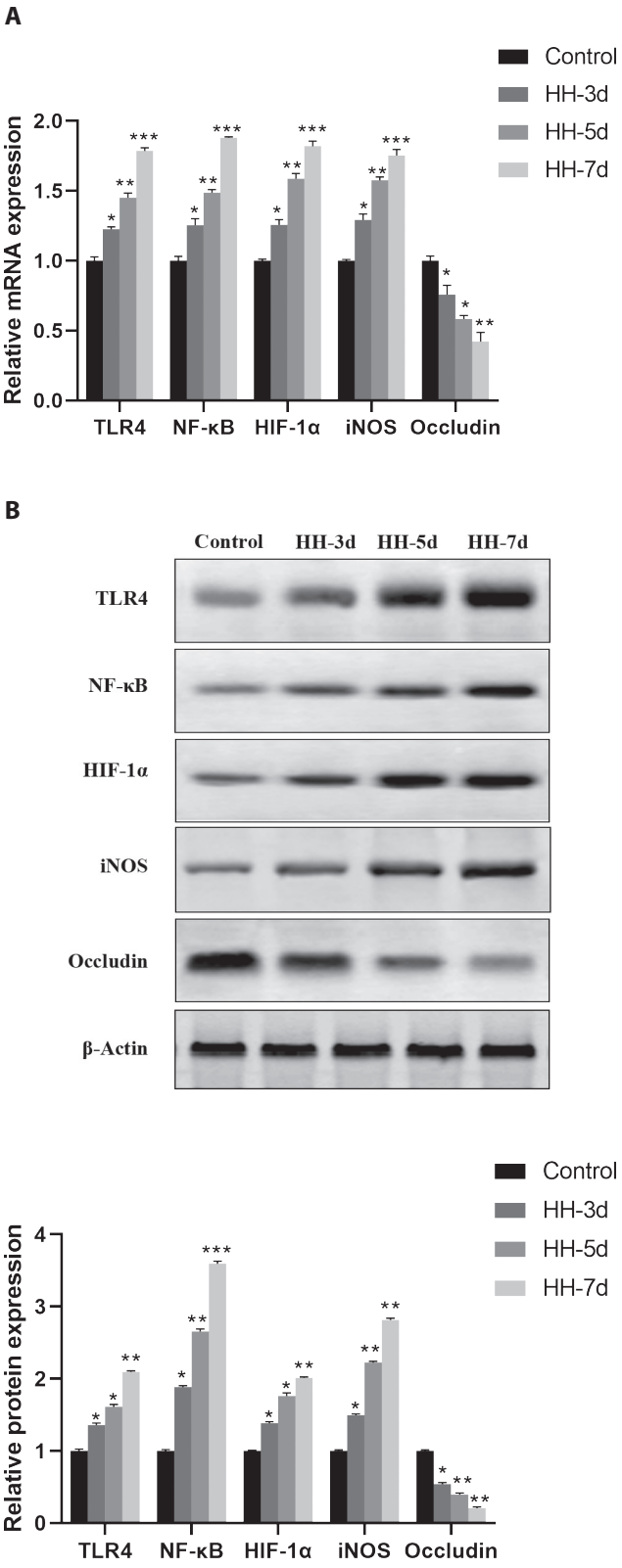

Figure 5. mRNA (**A**) and protein (**B**) expression of TLR4, NF-κB, HIF-1α, iNOS, and occludin in rat small intestine. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Control. For abbreviations, see Fig. 1.

tran sodium sulfate can be prevented, thus alleviating colon inflammatory response (Liu et al. 2017) . The above studies suggest that excessive activation of TLR4/NF-κB signaling pathway is a key factor causing intestinal inflammatory injury. Current research showed that in rats exposed to HH, TLR4 and NF-κB expression in small intestine significantly increased, and expression levels of serum inflammatory factors TNF-α, IL-1β and IL-6 all significantly enhanced, with certain time-dependent changes. The results suggested that HH exposure may induce excessive activation of TLR4/NF-κB signaling pathway in small intestine of rats, and eventually lead to inflammatory injury in small intestine. Under anoxic conditions, cells can lead to activation of HIF transcription factors (HIF-1α, HIF-2α and HIF-3α) and regulate cell survival, metabolism and tumor formation (Semenza 2001). HIF-1α, as an oxygen sensor, plays a key regulatory role in hypoxia-related diseases such as inflammation and ischemia (Brown et al. 2020). Existing research has proved that activation of HIF-1α in myeloid cells contributes to the progression of inflammatory bowel disease (Kim et al. 2018), while inhibition of HIF-1α activity is helpful to alleviate intestinal inflammation (Liu et al. 2011). In our study, HH exposure significantly increased the expression level of HIF-1α in small intestine of rats and showed a time-dependent increase. Our findings suggest that HH exposure induces hypoxia microenvironment in rat small intestine and promotes the occurrence of inflammatory response. Moreover, nitric oxide exerts an important mediating effect on the cellular perception and response to oxygen and participates in the regulation of HIF oxygen-sensing pathway (Jeffrey Man et al. 2014). At present, the expression of iNOS had been detected, and the result was consistent with the change of HIF-1α. The above results further provide evidence for the conclusion that HH exposure induces excessive activation of TLR4/NF-κB signals in rat small intestine, leading to inflammatory injury in small intestine.

Inflammatory response will lead to an increased CD4+/CD8+T cell ratio and increased intestinal permeability. In current study, HH exposure significantly increased the ratio of CD4+/CD8+T cells in rat small intestine, increased the level of Zonulin in serum, and reduced the expression level of occludin in small intestine, all in a time-dependent manner. It is suggested that HH exposure causes intestinal inflammation, and the longer the exposure time, the more severe the inflammatory injury. In addition, at the onset of inflammation, NK cells can promote the generation of subsequent adaptive immune response by regulating antigen-presenting function of dendritic cells and macrophages (Hall et al. 2013). Our study found that HH exposure significantly increased the proportion NK cells in the small intestinal of rats and showed a time-dependent increase. The above results show that HH induces intestinal inflammatory response in rats and may destroy the balance of intestinal microbial environment, resulting in adaptive response and antibacterial response to inflammation.

In conclusion, HH exposure significantly induces small intestinal mucosal injury in rats, and the degree of injury showed a time-dependent increase. Meanwhile, HH exposure significantly induces activation of inflammatory signals in rat small intestine, resulting in increased intestinal permeability. Current research results showed that HH exposure may cause intestinal mucosal injury by stimulating inflammation activation.

Conflict of interest. The authors claim that there is no conflict of interest between them.

Authors' contribution. Study concept and design: YW, LH; acquisition of data: YW; analysis and interpretation of data: LH; drafting of the manuscript: YW, LH; critical revision of the manuscript for important intellectual content: YW, LH; statistical analysis: YW, LH. All authors have read and approved the manuscript.

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