Original Article
A rat model of sustained hypobaric hypoxia-induced pulmonary hypertension

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Abstract: Objectives: High-altitude pulmonary hypertension (HAPH) is a worldwide public health issue in mountainous areas, while the underlying mechanism of it is still unclear. This study aims to establish a rat model of hypobaric hypoxia-induced pulmonary hypertension through sustained exposure to a simulated high altitude atmospheric environment in plain area. Methods: Twelve healthy male SD rats were randomly and equally divided into a model group and a control group. The model group was housed in an automatic adjusting hypobaric hypoxia chamber for 4 weeks, and the control group was housed under normobaric normoxic condition in the same room. The mean pulmonary arterial pressure (mPAP), right ventricular pressure (RVSP), the right ventricle (RV) weight, left ventricular (LV) weight, interventricular septum (S) weight, right ventricular hypertrophy index (RVHI), hematoxylin-eosinstaining, elastic fibers staining, ratio of the thickness of vascular wall to its outer diameter (MT%), ratio of the cross-sectional area of the middle vascular wall to the total vascular cross-sectional area (MA%), α-SMA positive ratio were detected to evaluate the pulmonary hypertension. Results: As compared to that in control group, rats in model group showed significant increases on mPAP (36.39 ± 4.26 mmHg versus 16.27 ± 6.99 mmHg, $P<0.001$), RVSP (45.29 ± 6.09 mmHg versus 28.12 ± 4.67 mmHg, $P<0.001$), RVHI (0.44 ± 0.08 versus 0.18 ± 0.05, $P<0.001$), MT% (44.50 ± 8.96 versus 19.50 ± 4.88, $P<0.001$), MA% (64.00 ± 6.66 versus 40.45 ± 7.08, $P<0.001$), α-SMA positive% (57.00 ± 6.87 versus 30.50 ± 6.83, $P<0.001$). Conclusion: The rat model of hypobaric hypoxia-induced pulmonary hypertension has been successfully established by automatic adjusting hypobaric hypoxia chamber. Sustained exposure to a low oxygen environment at a simulate-altitude of 5,000 meter for 4 weeks have caused the pathological remodeling of pulmonary vascular walls and pulmonary hypertension, and further led to a series of pathological changes, including right ventricular hypertrophy. This model is easy to be replicated with good reproducibility and can be widely used in further studies.

Keywords: High-altitude pulmonary hypertension, hypobaric hypoxia-induced pulmonary hypertension, rat model

Introduction

High-altitude pulmonary hypertension (HAPH) is a specific disease affecting populations that live at high elevations [1]. It is characterized by increased pulmonary vascular resistance secondary to hypobaric hypoxia-induced pulmonary vasoconstriction and vascular remodeling of pulmonary arterioles [2, 3]. According to the latest clinical classification of pulmonary hypertension (PH), HAPH falls into the group 3 pulmonary hypertension [2, 4]. Besides the substantial local residents, an increasing number of temporary tourists have come to be exposed to

the effects of high altitude [5]. HAPH has become a nonnegligible public health issue in mountainous areas throughout the world [1]. Despite extensive research, the precise mechanisms underlying HAPH are still unclear [6]. To explore the pathogenesis of HAPH and thus reveal novel therapeutic targets, a reliable animal model that mimics the hypobaric hypoxia-induced pathophysiological course is needed. However, the most commonly used rodent models of PH, including monocrotaline model, SU5416 plus chronic hypoxic model, chronic normobaric hypoxic model etc, cannot completely reproduce the pathophysiological

features of HAPH. Based on our former work [7], this study aims to establish a rat model of hypobaric hypoxia-induced pulmonary hypertension through sustained exposure to a simulated high altitude atmospheric environment.

Materials and methods

Animals

The experimental animals were reared in the Experimental Animal Center of Beijing Shijitan Hospital. This study was performed in accordance with the national guidelines and with the permission of the institutional animal ethics and welfare committee of Beijing Shijitan Hospital. 12 healthy male Sprague Dawley rats (SD, 8 week old, 280-320 g, purchased from Beijing Vital River Laboratory Animal Center) were randomly divided into a model group (n=6) and a control group (n=6): the model group housed in an automatic adjusting hypobaric hypoxia chamber (gradually decrease FiO2 over 3-5 days to acclimate rats and then maintain the oxygen concentration of 10%, atmospheric pressure of about 50 kPa, simulating the altitude of 5,000 meters), the control group housed under normobaric normoxic conditions in the same room. Rats in each group were housed 3 per cage with a 12: 12-h light-dark cycle. Food and water were available ad libitum. Temperature of 18 to 25°C and humidity of 55% to 65% were maintained. The chamber and cages were opened every 2 or 3 days for cleansing and food supplement, which last for 2 hours each time. End of study measurements were conducted at the end of the 4th week.

Hemodynamic measurement

Prior to the experiment, and on the day of sacrifice, bodyweight and systemic of the animals were measured. 1% sodium pentobarbital (40 mg/kg) was administered intraperitoneally to all rats for anesthetization, after which the right external jugular vein was isolated. A 2F microtipcatheter (Millar Instruments) was connected via a pressure control unit (Millar Instruments, Houston, TX) to a physiological recorder (Powerlab ML786) and viewed using Chart5 computer software (AD Instruments, Colorado Springs, CO). The catheter was guided into the right ventricle (RV) and pulmonary artery (PA) to obtain pressure measurements after calibration, as described previously [8]. An average of

20 pressure cycles was utilized to obtain final pressure value. The right ventricle systolic pressure (RVSP) and mean pulmonary artery pressure (mPAP) were calculated through Chart5 computer software.

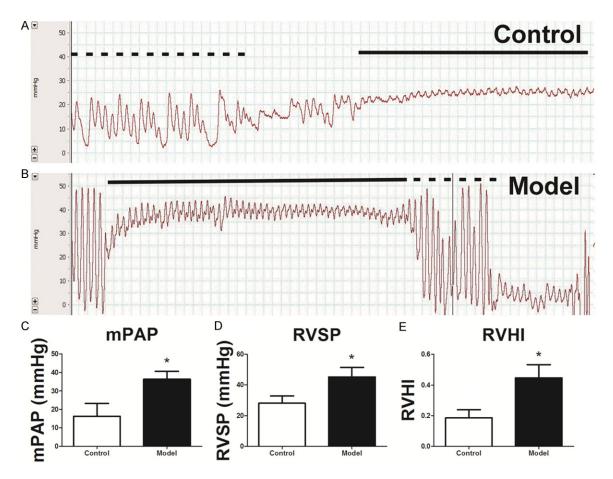### Assessment of right ventricular hypertrophy

After hemodynamic evaluation, rats were immediately euthanized with an overdose of pentobarbital. Then thoracotomy was performed to obtain the lungs and hearts. Immediately after the fresh heats were detached from rats, each of them was separated into 3 parts, right ventricle (RV), left ventricular (LV) and interventricular septum (S), along the right edge of the interventricular septum carefully and quickly. Then after dried by filter papers, they were weighed respectively by an electronic scale. Calculated under the formula (RV/LV+S), the right ventricular hypertrophy index (RVHI) was obtained to evaluate right ventricle hypertrophy.

Morphometric analysis of pulmonary arteries

The fresh rats' lung tissues were extracted along the hilar cross section, and then fixed in 10% formalin solution for 48 hours. The tissues were dehydrated paraffin-embedded and 5 μm conventionally sectioned for hematoxylin-eosin (HE) staining and Verhoeff-Van Gieson elastic (EVG) staining (Elastic Stain Kit, HT25A; Sigma Aldrich, St. Louis, MO, USA) in accordance with the package inserts. 5 EVG-stained sections were randomly selected from each sample for morphometric analysis, and 6 pulmonary arterioles with a diameter less than 300 μm were randomly selected from each sectionunder an optical microscope (Olympus Corporation, Shinluku, Japan). The outer diameter, wall thickness, wall area, lumen area and total wall area of pulmonary arterioles were measured by MPIAS-500 multimedia color pathological image analysis system (Shanghai Tongji Medical University, China), through which the medial wall thickness/outer diameter (MT%), medial wall cross-sectional area/total wall area (MA%) ratios were calculated automatically.

Immunohistochemical analysis

Immunohistochemistry analysis for α-smooth muscle actin (α-SMA) expression in the blood vessels wall was performed per the manufac-

Figure 1. Assessment of pulmonary hemodynamics and right ventricular hypertrophy. The high-fidelity trace from RV (dashed line) and PA (solid line) are demonstrated in (A) (control group) and (B) (model group). The bar graphs represents that the mean level of mPAP (C), RVSP (D) and RVHI (E) in model group are significantly higher than that in control group. *$P<0.001$, n=6, per group.

turer's instructions. Briefly, 5 µm paraffin sections were deparaffinized in xylene and rehydrated through a series of alcohol to water. Then sections were incubated in 3% hydrogen peroxide for 10 minutes, after which they were rinsed with distilled water 3 times and soaked in phosphate buffered saline (PBS). Subsequently, sections were blocked in 5% skim milk for 2 hours before incubated with diluted anti-α smooth muscle actin (α-SMA, A2547; Sigma Aldrich, St. Louis, MO, USA) overnight at 4°C. Following rinsed with PBS 3 times, sections were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using a 3, 3'-diaminobenzidine (DAB) kit (Maixin Biotech, Fuzhou, China). 5 sections were randomly selected from each sample for observation, and 5 high power fields of each section were randomly selected and analyzed by the MPIAS-500 multimedia color pathologi-

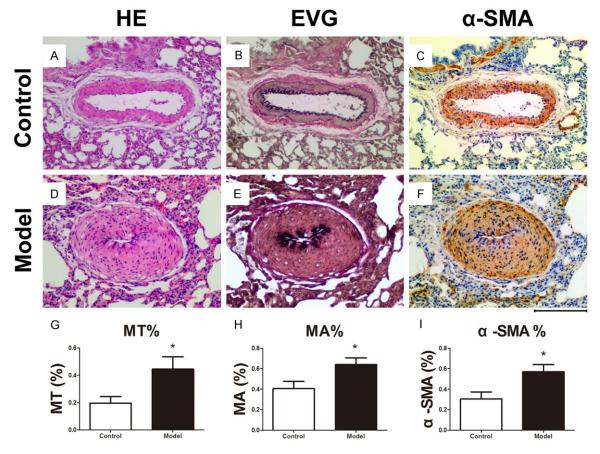cal image analysis system to calculate the expression rate of smooth muscle actin in pulmonary arterioles.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. All statistics and experimental data were finally evaluated by the formula of (mean ± SD). T-test was used to detect the differences in mPAP, RV/LV+S, MA%, MT% and α-SMA. All analyses were two-tailed with a significance level of 0.05.

Results

The general status of rats

Rats in the control group exhibited normally in activity with smooth and shiny fur, steady breath and increased weight. While, after sub-

Figure 2. Assessment of pulmonary vascular morphometry and α-SMA expression. Different from the control group (A: HE; B: EVG; C: α-SMA), increased thickness of the medial wall, luminal stenosis, and increased α-SMA expression can be observed in the sections of the model group (D: HE; E: EVG; F: α-SMA). The scale bar is 100 μm. The bar graphs represents that the mean level of MT% (G), MA% (H) and α-SMA expression (I) in model group are significantly higher than that in control group. *$P<0.001$, n=6, per group.

jected to chronic hypobaric hypoxia for 4 weeks, rats in the model group were less active and inclined to lying down with dry and frizzy fur, frequent wheeze and weight loss. During the experiment period, there were no deaths in either group.

Pulmonary hemodynamics and right ventricular hypertrophy

To exam the pulmonary haemodynamics, through a polyethylene microcatheter inserted from the right external carotid artery to the right ventricle and further advanced to the pulmonary, we obtained the high-fidelity trace from RV and PA in control group (**Figure 1A**) and model group (**Figure 1B**). In the model group, mPAP was 36.39±4.26 mmHg, increased by 20.12 mmHg as compared to 16.27±6.99 mmHg in control group (*P*<0.001, **Figure 1C**). Accordingly, the

RVSP and RVHI were 45.29±6.09 mmHg and 0.44±0.08 in the PAH group, respectively, either of which was significantly higher than that in the control group (28.12±4.67 mmHg and 0.18±0.05, *P*<0.001, **Figure 1D** and **1E**).

Pulmonary vascular morphometry

On the sections of the control group, HE staining demonstrated evenly-distributed endothelial cells within thin and sustained arteriole wall (**Figure 2A**). While, sections of the model group displayed thickened pulmonary arteriole walls and decreased lumen diameter with proliferating smooth muscle cells (**Figure 2D**).

EVG staining demonstrated observably increased width between the inner and outer elastic layer on the sections of the model group (**Figure 2E**) when compared to the control group (**Figure 2B**).

MT% increased to 44.50±8.96 in the model group, which was significantly higher than that of the control group (19.50±4.88, *P*<0.001, **Figure 2G**). MA% increased to 64.00±6.66 in the model group, while in the control group it was 40.45±7.08 (*P*<0.001, **Figure 2H**).

α-SMA immunohistochemical analysis

As shown in **Figure 2F** and **2C**, the α-SMA expression of rats in model group was significantly higher than that of the control group (57.00±6.87 versus 30.50±6.83). It was of statistical significance when comparing the actin expression of the model group with that of the control group (*P*<0.001, **Figure 2I**).

Discussion

In this study, a rat model of sustained hypobaric hypoxia-induced pulmonary hypertension has been successfully established. In accordance with the hemodynamic definitions of pulmonary hypertension [2, 4], right heart catheterization is required to establish the diagnosis of PH and HAPH as a gold standard. As is shown above, after a sustained exposure to hypobaric hypoxia environment, the level of mPAP and RVSP of the model group were significantly higher than that of the control group. In light of the sustained hypobaric hypoxia induction process and pulmonary hemodynamics, this animal model can meet the study requirement of PH and HAPH.

Furthermore, typical pathologic features of PH and HAPH have been observed in this rat model of sustained hypobaric hypoxia-induced pulmonary hypertension. HAPH is characterized by increased pulmonary vascular resistance secondary to hypobaric hypoxia-induced pulmonary vasoconstriction and vascular remodeling of pulmonary arterioles. The vascular alterations involve all elements of the vessel wall and include endothelial dysfunction, extension of smooth muscle into previously non-muscular vessels and adventitial thickening [1, 9, 10]. Sites of hypoxic pulmonary vasoconstriction are small pulmonary arterioles and veins with a diameter of <900 μm [11]. These structural changes in the pulmonary vasculature are due to hypoxia-associated smooth muscle cell proliferation and increased pulmonary vascular tone [12, 13]. Finally, pulmonary vascular remodeling results in the persistent hypoxic

pulmonary hypertension, which will bring about increasing pressure load and hypertrophy of the right ventricle and may even cause severe right heart failure or death [14, 15]. In this study, sustained exposure to hypobaric hypoxia environment at the simulate-altitude of 5,000 meter for 4 weeks caused the pathological remodeling of pulmonary vascular walls mentioned above, including the increased thickness of the medial wall, luminal stenosis, increased α-SMA expression and smooth muscle cell proliferation, and further led to right ventricular hypertrophy.

Additionally, this rat model of sustained hypobaric hypoxia-induced pulmonary hypertension is more suitable for HAPH study. Among the present most commonly used rodent models of PH, including monocrotaline model, SU5416 plus chronic hypoxic model, chronic normobaric hypoxic model etc, no single model can perfectly recapitulate HAPH [16-20]. As described in our previous work [7], we have always been studying on rat model of hypobaric hypoxia-induced pulmonary hypertension in that it simulates the main pathogenic factor of HAPH to trigger PH. Though traditionally hypoxia is thought to be the main cause of pulmonary hypertension, hypobaric infrabar is also a non-negligible part of HAPH pathogeneses in that it cannot be get rid of in the HAPH disease course. We have successfully established the rat model of intermittent hypobaric hypoxia induced hypertension [7], and found out that the development of hypobaric hypoxia-induced pulmonary hypertension could be greatly influenced by the increase of altitudeand time duration of hypobaric hypoxia. As observed in the intermittent hypobaric hypoxia induced hypertension model [7, 21], the pulmonary vascular pathophysiological characteristics of rat models exposed to the stimulate-altitude of 5,000 meter for 4 weeks were much more consistent with the pathophysiological characteristics of high-altitude pulmonary hypertension, so we adopted the same altitude and time duration in this study. Differently, we used the sustained exposure to hypobaric hypoxia environment instead of intermittent exposure in this study, considering that sustained exposure is more in line with the pathogeneses of HAPH. And the detailed comparison of sustained hypobaric hypoxia induced hypertension model and intermittent hypobaric hypoxia induced

hypertension model need to be done next. Furthermore, to explore the role of hypobaric infrabar in the pathogeneses of HAPH, the comparison of normobaric hypoxic model and hypobaric hypoxic model will also be a study point.

Conclusion

The rat model of hypobaric hypoxia-induced pulmonary hypertension has been successfully established by automatic adjusting hypobaric hypoxia chamber. Sustained exposure to a low oxygen environment at a simulate-altitude of 5,000 meter for 4 weeks have caused the pathological remodeling of pulmonary vascular walls and pulmonary hypertension, and further led to a series of pathological changes, including right ventricular hypertrophy. This model is easy to be replicated with good reproducibility and can be widely used in further studies.

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Disclosure of conflict of interest

None.

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