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Full Length Research Paper

Increased permeability of blood-brain barrier caused by inflammatory mediators is involved in high altitude cerebral edema

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Studies have shown that inflammatory mediators can result in increased permeability of blood-brain barrier (BBB) and even cerebral edema, but there is no report on whether high altitude hypoxia exposure can increase the permeability of BBB. In this study, the levels of inflammatory mediators, including tumor necrosis factor-alpha (TNF-α) endothelin (ET), nitric oxide (NO) superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) and glutathione-peroxidase GSH-PX in the brain, the permeability of BBB, brain water content, and histology and ultrastructure of the brain were detected. Our results showed the levels of TNFα, ET, NO, GSH and MDA, and SOD activity in the brain were increased in an altitude and exposure time dependent manner, which peaked after 9 days of exposure at 5000 m. Meanwhile, the permeability of BBB and brain water content was also elevated. Histology and ultrastructure detection showed lanthanum nitrate granules leaking from the vessels and distributing in the brain. Furthermore, the positive relationship was noted between the levels of TNFα, ET, NO, GSH and MDA, and SOD activity and brain water content. These results suggested TNFα, ET, NO, SOD, MDA and GSH played important roles in the increased permeability of BBB, which was involved in the high altitude cerebral edema.

Key words: High altitude, blood-brain barrier, permeability, inflammatory mediators, cerebral edema.

INTRODUCTION

Evidence showed the incidence of acute mountain sickness is about 68% at high altitude regions above 4000 m, and that of high altitude cerebral edema is 31% (Basnyat et al., 2000). The mortality of high altitude cerebral edema remains as high as 5%. It is a great threat to the life and health of people entering rapidly the high altitude. The pathogenesis of high altitude edema remains still unclear. Traditionally, it is believed that high altitude edema mainly refers to cytotoxic cerebral edema, in which disturbance of energy metabolism of cells plays a key role (Wang and Wang, 1994; Dragomir et al., 1978). However, the disturbance of metabolism only leads to cellular edema, and high altitude edema is mainly manifested by interstitial edema. Therefore, disturbance of energy metabolism can not satisfactorily explain the pathogenesis of high altitude edema. In recent years, increasing evidence shows that high altitude edema is a vasogenic brain edema instead of cytotoxic cerebral edema (Hackett and Roach, 2004), and increase in the permeability

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of blood-brain barrier (BBB) plays an important role in the occurrence of high altitude edema. BBB consists of cerebral capillary endothelial cell, basal lamina and foot process and is important to the stability of brain micro-environment. The permeability of BBB mainly depends upon the tight junction between endothelial cells. Tight junction is not only an intercellular barrier, but a membrane protein structure that controls the development and signal transduction of cells. Tight junction is a complex that consists of many types of transmembrane proteinse, membrane-related proteins and junction adhesion molecules, among them occludin is the most important transmembrane protein in tight junction (Gonzalez-Mariscal et al., 2003). Recent studies have found that Hypoxic and reperfusion can increase the microvascular permeability of the blood-brain barrier (BBB) by altering the occludin oligomeric assemblies of tight junctions that may lead to cerebral edema (McCaffrey et al., 2009). The study also found that hypoxia and reoxygenation also can influence the permeability of the BBB as well as the critical tight junction protein occludin as oxidative stress increases blood-brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation (Lochhead et al., 2010). Not only that, Hicks found that hypoxia-induced disruption of the blood-brain barrier may be through hypoxic stress triggers alterations to cytoskeletal structure that contribute to BBB disruption and that calcium influx through transient receptor potential C channels contributes to these events (Hicks et al., 2010). The study found that acute hypoxia and hyperthermia can induce the permeability increase of the blood-brain barrier in adult rats (Natah et al., 2009). Willis found that Hypoxia also may through the protein kinase C signaling mediates tight junctions protein disruption resulting in increased BBB permeability (Willis et al., 2010).

The aforementioned studies have shown more that hypoxia can result in increased permeability of BBB, but the exact mechanism remains still poorly understood. Therefore, we need to further explore why the permeability of BBB is increased by hypoxia exposure? What kind of substance leads to the increased permeability? What is the relationship between increased permeability of BBB and high altitude cerebral edema? Studies have shown that some inflammatory mediators played an important role in the increased permeability of blood vessels and tissue edema after trauma and infection, but there is no report on their effect on the permeability of BBB during high altitude hypoxia exposure. The present study aimed to investigate the relationship between the BBB permeability and inflammatory mediators during high altitude exposure. Our results suggested that tumor necrosis factor-alpha (TNF-α) endothelin (ET), nitric oxide (NO) superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) played important roles in the increased permeability of BBB, which was involved in the high altitude cerebral edema.

MATERIALS AND METHODS

Experimental animals and hypoxia exposure

A total of 180 Wistar rats (ratio of male to female was 1:1) weighing 180 to 230 g, were purchased from Huaxi Experimental Animal Center, Sichuan University. These rats were randomly divided into 15 groups (n =12 per group): Low altitude group (LA, 500 m), middle altitude group (MA, 2880 m), high altitude group (HA, 4200 m), and ultra-high altitude group (SHA, 5000 m). Rats in SHA groups were subdivided into 11 groups in which rats were exposed to ultra high altitude (5000 m) for different durations (1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 days). Rats were transported with a vehicle to specific site, and experiments were performed on the 1st day. Rats were given *ad libitum* access to food and water. All animal study protocols were approved by the Commission of Experimental Animal Care and Use of the university.

Detection of brain water content

Four rats of each group were anesthetized with enflurane, and the brain was obtained. The right brain was weighed and then dried in an oven at 105ºC followed by weighing dried brain. The brain water content was calculated with the Elliot formula. Water content (%) = (wet weight - dry weight)/wet weight×100%.

Detection of BBB permeability

Four rats of each group were anesthetized with enflurane, and then 1% evans blue (EB) solution was injected through external jugular vein. Thirty minutes later, 4℃ PBS was infused through left ventricle for 10 min until the fluid became clear. The perfusion pressure was maintained at 40 mmHg (1 mmHg = 0.133 kPa), and 8 ml of mixed fixation solution was injected. The brain was obtained and immediately put into liquid nitrogen. Five minutes later, the brain was cut into 4 mm coronal sections under frozen condition. Cortical brain section (500 mg) were weighed and put into 5 ml of 0.5 mol boric acid buffer (pH 5.0) for homogenation. Then centrifugation was performed at 4℃ for 5 min at 3000 rpm and 1 ml of supernatant was added into another tube, followed by supplement with 0.5 ml of anhydrous ethanol. This mixture was kept at room temperature for 15 min to precipitate proteins. Centrifugation was performed at 13000 rpm for 15 min at 4℃, and precipitation for 5 min. Then, 1 ml of supernatant was mixed with 0.5 ml of anhydrous ethanol followed by measurement of absorbance with a spectrofluorophotometer at 620 nm excitation wavelength and 680 nm scattering wavelength. Normal brain tissue was used as blank control and same procedures were performed. Finally, the permeability of BBB was determined according to absorbance, which was expressed by the EB content (μg) in per gram wet brain tissue (Deng et al., 2006).

Measurement of cytokine contents

A total of 500 mg of cortex were mixed with 4.5 ml of ice cold normal saline followed by homogenization. Centrifugation was performed at 4000 r/min for 10 min at 4℃ and 1 ml of supernatant was put into another tube, blend and kept still for 15 min at room temperature to precipitate proteins. Centrifugation was performed at 4000 r/min for

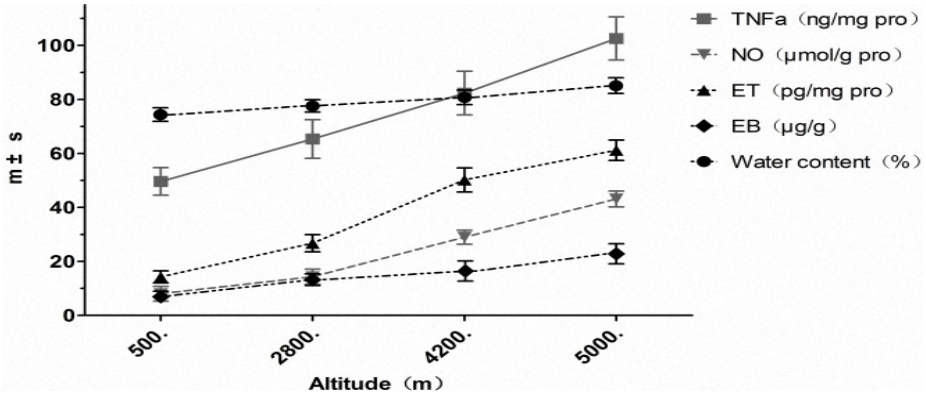


Figure 1. Inflammatory mediators and EB content in rat brain at different altitudes.

10 min at 4℃ and cytokine content was measured through radioimmunoassay. The radioimmunoassay kit was produced by East Asia Immunology Research Institute of Beijing, and cytokine content was measured with an automatic γ counter by professionals. Measurement of NO content in the brain was performed with a chemical method. Absorbance (OD) was determined with a microplate reader (MODEL 550) at 550 nm and distilled water served as blank control. The NO content of sample was calculated according to standard curve. All ODs of samples should subtract corresponding ODs of blank controls. Concentration of total proteins was measured by Doumas method, and the kit was purchased from Mike Company Sichuan. In addition, chemical method was applied to detect the activities of SOD and GSH-PX, and levels of MDA and GSH. Kits were purchased from Nanjing Jiancheng Bioengineering Institution.

Preparation for TEM

The remaining 2 rats in each group were weighed and anesthetized with an intraperitoneal injection of 10% urethane. Thoracotomy was conducted and transfusion was performed with 4℃ heparinized saline followed by 200 ml of lanthanum nitrate stationary liquid. The perfusion pressure was maintained at 120 mmHg for 20 min. Then, craniotomy was performed, and the middle cortex of left hemisphere was obtained followed by fixation in 4℃ lanthanum nitrate mixed stationary liquid (2% lanthanum, 2% glutaraldehyde, 1% paraformaldehyde, and 2.5% sucrose in 0.1 mol/L potassium arsenite buffer, pH = 7.7) (Liu et al., 1997). Then, front cortex (1 mm^3) was fixed in lanthanum nitrate mixed stationary liquid for at least 2 h. Tissues were rinsed with potassium arsenite buffer containing 0.1 mol/L lanthanum three times (3 × 10 min), and then fixed in 1% osmic acid. After washing with potassium arsenite buffer, tissues was stained with uranium and embedded with neutral resin. Then, tissues were cut into ultra-thin sections which were observed under a transmission electron microscope (Model TEM-2000Ex).

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software for Windows (SPSS Inc, Chicago, IL). Data were presented as means ± standard deviations. One-way ANOVA followed by Bonferroni test was used for multiple comparisons between means. The methods of multiple Logistic regression and linear regression were used for correlation analysis of oxygen free radicals and the brain water content. A value of $P<0.05$ was considered statistically significant.

RESULTS

Levels of inflammatory mediators and content of EB in the brain at different altitudes

The contents of TNFα, ET, NO, SOD, MDA and GSH activity in the brain at different altitudes were increased with altitude dependent manner. The higher the altitude was, the higher the contents were. The GSH-PX activity in the brain was degraded with altitude increasing (Figures 1 and 2).

Change of inflammatory mediators and evans blue content in rat brain exposure to different time at high altitude regions

Rats entered high altitude region from low altitude region, and the levels of TNFα, NO, and ET increased and peaked on day 9. The evans blue (EB) content in rat brain increased gradually with exposure time prolong and reached the peak level after 9 days of exposure at 5000 m. Brain water content of rat also increased with the prolongation of high altitude exposure, and peaked on day 9 at high altitude regions. Analysis showed brain water content was positively correlated with the levels of TNFα, NO, and ET in rat brain after different durations of high altitude exposure ($r = 0.921$, 0.897 and 0.837, respectively) (Figure 3)

The levels of oxygen free radicals in rat brain exposure to different time at high altitude regions

The SOD activity increased significantly after the rats entered high altitude area from low altitude region, and

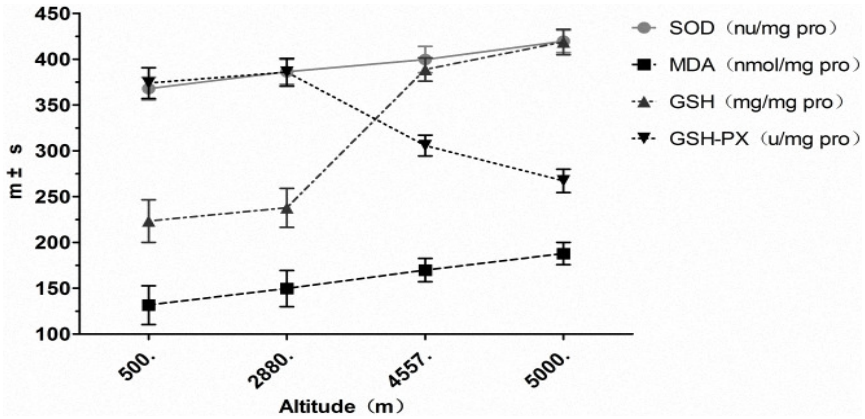
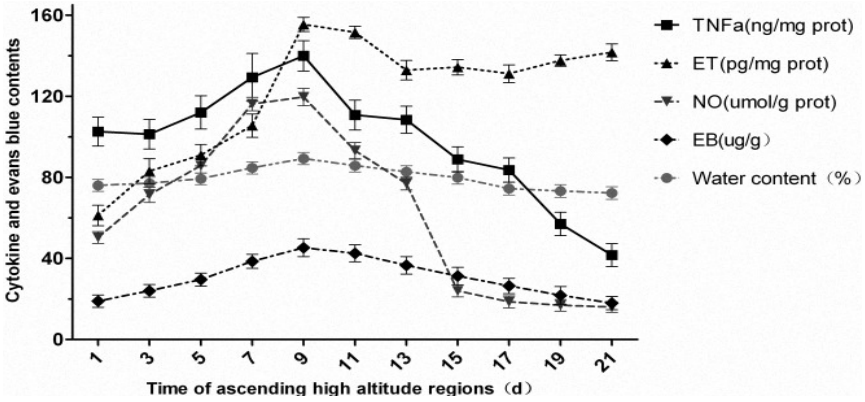


Figure 2. Oxygen free radicals in rat brain at different altitude exposure.



Figuure 3. Levels of inflammatory mediators and EB content in rat brain exposure to different time at high altitude (5000 m).

SOD activity on day 9 was remarkably different from that on day 1 ($P < 0.01$). The MDA level on day 15 was significantly different from that on day 3 ($P < 0.01$); The GSH level was increased, and its levels on day 9, 13 and 17 were higher than those on day 1, 3 and 5 ($P < 0.01$). However, the level of GSH-PX was obviously decreased and reached a minimal level on day 15, which was profoundly different from that at early days at high altitude ($P < 0.01$). Analysis showed that brain water content was positively related to SOD activity, and levels of MDA and GSH after different durations of high altitude exposure ($R = 0.781$, 0.821 and 0.878, respectively). But brain water content was negatively associated with GSH-PX level in rat brain ($R = 0.887$) (Figures 4 and 5).

Permeability of cerebral vessels and BBB after different durations of high altitude exposure

In order to evaluate the permeability of cerebral vessels, EB content in rat brain after different durations of high altitude exposure was detected. Results showed EB content increased gradually with the increase in altitude and exposure time. The EB content in rat brain reached the maximum on day 7 to 9 at an altitude of 5000 m (Figures 5 and 6).

Ultrastructure of brain tissue

At low altitude, electron microscopy showed most of lanthanum nitrate granules in cerebral vessels and less in the cortex (Figure 7A). However, a large number of lanthanum nitrate granules leaked from the vessels at high altitude, and distributed in the cortex, which was more evident at ultra high altitude group (Figure 7B). These results showed that BBB was severely damaged after high altitude exposure, and macromolecules could leak from cerebral vessels. Glial cell degeneration, astrocyte swelling and increased processes of astrocytes,

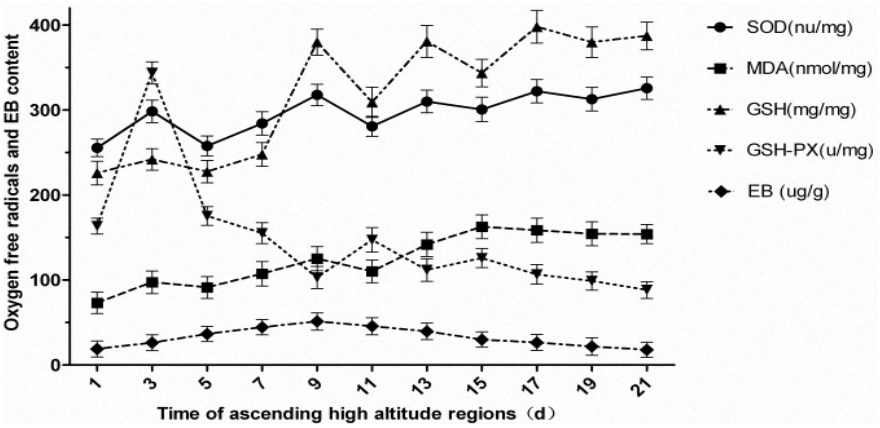


Figure 4. Oxygen free radicals and EB content in rat brain exposure to different durations at high altitude regions (5000 m).

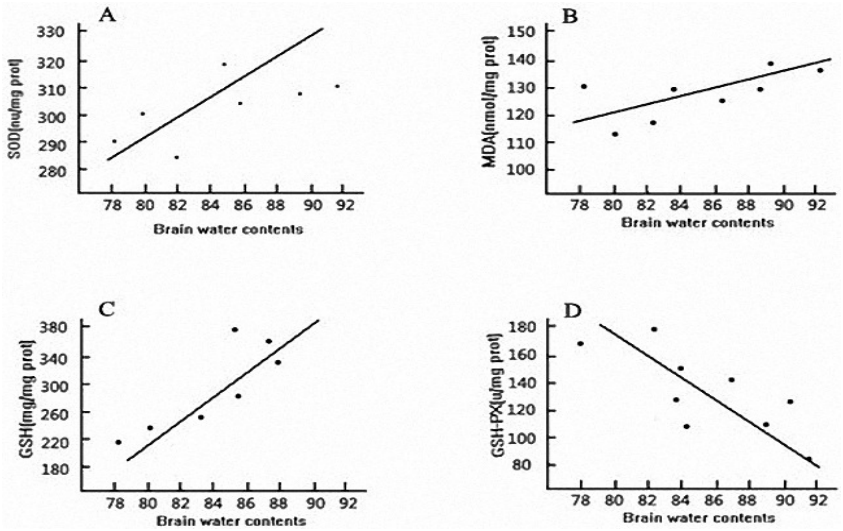


Figure 5. Relationships between oxygen free radicals and brain water content.

lysis or disappearance of neurofibrils and vacuolization or varicose-like thickening of dendrites (Figure 7C), synaptic swelling, degeneration and detachment of myelins, mitochondrial swelling, reduced ribosome, and swelling of rough endoplasmic reticulum were also observed under electron microscope (Figure 7D).

DISCUSSION

Effect of inflammatory mediators on the permeability of BBB during high altitude exposure

Inflammatory mediators are key factors in systemic inflammatory response (syndrome). They can not only induce tissue injury but also increase the permeability of blood vessels leading to vascular leakage (Koussoulas et al., 2006). But the effect of inflammatory mediators on the BBB permeability at high altitude is still unclear. In this study, we found that the contents of TNFα, NO, ET and MDA and SOD activity in rat brain increased with the increase in altitude and exposure time. These results indicated TNFα, NO, ET, SOD and MDA played important roles in the increased permeability of BBB. TNFα serves as an initiator of systemic inflammatory response (syndrome) and a mediator activating the cytokine cascade. It increases immediately after ascending to high altitude, and reaches the peak level rapidly. Once it is

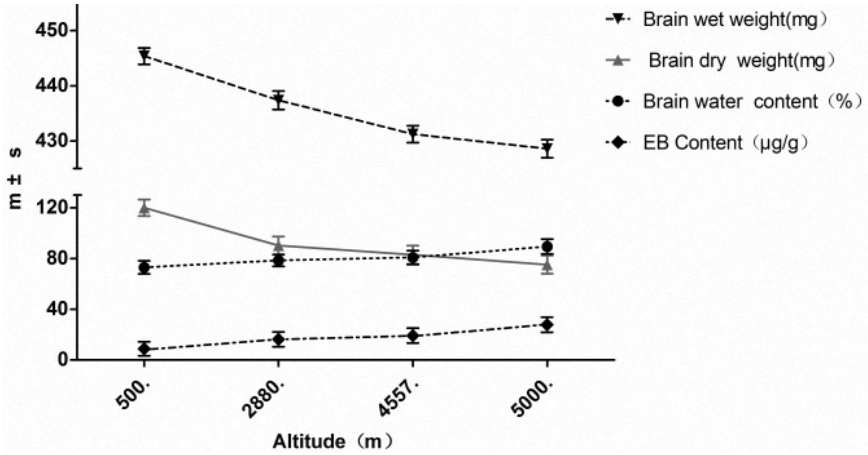


Figure 6. Brain water content and EB content at different altitude exposure.

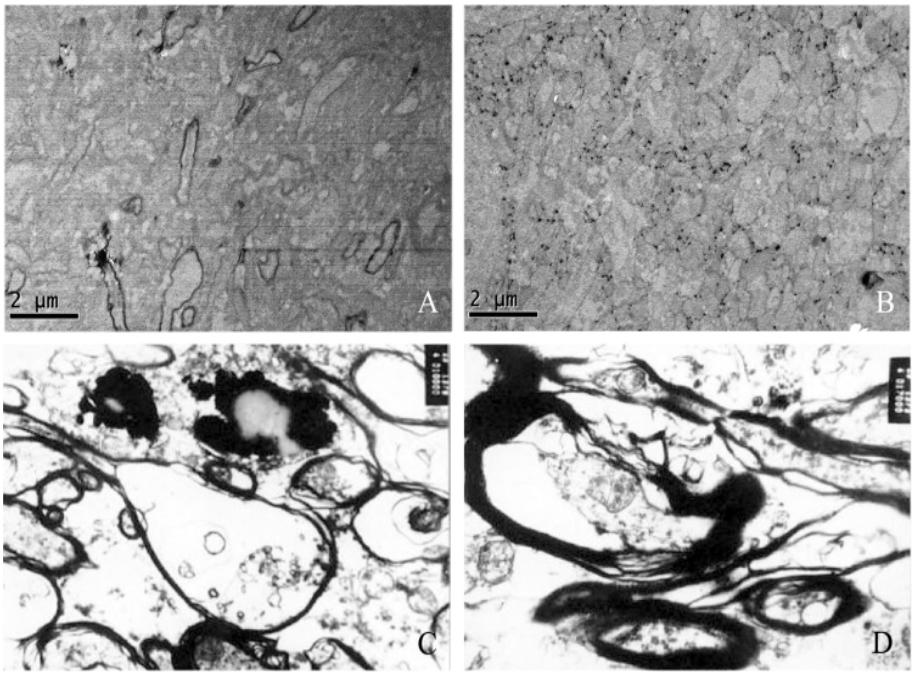


Figure 7. Ultrastructural changes of brain tissue. (A) A few lanthanum nitrate granules were observed in the cortex after low altitude exposure, and most of lanthanum nitrate granules were in micro vessels (TEM ×8900). (B) There were many lanthanum nitrate granules leaking from vessels after high altitude exposure, which were dispersed in the cortex (TEM ×8900). (C) Lysis or disappearance of neurofibrils was observed in rat brain, and the myelins became extremely thin and detached (TEM×11000). (D) Axons became atrophy and thin. Mitochondrial crista was decreased and homogeneous-like. Fusion of myelins was also found (TEM ×17000).

released, a great number of secondary factors, such as IL-1, IL-6 and IL-8, are produced. When TNFα binds to the related receptor on the endothelial cells, the structure and function of cytoskeleton proteins are altered, and endothelial cells contract and become round, followed by widening of intercellular space and subsequent increased permeability of BBB. TNFα and other factors, such as IL-6, can stimulate macrophages to produce a great amount of NO which may increase the permeability of BBB by dilating vascular smooth muscle cells and vascular

endothelial cells. ET can not only increase the contraction of cerebral vessels, but also induce the production of arachidonic acid by stimulating phospholipase A_2 in cell membrane, and enormous oxygen free radicals to destroy the integrity of biomembrane, resulting in increased permeability of BBB. It has been reported that ischemia or hypoxia induced ET-1expression can result in strong contraction of cerebral vessels and decreased expression of tight junction proteins in endothelial cells. These findings suggest that over-expression of ET can lead to brain water accumulation and cerebral edema by destroying BBB and increasing its permeability (Lo et al., 2005). In addition, experiments also reveal that IL-1 from astrocytes together with ET-1 and TNFa can affect the permeability of cerebral micro-vessels, indicating that the paracrine of ET-1, TNFα and IL-1 is also involved in destruction of BBB and inflammatory response of central nervous system (Didier et al., 2003). Hypoxia can increase not only TNFα level, but also the secretion of NO and ET. These factors interact with each other, and lead to increased permeability of BBB. Thus it can be seen that inflammatory mediators have only the function of increasing the permeability of BBB. But under hypoxic environment at high altitude, this function is significantly reinforced. Therefore, the generation of inflammatory mediators is a key contributing factor inducing increased permeability of BBB at a high altitude.

Relationship between permeability of BBB and high altitude cerebral edema

BBB is an important structure to maintain the homeostasis of central nervous system. Increased permeability can lead to sodium and water retention of brain inducing edema. Now, it has been found that increased permeability of BBB can be caused by not only severe ischemia, but severe hypoxia. Our study showed that during high altitude exposure, the permeability of BBB increased with the increment in altitude and exposure time. With the increase in the permeability of brain micro-vessels, brain water content increased profoundly. It is clear that brain water content is correlated with the permeability of BBB. With magnetic resonance imaging, Hackett et al. (1998) have proved that high altitude cerebral edema is mainly found in cerebral white matter, and edema in grey matter was extremely mild. Therefore, Hackett postulated that blood vessel related factors may play an important role in high altitude cerebral edema which does not belong to cytotoxic cerebral edema. Fischer et al. (2002) revealed that severe hypoxia could injure BBB, and resulted in increased permeability of cerebral vessels and abnormal leakage between cells. It is shown that increased permeability of BBB is important for the occurrence of high altitude cerebral edema. Nevertheless, the mechanism underlying the increased permeability of BBB under high altitude hypoxia is still unclear. Hackett and Roach (2004) postulated that factors influencing the permeability of BBB were mainly chemical mediators. Their study also proved that inflammatory mediators were a key factor inducing the changes in permeability of BBB. Apart from inflammatory mediators, VEGF has the strongest effect among these chemical mediators. VEGF is a potent leakage promoting factor. Increase in VEGF release can lead to increased permeability of BBB, and subsequent cerebral edema (Zhou et al., 2009). The mechanism of increased permeability of BBB caused by VEGF may be related to the decrease and arrangement disorder of occludins by VEGF. Kaner et al. (2004) proved that once VEGF bound to its receptor, the expression of occludins would decrease, and the permeability of capillary is increasing. Besides these, hypoxia can lead to expression, abnormal distribution, rearrangement and redistribution of occludins and actin (Brown and Davis, 2005; Kago et al., 2006), and causing expansion of intercellular space in cerebrovascular endothelial cells, and resulting in increased permeability of BBB. Additionally, extracellular matrix is also a key factor maintaining endothelial cell functions. In recent years, researchers have found that cerebral hypoxia can also activate MMPs to increase the permeability of BBB leading to liquid leakage (Lee et al., 2004). The balance of brain water is greatly dependent upon the transmembrane transport of aquaporin. The decrease in water transmembrane transport could be caused by not only cerebral ischemia, but cerebral hypoxic injure, leading to cerebral edema (Meng et al., 2004). Thus, hypoxia can not only cause abnormal expression of tight junction proteins, but also damage extracellular matrix, and disrupt normal aquaporins, leading to increased permeability of BBB and subsequent cerebral edema. Therefore, high altitude cerebral edema may result from comprehensive effects of many factors including cytotoxic cerebral edema, and vasogenic cerebral edema. But at ultra high altitude regions, the cerebral edema is mainly vasogenic cerebral edema which was also demonstrated by our study. Therefore, it is important to find strategies to prevent the increase in permeability of BBB, which may be beneficial for the prevention and therapy of high altitude cerebral edema.

Early prevention and treatment of increased permeability of BBB at high altitude

Early prevention of increased permeability of BBB at high altitude is of great importance to the prevention and treatment of high altitude cerebral edema. Considering that the permeability of BBB can be increased by inflammatory mediators, we applied granulocyte elastase inhibitor-sivelestat sodium as an intervention, and results revealed that sivelestat sodium could significantly protect BBB from

being damaged at high altitude (Ma et al., 2009) alleviating brain edema. In addition, it has been found in recent years that estrogen can not only protect neurone, but also influence water transporter across BBB, inhibit the activation of MMPs and reduce the permeability of BBB after cerebral ischemia and cerebral hypoxic injure (Liu et al., 2005). Estrogen could also through inducing the expression of AQP4 mRNA in astrocytes around blood vessels of brain parenchyma, to prevent the endotoxin induced increase in the permeability of BBB and cerebral edema. In addition, estrogen still can alleviates BBB injury caused by brain trauma, and reduces the incidence of cerebral edema through inhibiting lipid peroxidation, promoting NO generation, improving cerebral blood flow and regulating the expression of AQP (O'Connor et al., 2005). It is evident that estrogen obviously contributes to the prevention of increased permeability of BBB caused by ischemia and brain injury, as well as brain edema. Sharma et al. (2003) showed ginkgo biloba extract-ginkgocide B could exert neuroprotective effects via down-regulating cNOS, iNOS and heme oxygenase and reducing the permeability of BBB, which then alleviated brain edema and cell injury. Felinski and Antonetti, (2005) studied brain tumor induced edema and results showed that glucocorticoids (GCs) could directly affect the functions of endothelial cells, and consequently serve to regulate BBB permeability through inhibiting inflammatory response and regulating the expression, and phosphor-ylation of tight junction proteins. Recent research reveals that Chinese medicine, 'Aescin' is very effective in con-trolling inflammation, edema and oxidation, scavenging oxygen free radicals, restoring normal permeability of capillary, inhibiting inflammation, and improving circulation.

The anti-inflammation and anti-leakage activities of Aescin are several times higher than hormones (Kimura et al., 2006; Ogawa et al., 2008), and aescin is widely used in the treatment of traumatic and/or hemorrhagic brain edema. From what is previously mentioned, estrogen, extract of ginkgo bilola and aescin can confer protective effects through reducing brain vessel permea-bility caused by trauma and ischemia, and alleviating brain edema. But it still remains unclear how these substances improve the permeability of BBB during high altitude exposure, and the mechanisms underlying their effects in preventing high altitude edema are still required to be confirmed by further researches.

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