Effects of Plateau Air Quality and Low Oxygen Content on Platelet mtDNA Methylation and High Altitude Pulmonary Edema

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ABSTRACT

In 2020, the monitored AQI (Air Quality Index) in plateau—Tibet varied from 24 to 98 and averaged 48, while the PM_{2.5} concentration ranged between 3.0 and 31 $\mu\text{g m}^{-3}$ and with an average of 10 $\mu\text{g m}^{-3}$. The plateau—Tibet, had a good air quality, but with a lower atmospheric pressure and low oxygen concentration. As the important characteristic of plateau atmospheric environment, hypobaric hypoxia (HBH) is the key risk factor causing high altitude pulmonary edema (HAPE). It has been reported that the abnormal platelet function caused by HBH is an important pathogenesis of HAPE. In this preliminary study, male Sprague-Dawley rats were randomly divided into control group and HBH 24, 48, and 72-hour exposed groups. A multi-functional hypoxic chamber was used to simulate HAPE model with an altitude of 6000 m, oxygen concentration of 9.46% and partial oxygen pressure of 9.6 KPa. We measured pulmonary wet to dry (W/D) weight ratio, *MT-Cox1*, *MT-Cox2* and *MT-Cox3* methylation levels by pyrosequencing on blood samples from all rats and the levels of plasma inflammatory mediators by Luminex liquid chip technology. Results showed that the W/D ratio increased in the HBH-48h and HBH-72h exposed groups significantly ($p < 0.05$). The mtDNA methylation levels in all HBH exposed groups were lower than pre-exposure (all p value < 0.05). The levels of *MT-Cox1*, *MT-Cox2_Pos2* and *MT-Cox2_Pos3* methylation were correlated with W/D ratio significantly ($r = 0.16\text{--}0.20$, $p < 0.05$). And a strong correlation was found between some inflammatory cytokines (IL 4, IL 6, IL 10 and IL 12p70) and *MT-Cox2* methylation levels ($r = 0.09\text{--}0.30$, $p < 0.05$). In conclusion, HBH exposures can induce alterations in platelet mtDNA methylation in HAPE, which may be related to unfavorable effects on HAPE caused by the inflammation through the regulation of inflammatory response.

Keywords: Air quality, Hypobaric hypoxia, High altitude pulmonary edema, Platelet, Mitochondrial DNA methylation

1 INTRODUCTION

Many studies suggested that the characteristics of atmospheric environment are pivotal in air quality and human health. The genetic landscape of all populations inhabiting nature world is shaped by the environment. People have encountered numerous novel environments with

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various temperature and climate as human populations have expanded across the world. High altitude (HA) is one of the most challenging environments humans have inhabited (Witt and Huerta-Sanchez, 2019). It is well known that atmospheric dust aerosols could affect the climate and weather systems, air quality as well as public health (Zhang *et al.*, 2021). For example, external forces such as aerosols and greenhouse gases that change the climate on the Tibetan Plateau in response to teleconnection are elementary problems now arise (Ma *et al.*, 2020). It has been reported that the atmosphere in Lhasa, the largest city in Tibetan Plateau, had been severely influenced by local emissions due to the current rapid urbanization and industrialization (Chen *et al.*, 2018). However, the air quality was relatively better in Lhasa in comparison with other provincial capital cities in China (Yin *et al.*, 2019). In addition, the air quality standards for particulate matter (PM) in HA urban areas from different countries must take temperature and pressure into account. According to health authorities, cases of asthma and allergies have increased dramatically in proportion to population growth in HA urban areas in many countries (Bravo Alvarez *et al.*, 2013). It is necessary to understand the link between long-term air quality data with high temporal and spatial resolutions and some processes that influence air quality and correspond environmental and healthy effects (Chen *et al.*, 2019). In this context, more and more attention has been paid to the air quality in HA environments, the effects of aerosols on regional climate change as well as the effects of these changes caused by the characteristics of HA environments on human health. As the foremost characteristic of atmospheric environment in HA, hypobaric hypoxia (HBH) refers to the consequent decrease of oxygen (O_2) proportion partial pressure in the inhaled air which results from lower pressure of atmosphere (Ortiz-Prado *et al.*, 2019). It is well learned that HBH is the primary factor causing physiological responses to HA. Barometric pressure sets the partial pressure of alveolar O_2 pressure in conjunction with alveolar ventilation, thus directly determining the partial pressure of inhaled O_2 (Parati *et al.*, 2018). The tissue hypoxia and subsequent hypoxemia trigger a considerable number of regulatory mechanisms, which in most occasions are conducive to adaptation, but sometimes it may evolve into pathological conditions such as acute mountain sickness (AMS) (Bilo *et al.*, 2019).

High altitude pulmonary edema (HAPE), as a potentially fatal AMS, occurs within 2–4 days after reaching an altitude of 3000 m or above (He *et al.*, 2017). HAPE is a severe lung injury caused by acute HBH and its pathogenesis is still not fully clarified, which seriously impedes the development of clinical research from preventing and treating HAPE (Jin *et al.*, 2019). As shown in the previous studies, pulmonary hypertension (Eichstaedt *et al.*, 2020), hypoxic contraction of pulmonary blood vessels (Swenson, 2020), increased capillary pressure (Zhang *et al.*, 2018), blood gas barrier injury (Solaimanzadeh, 2020) and increased pulmonary vascular permeability (He *et al.*, 2018) are the main pathophysiological factors of HAPE.

According to the reports, the degranulation of perivascular triggered by platelets would thus lead to tissue injury and inflammatory response, which are related to the pathogenesis of HAPE. Platelets that are circulating and non-nucleated cells play an important role in thrombosis, hemostasis and wound healing (Eicher *et al.*, 2018). Function as a main site to produce terminal platelet, lung contributes substantially to nearly half of the total production of platelet biogenesis (Lefrancais *et al.*, 2017; Demopoulos *et al.*, 2020).

Mitochondria, as the main power supply organelle in platelets, play a crucial part in maintaining the normal structure and function of platelets (Sledz *et al.*, 2020). Mitochondrial DNA (mtDNA), a circular double-stranded genomic DNA, exists independently in cells, which encodes 13 oxidative phosphorylation complex (OXPHOS) polypeptides located in the inner mitochondrial membrane (Sharma and Sampath, 2019). mtDNA could act as a feedback mechanism to check for changes in gene sequence in the short term because of its quick response to the stimulation of outside environment. Due to the lack of histone protection, mtDNA is vulnerable to oxidative stress damage, resulting in mtDNA methylation (Singer, 2019), which is the most extensively studied epigenetic mechanism. Epigenetics refers to the heritable changes that lead to gene expression without alteration of the original DNA sequence. There are mounting evidences indicating that epigenetic mechanisms may also contribute to regulate the replication and transcription of mtDNA (Sharma *et al.*, 2019).

It has been suggested that epigenetic variations may interplay with platelet functions (Danese *et al.*, 2021) and the signals from platelets were conferred epigenetic changes, including secretion of potent growth factors and upregulation of oncoproteins in circulating tumor cells, which may

contribute to promote metastatic outgrowth, mitogenesis and angiogenesis (Tao *et al.*, 2021). The platelet activity and apoptosis dysfunction in many diseases may be caused by mitochondrial dysfunction, such as cardiovascular disease (CVD), Alzheimer's and Parkinson's, sepsis and diabetes mellitus, which indicates that mitochondria play an indispensable role in platelet activation, metabolism, and apoptosis (Wang *et al.*, 2017). Therefore, the epigenetic regulation of platelet mtDNA is of extraordinary significance. However, so far there have been no research in the effect of environmental HBH exposures on the levels of platelet mtDNA methylation.

Recent studies showed that hypoxia could cause mitochondrial dysfunction, which could participate in the occurrence of HAPE via multifarious pathways, such as excessive production of reactive oxygen species (ROS) (Michelakis *et al.*, 2004), contraction of pulmonary vascular smooth muscle cells and obstacles to pulmonary water clearance (Fig. 1). Mitochondria may be the core element for HA adaptation and altitude sickness, and mtDNA mutations may be associated with the occurrence of HAPE. Previous studies have found that mitochondrial epigenetics might play an important role in HAPE apart from genetic variation (Chanana *et al.*, 2020). ROS is mainly generated by the mitochondrial electron transport chain complexes (McElroy and Chandel, 2017). Respiratory chain complexes are made up of some subunits, most of which are encoded by nuclear genes, except for some of the subunits of complex I, III, and IV that are encoded by mtDNA and synthesized on mitochondrial ribosomes (Das *et al.*, 2012). The main function of complex IV is OXPHOS, which is located at the end of the respiratory electron transfer chain and also known as cytochrome c oxidase (Cox) (Xiang *et al.*, 2016). What's more, studies have reported that inflammation is a vital pathogenic feature of HAPE and a series of inflammatory factors are clearly involved in

Fig. 1. Proposed conceptual model linking HBH exposure, platelet mtDNA methylation, and HAPE. PM, particulate matter; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; NAD, nicotinamide-adenine dinucleotide; FMN, flavin mononucleotide; FeS, ferrous sulfide; Cyt, cytochrome; FAD, flavin adenine dinucleotide; MMP, mitochondrial membrane potential.

the pathogenesis of HAPE (Zhou *et al.*, 2017). It has been proven that mitochondria are both targets and regulators of inflammatory pathways, inflammatory cytokines could promote mitochondrial dysfunction (Dikalova *et al.*, 2020).

The air quality index (AQI) and oxygen concentration in Tibet were compared and analyzed, which further proved that HBH was the most significant and important characteristic of the atmospheric environment in HA. Therefore, we assumed that HBH could induce platelet mitochondrial dysfunction via affecting respiratory chain, which in turn trigger inflammatory response and ultimately induce lung injury or HAPE. To simulate human experiments and examine our hypothesis, 32 male Sprague-Dawley rats were placed in the multi-functional hypoxic chamber to simulate HAPE model with altitude of 6000 m, oxygen concentration of 9.46% and partial oxygen pressure of 9.6 KPa. We quantified the levels of *MT-Cox1*, *MT-Cox2*, and *MT-Cox3* methylation that encode complex IV genes in plasma platelet mtDNA in rat model of HAPE, and we detected the levels of a series of inflammatory factors in plasma. The aim of this study is to investigate the effect of plateau air quality and low oxygen content on platelet mtDNA methylation and HAPE.

2 METHODS

2.1 Animals and Breeding Conditions

SPF grade adult male SD rats, weighing 200 ± 20 g, were purchased from the Beijing Hua Fukang Biotechnology Co., LTD, with the production license number of SCXK (Jing) 2020-0004. All the rats were exposed to an endocellular environment at 20–26°C and 40–70% humidity for 2 days in advance. All the animal experiments were conducted in accordance with Tianjin Medical Experimental Animal Care guidelines, and animal protocol was approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Co., Ltd.

2.2 Multi-functional Hypoxic Chamber

The instrument used in this research is DSF580I multi-functional hypoxic chamber (Fig. 2) with 4 modes of hypobaric hypoxia, hypobaric normoxia, normobaric hypoxia and low O₂ and carbon dioxide (CO₂). One of four modes or compound patterns can be selected according to the experimental requirement. The multi-functional hypoxic chamber mainly consists of the cabin

Fig. 2. Multi-functional hypoxic chamber. (a) The main body of the multi-functional hypoxic chamber; (b) Control part; (c) Parameter settings of HBH; (d) Running interface.

and the control part whose interior is large enough to hold 4–5 rat cages (Fig. 2(a)). Cameras are mounted on the door and the inside of the cabin, which can be used to monitor the animals inside through the external display screen. There is a touch screen and several control buttons including regulating O₂ and nitrogen (N₂) in the control part (Fig. 2(b)). The working mode and the system parameters are set by the touch screen.

2.3 Experimental Design and the Simulation Conditions of HA Atmospheric Environment

An overview of the study is provided in Fig. 3. Thirty-two SD rats were randomly divided into four groups: the control group (n = 8) and the exposed groups (n = 24). The exposed groups were divided into three groups according to different exposure levels (24, 48, and 72 hours) (n = 8, respectively). The multi-functional hypoxic chamber (Fig. 2) described above was used to simulate HA atmospheric conditions to obtain HAPE model, the altitude was set at 6000 m according to the previous research. Other parameter settings include oxygen concentration of 9.46%, partial oxygen pressure of 9.6 KPa, culture time (1440 min, 2880 min and 4320 min), pump switching time (720 min), rate of elevation (10 m s⁻¹), rate of descent (5 m s⁻¹) and cabin temperature (–20°C) (Figs. 2(c–d)).

2.4 Lung Wet/Dry (W/D) Weight Ratio

The magnitude of pulmonary edema was determined by weighing and computing W/D ratio of the pulmonary tissues. The left lobe of the lung was excised, washed several times with 0.9% NaCl, and the excess moisture on the surface of the lung tissue was blotted gently using a clean filter paper. The lung tissue was weighed to obtain wet weight and then placed together with the foil in an electric thermostatic oven at 60°C for 48 h, finally the weight was recorded as the dry weight. The W/D ratio of the lung tissue was calculated to assess the degree of pulmonary edema.

2.5 Hematoxylin and Eosin (HE) Staining

Lung tissues were fixed in 4% paraformaldehyde for 48 h and then sectioned and stained. Eight different areas were randomly selected from the control group and the exposed groups. Lung tissue structure, lung septum, and red blood cell exudation were observed under light microscopy. Inflammatory cell infiltration and pulmonary edema images were collected by optical microscope. The severity of lung injury was based on the following scoring criteria: no damage = 0 point; 25% injury area = 1 point; 50% injury area = 2 points; 75% injury area = 3 points; total lung injury = 4 points (Smith *et al.*, 1997). The extent of lung tissue damage was observed and scored using the double-blind method by two pathologists.

Fig. 3. Overview of experimental approach. Rats were assigned to control (NC), hypobaric hypoxia (HBH)-24h, HBH-48h and HBH-72h exposed groups. The altitude was set at 6000 m, and the pump operating time was 720 min.

Table 1. PCR and pyrosequencing primer sequences.

Gene name	Primer	Sequence
MT-Cox1	Forward primer (5' to 3')	AGTTGGAGTTGGAATAGGATGAATA
	Reverse biotin primer (5' to 3')	TAACCTCTAAAATAAAAAACACCCC
	Sequencing primer (5' to 3')	ATATTTTTTTTTTAGT
MT-Cox2	Forward primer (5' to 3')	TAATGATTTAAAATTAGGTGAATTT
	Reverse biotin primer (5' to 3')	TAACCCTAATAAAAAATAACTCATAAATA
	Sequencing primer (5' to 3')	ATAGAATTTTAAATT
MT-Cox3	Forward primer (5' to 3')	GTTATTATATTTTTATTGTATAAAAAGGTT
	Reverse biotin primer (5' to 3')	AAATAATAAAATACTCAAAAAAATCC
	Sequencing primer (5' to 3')	GTATAAAAAGGTTTT

2.6 Platelet mtDNA Methylation

The whole blood was centrifuged to obtain plasma, the samples were frozen and stored at -80°C until analysis. Platelet mtDNA was extracted from plasma samples, and methylation levels of platelet mtDNA candidate sites for platelet mtDNA associated with lung function were detected by pyrosequencing. PyroMark Q96 MD pyrosequencing system (QIAGEN, Germantown, MD) was used for pyrosequencing. The methylation levels of *MT-Cox1*, *MT-Cox2* and *MT-Cox3* genes encoding cytochrome C enzyme were determined by bisulfite pyrosequencing. The Meth Primer program was used to design primer sequences based on the GeneBank: J01415.2 (L-strand) mitochondrial genome sequence and the specific information was supplied in Table 1. In detail, one CpG position for *MT-Cox1* region; two CpG positions for *MT-Cox3* region and three CpG positions for *MT-Cox2* region were quantified.

2.7 Measurement of Plasma Inflammatory Cytokines

The Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay was used to detect plasma inflammatory mediators in all groups (Bio-Rad, Hercules, CA). Luminex Bio-Plex Pro Rat Cytokine Th1/Th2 Panel 12-Plex based on the coupled magnetic beads allowed the simultaneous quantification of 12 cytokines from previously stored plasma, including IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, TNF- α , GM-CSF and IFN- γ . Twelve groups of fluorescently staining beads were coupled with target-specific reactants to trigger biochemical reactions with target biomolecules, which were detected by Bio-Plex MAGPIX System (Bio-Rad). A high-speed digital signal processor was used to process the fluorescent output, and the Bio-Plex Manager™ software was used to calculate cytokine concentrations automatically based on a standard curve.

2.8 Statistical Analysis

Analyses were performed using the GraphPad Prism 8.0.1 software program. Paired t-test were used to calculate the significant differences in the methylation levels of a given gene in platelets between the control and exposed groups. Significance of the difference in methylation exposure at each site was calculated by Brown-Forsythe and Welch one-way analysis of variance (ANOVA). One-way ANOVA was used for comparisons between groups of lung injury score and the W/D ratio. The significance analysis and degree of relevance were performed to determine by linear regressions. P value < 0.05 represented statistically significant.

3 RESULTS AND DISCUSSION

3.1 AQI and PM_{2.5} Concentrations in Tibet

The monthly AQI and PM_{2.5} concentrations in Tibet included seven cities during 2020 were processed and shown in Fig. 4. The data were derived from the PM_{2.5} Historical Data Network (<https://www.aqistudy.cn/historydata/>). In 2020, the monitored AQI in Tibet varied from 24 to 98 and averaged 48, which was below 50, indicating that the air quality in Tibet was quite excellent (Bhaskar *et al.*, 2017). The annual PM_{2.5} concentrations ranged between 3.0 and 31 $\mu\text{g m}^{-3}$ and with an average of 10 $\mu\text{g m}^{-3}$, which was lower than the National Ambient Air Quality Primary

Standard of $\text{PM}_{2.5}$ ($15 \mu\text{g m}^{-3}$) (GB3095-2012) (Cao *et al.*, 2019).

In 2020, the April, May, and June were with the highest AQI and were 52, 63, and 54, respectively; while the highest $\text{PM}_{2.5}$ concentrations were occurred in winter, including January ($12 \mu\text{g m}^{-3}$), November ($14 \mu\text{g m}^{-3}$) and December ($16 \mu\text{g m}^{-3}$). The above results indicated that the O_3 and NO_x other than $\text{PM}_{2.5}$ were the major indicatory pollutants for AQI during April, May and June, which were owing to solar radiation and temperature (Cifuentes *et al.*, 2021) and the emission from vehicles (Peng *et al.*, 2021). Among the sub-cities in Tibet, as to the AQI, the Ali area had the highest average, while the Nyingchi had the lowest, and they were 58 and 40, respectively. However, the Nagqu had the highest average $\text{PM}_{2.5}$ concentration and the Ali area had the lowest one; they were 18 and $6.0 \mu\text{g m}^{-3}$, respectively.

3.2 The Oxygen Concentration and Partial Oxygen Pressure in Different Altitudes and the Composition of Atmosphere

It is well known that the atmosphere is consist of many kinds of components, such as N_2 , O_2 , inert gases, CO_2 and other gases. The volume fraction of oxygen in atmosphere is 21%, which cannot change with the increased altitude. However, the partial pressure of atmospheric oxygen falls progressively as barometric pressure decreases with increasing altitude (Grocott *et al.*, 2009). As displayed in Fig. 5(a), the oxygen concentration and partial pressure of oxygen in Tibet (4000–5000 m) decreased significantly compared with the plain areas (less 50 m above the mean sea level).

It can be demonstrated that the overall environmental air quality in HA areas was relatively better compared with plain areas when coupled with the graphic information (Figs. 4 and 5). On the other hand, the bar chart showed a remarkable decline in the oxygen concentration and partial oxygen pressure from plain areas to HA areas. Meanwhile, the partial pressure of inspired oxygen (PiO_2) would diminish with the decreased availability of atmospheric oxygen for aerobic respiration (Grocott *et al.*, 2009), which could cause the tissue hypoxia and subsequently trigger a considerable number of regulatory mechanisms. Therefore, it can be further indicated that the relatively larger contribution of HBH in the atmospheric environment to the health of people entering the HA.

3.3 The Methylation Levels of Platelet mtDNA Before and After Exposure

The methylation levels of platelet mtDNA of three genes were measured in the plasma platelets of rats exposed to HBH for 24 h, 48 h and 72 h in the multi-functional hypoxic chamber: *MT-Cox1*;

Fig. 4. The AQI and $\text{PM}_{2.5}$ concentration in Tibet in 2020.

Fig. 5. The oxygen concentration and partial oxygen pressure in (a) different altitudes and (b) the composition of atmosphere.

MT-Cox2 and *MT-Cox3*. The basal DNA methylation level of each gene included different CpG sites varied between the three gene regions studied here. Following HBH exposure for 24 h, *MT-Cox1*, *MT-Cox2* and *MT-Cox3* methylation levels were all significantly decreased. Following HBH exposure for 48 h, *MT-Cox1*, *MT-Cox2* except for *MT-Cox2_Pos2* and *MT-Cox3* methylation levels were significantly decreased. Following HBH exposure for 72 h, *MT-Cox1*, *MT-Cox2_Pos1* and *MT-Cox3* methylation levels were significantly decreased. The levels of *MT-Cox2* methylation in other CpG sites decreased with HBH exposure although did not achieve statistical significance (Fig. 6).

3.4 mtDNA Methylation Changes in *MT-Cox* Genes between Before and After HBH Exposure

The levels of HBH exposure were negatively correlated with the absolute changes in methylation of *MT-Cox1*, *MT-Cox2* and *MT-Cox3* genomic regions of platelet mtDNA. We did not observe an ascending effect upon absolute changes in mtDNA methylation following HBH exposure between three exposed groups. On the contrary, there was marginally significant reduction in the change of *MT-Cox2_Pos3* methylation in HBH-24h compared with the HBH-48h exposed group ($p < 0.05$). The results indicated that the HBH exposure time had a negative relation with the *MT-Cox* methylation levels (Fig. 7).

As extensive studies have indicated that changes in epigenetic levels between environmental factors and altered gene expression may be indirect factors leading to the emergence of some disease phenotypes. Environmental exposures to physical, chemical and nutritional factors can cause epigenetic changes potentially by altering gene expression and modifying individual illness susceptibility in multiple pathways (Jirtle and Skinner, 2007). For instance, it has been found that exposure to PM could increase the risk of lung cancer in individuals through touching off systemic inflammation and DNA hypomethylation of leukocyte (Guo *et al.*, 2014; Chao *et al.*, 2018). Associated with particle-containing welding fumes exposure, a recent study of 101 welders from southern Sweden showed that the *D-loop* and *MT-TF* genes became hypomethylated, indicating that oxidative stress was related to the exposure (Xu *et al.*, 2017). Yang *et al.* (2016) suggested that the *MT-RNR1* and *MT-TF* genes in mtDNA methylation were decreased whereas the *LINE-1* gene in nDNA remained unaltered in chrome plating exposed workers. And the findings from Sanyal, *et al.* (2018) concluded that mtDNA methylation could be impacted by chronic arsenic toxicity, meanwhile, the expression of certain mitochondrial gene enhanced, and mitochondrial biogenesis increased in arsenic-exposed population compared with individuals who were not exposed to arsenic. In our study, we identified hypomethylation of *MT-Cox* after HBH exposure, indicating a mechanism by which HBH exposure could affect mitochondrial functions such as mitochondrial gene expression and oxidative stress. Moreover, the levels of HBH exposure were negatively correlated with the absolute changes in methylation of *MT-Cox1*, *MT-Cox2* and *MT-Cox3* genomic regions of platelet mtDNA. However, the absolute changes did not reach statistical significance between three HBH exposed groups except for the absolute change of HBH-24h exposed group

Fig. 6. Methylation changes after HBH exposure for 24, 48, and 72 hours in rats platelet mtDNA. Platelet mtDNA methylation changes in (a) *MT-Cox1*; (b) *MT-Cox2_Pos1*; (c) *MT-Cox2_Pos2*; (d) *MT-Cox2_Pos3*; (e) *MT-Cox2*; (f) *MT-Cox3_Pos1*; (g) *MT-Cox3_Pos2*; (h) *MT-Cox3* in the plasma platelet mtDNA of control and exposed rats. Box: median. Bars: min and max values. C: control and E: exposed group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicated the significance of the estimated effect value. HBH: hypobaric hypoxia exposed group.

compared with HBH-48h in *MT-Cox2_Pos3*. We speculate that the alteration in *MT-Cox3* methylation in HBH-24h and HBH-48h exposed groups may be implicated in different HBH exposure levels, although further studies are required to analyze this correlation in detail. HBH-induced oxidative stress affects nitric oxide (NO), an endothelium derived mediator, leading to the accumulation of harmful mediators such as peroxy-nitrite, which limits vasodilating potential mediated by NO in the bodies, restraining the activation/aggregation of platelet consequently (Faiss *et al.*, 2013; Gresele *et al.*, 2019). Concurrently, mtDNA is one foremost target of oxidative stress generated by environmental exposure (Xu *et al.*, 2017), therefore, future studies are warranted to determine whether *MT-Cox* hypomethylation after HBH exposure is related to mitochondrial oxidative stress and gene expression.

3.5 Analysis of W/D of Lung Tissue

All groups were randomly selected, and W/D ratio was significantly higher in exposed groups with increased HBH exposure level. Compared with the control group, W/D ratio and lung injury score were elevated significantly in HAPE rats after HBH exposure ($p < 0.05$). However, the difference between HBH-24h exposed group and control group was not observed (Fig. 8(a)).

Fig. 7. Association between three HBH exposure levels and the absolute changes (Δ) in levels of platelet mtDNA methylation in all HAPE rats in the multivariable linear regression models. (a) *MT-Cox1*; (b) *MT-Cox2_Pos1*; (c) *MT-Cox2_Pos2*; (d) *MT-Cox2_Pos3*; (e) *MT-Cox2*; (f) *MT-Cox3_Pos1*; (g) *MT-Cox3_Pos2*; (h) *MT-Cox3*. Note: the methylation variables were defined by percentage changes (%) before to after the HBH exposure, and the 95% confidence interval was represented as vertical lines; * $p < 0.05$ suggested statistically significance of the estimated effect value. HBH: hypobaric hypoxic exposed group.

3.6 Pathological Changes in the Lung Tissue

HE staining of the rats lung tissue showed that the alveolar structure was integrated in the control group, the pulmonary septum was basically not thickened, erythrocyte distribution was observed and obvious edema was not observed in lung interstitium or the alveolar cavity. We observed significant pathological variations in the lung tissues of HBH-challenged rats, including pulmonary interstitial edema, pulmonary capillary congestion, considerable inflammatory cell infiltration into the alveolar space and lung interstitium, and thickened alveolar wall (Figs. 8(c–f)). The lung tissue score was significantly elevated in the exposed groups compared with the control group ($p < 0.05$) (Fig. 8(b)).

3.7 Correlation of mtDNA Methylation with W/D of Lung Tissue

The changes of W/D ratio with HBH exposure were remarkably related to *MT-Cox1* ($r = -0.1644$, $p = 0.0493$), *MT-Cox2_Pos2* ($r = -0.1935$, $p = 0.0315$) and *MT-Cox2_Pos3* ($r = -0.1695$, $p = 0.0456$) methylation. The remaining methylation sites of *MT-Cox* did not show significant correlations with W/D ratio (Fig. 9).

Change in mtDNA levels is increasingly applied to a biomarker of mitochondrial dysfunction (Sun *et al.*, 2021). As a vital and promising research, the role of mitochondrial dysfunction and its

Fig. 8. W/D ratio and HE pathological staining of lung tissue in rats ($\times 200$). The evaluation of the lung injury score was due to the pathology detected by using HE staining. (a) W/D; (b) Lung injury score; (c) Control group; (d) HBH-24h exposed group; (e) HBH-48h exposed group; (f) HBH-72h exposed group. NC: control group; HBH: hypobaric hypoxic exposed group.

Fig. 9. Correlation of changes in W/D ratio of lung tissue and *MT-Cox* methylation. The correlation between the W/D ratio and DNA methylation by HBH exposure is illustrated, with significant differences indicated. The values of *r* and *p* ($p < 0.05$) are shown in the figures above.

potential mechanistic in the evolution of lung diseases is highly acknowledged (Ten and Ratner, 2020). Platelets have interrelated activities with lung, which is a complex multicellular organ responsible for many physiologic processes, such as gas exchange and immune surveillance (Bozza *et al.*, 2009). It has been proven that platelets were a crucial element of acute lung injury (ALI), exerting synergistic action with fibrinogen to mediate endothelial damage via various pathways of signal transduction (Dixon *et al.*, 2012). The platelet-derived growth factor beta beta (PDGF- $\beta\beta$), a biomarker, is known to be associated with the smooth muscle proliferation and processes of exaggerated vasoconstriction that are hallmark characteristics of HAPE (Pandey *et al.*, 2016). Additionally, the link between elevation of W/D ratio and increased endothelium swelling or epithelium disruption indicated that the defeat of alveolar capillary barrier stress played a pivotal role in the development of HAPE, suggesting that the W/D ratio of lung was an indicator of overall pulmonary edema severity (Bai *et al.*, 2010; Wu *et al.*, 2019). There was a strong relation between decreased *MT-Cox1*, *MT-Cox2_Pos2*, *MT-Cox2_Pos3* methylation in the plasma platelets and increased W/D ratio after HBH exposure from our study. Although, the correlations between *MT-Cox2_Pos1* and *MT-Cox3* (including 2 CpG sites) methylation with W/D

ratio were decreased with HBH exposure, none of them was statistically significant. We conject that the changes in *MT-Cox1*, *MT-Cox2_Pos2*, *MT-Cox2_Pos3* methylation might be implicated with increase of W/D ratio. Given that platelet mtDNA methylation has been shown to contribute to mitochondrial dysfunction, platelet mtDNA methylation is a plausible epigenetic risk factor to investigate in many diseases. A previous study has proven that the development of CVD could be promoted by platelet mtDNA methylation through regulating platelet activity (Baccarelli and Byun, 2015). Concurrently, recent evidence supported that the variation of platelet mtDNA methylation might be involved in the complex multifactorial disease pathogenesis, such as the association between trimethylamine-N-oxide (TMAO), atherosclerosis and L-carnitine, which could be more complicated than already assumed (Bordoni *et al.*, 2020). Together above, we suggest that platelet mtDNA methylation might be involved in the process of HAPE.

3.8 Correlation of Platelet mtDNA Methylation with Inflammatory Mediators

A premade platform was used to detect 12 inflammatory cytokines levels of rats' plasma and analyze their correlation with mtDNA methylation of *MT-Cox* genes (Bio-Plex Pro Rat Cytokine Th1/Th2 Panel 12-Plex (Bio-Rad, Hercules, CA). As shown in Fig. 10, seven proinflammatory cytokines in plasma following HBH exposure were significantly associated with *MT-Cox2* methylation (including 3 GpG sites) ($r = 0.09-0.30$, $p < 0.05$). In contrast, *MT-Cox3_Pos3* methylation was inversely related to proinflammatory cytokines ($r = -0.1506$, $p = 0.0341$). *MT-Cox1* and the remaining methylation sites of *MT-Cox3* showed no significant correlation with cytokine levels at the same p value (Fig. 10).

Studies have shown that platelet function is closely related to inflammation. Sreeramkumar *et al.* (2014) suggested that dynamic recombination of neutrophil domains and receptors provided a rapid and effective regulatory mechanism in the early stages of inflammation by simultaneously interacting with vascular walls and activated platelets in circulation. A growing body of data revealed that platelets were active cell populations in atherosclerosis and a key cross-link between inflammation and thrombosis (Yin *et al.*, 2019). Additionally, platelets are capable of releasing mitochondria, and mtDNA is a recognized damage-associated molecular pattern detected in considerable inflammatory situations and in the concentrates of platelet, damage to which can cause inflammation and bioenergetic deficit (Mills *et al.*, 2017). Hyperreactive platelets with larger mitochondrial mass have the potential of exacerbating inflammatory responses by releasing higher amounts of mitochondrial DNA and other inflammatory mitochondrial components (Davizon-Castillo *et al.*, 2019). To sum up, there is an important association between inflammatory response and abnormal mitochondrial function of platelets.

Once facing pressure, mitochondria produce ROS and release mitochondrial components, such as mtDNA, into cytoplasm or extracellular matrix in order to control inflammation (Suarez-Rivero *et al.*, 2021). This phenomenon has been described before (Ma, 2015). that mtDNA was damaged as early as at the initiation of proinflammatory cytokine release and TNF- α induced DNA damage by increasing mitochondrial mtROS. Plenty of evidences suggested that mtDNA damage induced by inflammation might be involved in a wide variety of lung diseases. For example, Hu *et al.* (2015) suggested that inflammation could be activated and the release of TNF- α , IL-6, and IL-10 could be accelerated by mtDNA during the injury of liver ischemia-reperfusion. The complex of Cramp-mtDNA has been found to deteriorate the proinflammatory effect of neutrophils on septic mice and disadvantageous tissue damage could be further induced (Zuo *et al.*, 2019). And Jing and Hu *et al.* (2020) showed that CS up-regulated PINK1 expression, mitochondrial autophagy occurred due to the release of mtDNA, the TLR9/MyD88 pathway was activated, finally inducing inflammation and cell injury in ALI. Furthermore, Zhao *et al.* (2021) have emphasized that mtROS maintained mitochondrial dysfunction and inflammation that contribute to ischemic acute kidney injury through obstructing mtDNA mediated by TFAM. It has also been reported that increased cell-free mtDNA played a crucial part in the inflammatory and immune reactions during non-hemolytic transfusion reaction, severe trauma, virologically suppressed HIV-infected individuals and ischemia-reperfusion injury, which was a sign of continued inflammation and better neurocognitive function (Perez-Santiago *et al.*, 2017; Iske *et al.*, 2020). Observed in our present study, there were significant positive associations between seven cytokines levels (IL-1 α , IL4, IL6, IL10, IL12, INF- γ and TNF- α) and alterations in *MT-Cox2* methylation included 3 CpG sites following HBH exposure. While the methylation of *MT-Cox3_Pos3* was inversely correlated with IL13 after HBH exposure.

Fig. 10. Correlation of changes in inflammatory markers and MT-Cox methylation. The correlation between inflammatory cytokines and DNA methylation by HBH exposure is illustrated, with significant differences indicated. The values of r and p ($p < 0.05$) are shown in the figures above.

However, MT-Cox methylation of other sites did not show significant associations with inflammatory cytokines. It is well learned that the levels of alveolar macrophages, neutrophils and lymphocytes, albumin, low density lipoprotein cholesterol, and proinflammatory cytokines and chemokines were all increased in bronchoalveolar lavage fluid (BALF) in HAPE patients (Xiang *et al.*, 2016). As one of the most important immune cell types, macrophages are recruited to perform their physiological functions at the site of injury (Li *et al.*, 2021). Additionally, DNA hypomethylation can be exacerbated by ROS stress and inflammation. With these continuous stimuli, the stress/damage and methylation in the lung act in a positive feedback manner (Guo *et al.*, 2014). In this context, the results from our study supported the assumption that alterations of methylation could increase the risk of HAPE induced by inflammation.

As a major part of cellular oxygen consumption in mitochondrial respiratory chain (MRC), mitochondrial Cox is critical for the generation of aerobic energy in the form of ATP (Timon-Gomez *et al.*, 2018). MRC function is directly affected by Cox dysfunction and subsequently leads to increased leakage of mitochondrial electron (Liu *et al.*, 2018). In our current study, we found that *MT-Cox2*, which had a significant correlation with W/D ratio compared with *MT-Cox1* and *MT-Cox3*, had a stronger association with the inflammatory cytokines after HBH exposure. It can be inferred from our results that *MT-Cox2* may play a crucial role in the process of inflammatory cytokines expression and MRC dysfunction in HAPE. Cox subunit 2 (*Cox2*) is an integral component of Cox, which is closely related to the mitochondrial electron transfer and ATP synthesis (Heidari *et al.*, 2020). A previous experiment demonstrated that *Cox2* was significantly decreased in some patients whose Cox function was deficient (Brischigliaro and Zeviani, 2021) and in the ischemia-reperfusion animal models (Mariero *et al.*, 2019). Recent research reported that the *Cox2* expression and the apoptosis proportion were increased remarkably in a model of chronic obstructive pulmonary disease (Yang *et al.*, 2015). In addition, the production of ROS decreased due to the high expression of *Cox2* in a feedback manner (Xiang *et al.*, 2019). It was also reported that cigarette smoke extract (CSE) induced methylation and inactivation of the mitochondrial transcription factor (mtTFA) promoter, thereby reducing the expression of mtTFA, further resulting in the reduction of *Cox2* expression, subsequent MRC dysfunction and the cell apoptosis of endothelial and epithelial in the lung (Zhang *et al.*, 2013; Yang *et al.*, 2015). Consequently, further studies are required to explore HBH exposure-induced changes of *MT-Cox2* methylation and the potential mechanism of *Cox2* in HAPE based on its important role in MRC function.

3.9 Possible Mechanisms in HAPE after HBH Exposure

There are many special natural environmental features on the HA, such as HBH, high solar radiation, arid, variable climate, low temperature, and geochemical anomaly, which cause great effects on human physiology and health (Zhang and Li, 2015). The bodies have to carry out a series of adaptive adjustments to achieve the purpose of adapting to the life on the HA when living on the plain enter the HA and under the conditions of HBH. And the bodies clinically can appear some symptoms (also known as stress response) during the process of adjustment and adaptation (Sitko *et al.*, 2019). The feature of HBH in HA environment has a great impact on the lung diseases especially HAPE. Lung W/D ratio is commonly used as indicator of pulmonary vascular permeability (Wu *et al.*, 2019), which is an important characteristic of HAPE caused by HBH and pathological features of HAPE have been well known worldwide (Droma *et al.*, 2001), both the lung W/D ratio and HE pathological staining are important indicators to evaluate the degree of HAPE therefore. The increased lung W/D ratio and the lung injuries showed in HE pathological staining from our results suggested that HBH environmental exposures indeed caused the occurrence of HAPE in rats. As mentioned above, inflammation is a vital pathogenic feature of HAPE and HBH can cause mitochondrial dysfunction. The *MT-Cox* hypomethylation, the relationship between lung W/D ratio and the levels of *MT-Cox* methylation and the correlation between the levels of inflammatory cytokines and *MT-Cox* methylation indicated that *MT-Cox* hypomethylation after HBH environmental exposures may be related to mitochondrial oxidative stress and gene expression, and the changes of *MT-Cox* methylation were involved in the occurrence of HAPE and could increase the risk of inflammation-induced HAPE.

4 ADVANTAGES AND LIMITATIONS

There are some advantages in this study. First of all, mtDNA is an innovational selection to investigate because of its significance in the physiological pathogenesis of lung diseases. In addition, we have conducted numerous epigenetic analyses (mtDNA methylation levels and their relationship with inflammation and W/D ratio respectively) that have contributed an extend study of the epigenome in response to HAPE due to exposure to HBH. Finally, the design of our study has enabled the delineation of epigenetic changes related to HAPE.

There are also certain limitations that hamper us from the inferences which can be obtained from current observations. Firstly, we are unable to identify all genes influenced by HBH exposure owing to adopting a candidate gene pathway. Secondly, we cannot get rid of the possibility that

other regions of the mitochondrial epigenome may be differentially methylated due to exposure. For the reason that further targets need to be identified and analyzed through a microarray-based approach. Last but not least, the study of the functional impact on the observed epigenetic changes has been precluded due to the absence of rat behavioral studies and the lack of RNA and protein samples to analyze gene expression.

5 CONCLUSIONS

This research investigated the air quality in plateau-Tibet and studied the effect of acute HBH exposure upon platelet mtDNA methylation in rat model of HAPE. The major results were summarized as follows:

1. In 2020, the monitored AQI in plateau-Tibet varied from 24 to 98 and averaged 48, while the $PM_{2.5}$ concentration ranged between 3.0 and 31 $\mu g m^{-3}$ and with an average of 10 $\mu g m^{-3}$. The plateau-Tibet, had a good air quality, but with a lower atmospheric pressure and lower oxygen concentration.
2. This study explored the effect of acute HBH exposure upon platelet mtDNA methylation in rat model of HAPE. And the methylation levels of CpG positions in platelet mtDNA are measured in three genomic regions (*MT-Cox1*, *MT-Cox2* and *MT-Cox3*). To our knowledge, this is the first time that the methylation changes of platelet mtDNA have been observed in HAPE in response to HBH exposure. We also reported an association between platelet mtDNA methylation of the *MT-Cox* genes with W/D ratio as well as inflammatory cytokines in rat model of HAPE.
3. The exposure of HBH in HA atmospheric environment indeed affect the platelet mtDNA methylation and HAPE. Our findings strengthen the understanding of the relationship between HAPE and mitochondrial epigenetic changes and demonstrate that exposures to HBH can lead to hypomethylation of three *MT-Cox* genes of platelet mtDNA.
4. The levels of *MT-Cox2* methylation were also found to have a significant effect on inflammatory imbalance in HAPE.
5. The results of this study will help inform further studies in the treatment of HAPE, including the functional consequences of the observed mitochondrial epigenetic changes and how they related to air quality in HA and physiological responses to HA. Additionally, our findings may contribute to accelerate the production of biomarkers of HAPE.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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