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Research on rat models of hypobaric hypoxia-induced pulmonary hypertension

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Abstract. – OBJECTIVE: Rat models of hypobaric hypoxia-induced pulmonary hypertension are commonly used in studies of chronic mountain sickness, while there are few researches specially focusing on these rats model. This study aims to exploring possible pathogenesis of hypobaric hypoxia-induced pulmonary hypertension by experimenting on hypobaric hypoxia-induced PH rat models at different simulate- altitudes.

MATERIALS AND METHODS: 32 healthy male SD rats were randomly divided into six groups of different degree and time period of hypobaric hypoxia. The mean pulmonary arterial pressure (m PAP), right ventricular pressure (RVSP), the right ventricle (RV), left ventricular (LV), ventricular septal (S), the right ventricular hypertrophy index (RVHI) [calculated under the formula of RV / (LV + S)], hematoxylin-eosin staining, elastic fibers staining, the ratio of the thickness of vascular wall to its outer diameter (MT%), the ratio of the cross-sectional area of the middle vascular wall to the total vascular cross-sectional area (MA%); the α-SMA, and the Ki6 expressions were detected to evaluated the pulmonary hypertension.

RESULTS: There were significant differences of the mPAP, RVSP and RVHI value between the hypobaric hypoxia groups and the control group ($p < 0.05$). The mPAP, RVSP, RVHI, MT%, MA%, α-SMA, and Ki6 of rats in model groups at an altitude of 3KM were higher than those of the control group, which raised gradually with the number of weeks increasing. The mPAP, RVSP, RV / (LV + S) value, MT%, MA%, α-SMA, and Ki67 of the 5KM-4W group were significantly higher than those of the control group ($p < 0.05$).

CONCLUSIONS: Rat models with pulmonary hypertension at different altitudes have been successfully established by automatic adjusting hypobaric hypoxia chamber. Exposure to a low oxygen environment at a simulate-altitude of 3 km for 8 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 provides evidence for clinical trial of drugs.

Key Words:
Hypobaric hypoxia, Pulmonary hypertension, Animal models.

Introduction

Pulmonary hypertension is a disease that can induce pulmonary vascular remodeling and constriction and dysfunction of pulmonary circulation. It can give rise to continuous overloading of right ventricular pressure, causing the right heart failure and eventually leading to death[1-3]. High-altitude pulmonary hypertension falls into the third category of pulmonary hypertension. With a high morbidity and mortality rate, increasing attention has been being paid on it[4-7]. The sharp increase of pulmonary artery pressure causing by hypoxia-induced vascular constriction is one of the most important pathogeneses of high altitude pulmonary edema (HAPE)[8-10]. Due to the limitations of researches on human beings, studying on animal models of hypobaric hypoxia-induced pulmonary hypertension has become the key method of chronic mountain sickness researches. Therefore, right altitudes and successful preparation of animal models of hypobaric hypoxia-induced pulmonary hypertension play a key role in studying the pathogeneses of hypobaric hypoxia-

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induced pulmonary hypertension and its drug therapy. Currently, researches on rat models of hypobaric hypoxia-induced pulmonary hypertension are far from enough[11]. By studying on rat models at different simulate-altitudes, this study aims to explore the pathogenes1s of hypobaric hypoxia-induced pulmonary hypertension and provide a theoretical basis for its treatment.

Materials and Methods

Animals

32 healthy male Sprague Dawley (SD) rats, provided by Beijing Vital River Laboratory Animal Center, were randomly divided into six groups: one control group (CTRL group), one group exposed to hypobaric hypoxia at a simulated altitude of 3 kilometers for 4 weeks (3KM-4W group), one group exposed to hypobaric hypoxia at a simulate-altitude of 3 kilometers for 6 weeks (3KM-6W group), one group exposed to hypobaric hypoxia at a simulate-altitude of 3 kilometers for eight weeks (3KM-8W group), and one group exposed to hypobaric hypoxia at a simulate-altitude of 5 kilometers for four weeks (5KM-4W group). This animal experiment was conducted under the guidance of China Animal Management Committee and Peking University Animal Care Committee. The experimental animals were reared in the Experimental Animal Center of Beijing Millennium Monument Hospital, affiliated to the Capital University of Medical Sciences. Animals were kept in different cages, eight per cage, drinking tap water. Water and food were taken by rats freely. Keeping the fluorescent lights on for 12 hours per day; temperature of 17 to 23°C and humidity of 55% to 65% were maintained. The hypobaric hypoxia-3KM model groups were placed in an automatic adjustment hypobaric hypoxia chamber (simulated altitude of 3 km, atmospheric pressure of about 30 kPa, and oxygen concentration of 10%). The model group of hypobaric hypoxia-5KM was placed in another automatic adjustment hypobaric hypoxia chamber (simulated altitude of 5 km, atmospheric pressure of about 50 kPa, and oxygen concentration of 10%). All groups were exposed to a low oxygen environment intermittently for eight hours per day. The 3KM-hypobaric hypoxia model groups were kept under observation for 4 weeks, 6 weeks and 8 weeks respectively, while the 5KM-hypobaric hypoxia model group was observed for 4 weeks.

Hemodynamic Measurement

The 3KM-hypobaric hypoxia groups were released from the chamber at the 4th week, 6th week, and 8th week respectively. The 5KM-hypobaric hypoxia group left the chamber at the 4th week. The body weight (BW) of rats in each group was accurately measured after they got out of the chamber. After the measurement, intraperitoneal injections of 1% sodium pentobarbital (40 mg/kg) were given to the rats for anesthetization. The mean pulmonary artery pressure and right ventricular pressure were determined by the right heart catheter method.

Evaluation of Right Ventricular Hypertrophy

Hearts of the rat models were removed by thoracotomy after the hemodynamic test. The right ventricle (RV), left ventricular (LV) and interventricular septum (IVS) separated along the right edge of the interventricular septum from the heart were first dried by filter papers and then weighted respectively by electronic scales. The right ventricular index (RV/LV+S) was used to reflect the degree of right ventricle hypertrophy.

Morphometric Analysis of Pulmonary Arteries

After the hemodynamic test, the rats' lung tissues were extracted along the hilar cross section, and then fixed in the 10% formalin solution (produced by Jinan Baibo Biological Technology Co., Ltd., 500 ml/box, SDA License No. Guoyaozhunzi 1400105) for 48 hours. The tissues were dehydrated paraffin-embedded and 5 μm conventionally sectioned for HE staining and elastic fiber staining. Five elastic fibers stained slices of lung tissues of each rat were randomly selected. From each slice, 6-8 pulmonary arterioles with diameters less than 100 μm and a relatively round cross-sectional area were selected. The thickness of pulmonary arterioles (MT) and the cross-sectional area (MA) of the middle vascular walls were measured by PIPS-2020 type image analyzer, then based on which the MT% and MA% were calculated accordingly.

Immunohistochemical Analysis

Determination of vascular smooth muscle actin (α-SMA): lung tissues extracted along the hilar transaction were fixed in the 10% formalin-solution. Then they were dehydrated paraffin-embedded, 5 μm conventionally sectioned, dewaxed and dipped into water after-

wards. Subsequently, they were incubated in 3% hydrogen peroxide for 5-10 min, rinsed with distilled water and soaked in phosphate buffered saline (PBS). Next, after being blocked by 5% normal goat serum, the lung tissues were infused with diluted anti-a smooth muscle actin (α-SMA, A2547; Sigma-Aldrich, St. Louis, MO, USA), staying overnight at the temperature of 4°C and then were rinsed by PBS the next day. The biotin-labeled secondary antibodies were added into the solution, which was incubated for 20 min at the temperature of 37°C and then was rinsed by PBS. Peroxidase-labeled streptavidin horseradish was added dropwise into the solution. The lung tissues were incubated in the solution for 30 min at the temperature of 37°C and then were rinsed by PBS. After the DAB (diaminobenzidine) staining and hematoxylin redyeing, the lung tissues were dehydrated, hyalinized and mounted. Five lung tissue slices of each rat were randomly selected to observe the changes of smooth muscle actins at different altitudes in different weeks under the optical microscope. Five high power fields of each slice were randomly selected and analyzed by the PIPS-2020 image analyzer to calculate the expression rate of smooth muscle actions in pulmonary arterioles.

Determination of Ki67: Lung tissues extracted along the hilar transaction were fixed in the 10% formalin-solution. Then, they were dehydrated paraffin-embedded and 5 μm conventionally sectioned. After the section, the tissues were quickly transferred into xylene and ethanol to dewax. 0.03% hydrogen peroxide was used to eliminate the endogenous peroxidase activity. The tissues were dipped in PBS for 5 min and washed 3 times afterwards. Then the normal goat serum confining liquid (PBS 1:1000 diluted) was added dropwise and kept at room temperature for 20 min. Filtering out the serum and adding ki67 primary antibodies; keeping the solution overnight at the temperature of 4°C and rewarming it at 37°C for 1h the next day. The tissues were dipped into PBS for 5 min and washed 3 times. Then adding the secondary antibodies at room temperature, keeping it for 4h (protecting from light), dipping in PBS for 5 min and washing 5 times to get rid of the unbound fluorescent secondary antibodies. DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) was diluted by PBS at the ratio of 1:1000. The slice was dipped in PBS for 5 min and washed 5 times after the one-minute DAPI staining. Then, drying the slice and mounting it with the 50% buffered glycerol. The Ki67 expressions in pulmonary arteries at different altitudes in different weeks were observed under the fluorescent microscope. It would be considered as positive, if there was any red fluorescence appearing in the background. Five high power fields of each slice were randomly selected for observation. ImageJ software (version 1.48u, National Institutes of Health, Bethesda, MD, USA) was used to analyze the fluorescence intensity.

Statistical Analysis

All experiments were repeated at least 3 times. All statistics and experimental data were finally evaluated by the formula of (mean ± SEM). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. *t*-test and One-Way ANOVA test were used to detect the differences in measurement data among the six groups. When $p < 0.05$, the difference was of statistical significance.

Results

The General Situation In Rats

Rats in the control group were stout and active with smooth and shiny fur, steady breath and increased weight; compared with the blank control group, declined activity, loss of appetite, preference to lying down and depression of different degrees could be seen in rats of other model groups. The weight loss of rats in the 3KM-hypobaric hypoxia groups increased gradually as time went by. Weight loss or even wheeze appeared mostly in rats of the 5KM-hypobaric hypoxia group, who were less active and preferred to lie down. Their fur was always dry and frizzy.

Hemodynamic

Compared with the control group, the mean pulmonary artery pressure, right ventricular pressure, and right ventricular hypertrophy index of the 3KM-hypobaric hypoxia model groups were much higher, which would gradually increase as time went by. The differences in data between the 3KM-8W group and the control group were of statistical significance ($p < 0.05$). The mean pulmonary artery pressure, right ventricular pressure, and right ventricular hypertrophy index of

rats in the 5KM-4W group increased more than those of the control group and the differences between the two groups were statistically significant ($p < 0.05$) (Figure 1).

Morphometric Analysis of Pulmonary Arteries

Heart gross observation: hearts of rats in 3KM-hypobaric hypoxia groups and 5KM-hypobaric hypoxia group became much bigger than those of the control group. Their right ventricular area expanded with a blunter apex cordis and their anterior and posterior interventricular grooves invaginated a little bit; the infundibular part of their right ventricles began to bulge outward and upward; a significant increase could be seen in the inner diameter of the right ventricular outflow tract. The differences mentioned above became more significant with the increase of pressure.

Under the light microscope, thin pulmonary artery walls with smooth lining and flat endothelial cells could be seen in rats of the control group (Figure 2a, A, F) after the HE and elastic fibers staining (Figure 2). The hypertrophy of artery intima and media of rats in 3KM-hypobaric hypoxia model groups became more and more patent as time went by and their smooth muscle cell proliferation gradually increased, arraying disorderly with narrower lumen, which might be accompanied by infiltration of inflammatory wall cells of various degrees. A significant increase in thrombosis in rats of 3KM-8 week model group (Figure 2a, D, I) could be seen. The hypertrophy of artery intima and media was most evident in rats of 5KM-4 week group, whose smooth muscle cell proliferation increased significantly and cells arrayed disorderly with luminal stenosis, which were the typical features of pulmonary arteriole reconstruction. The inflammatory cell infiltration became more patent and a significant increase in luminal thrombosis could be seen.

The MT% and MA% value of the 3KM-hypobaric hypoxia model groups were much higher than those of the control group. As time went by, these two indexes of the model groups became much higher. The differences in MT% and MA% value between the 3KM-8 week groups and the control group were of statistical significance ($p < 0.05$) (Figure 2b). There was a significant difference in these two indexes between the 5KM-4 week group and the control group ($p < 0.05$) (Figure 2b).

α-SMA Immunohistochemical Analysis

The smooth muscle actin expressions of rats in the 3KM-4W, 3KM-6W, and 3KM-8W-hypobaric hypoxia model groups (Figure 3a, B, C, D) increased more than that of the control group (Figure 3a, A). The actin expression increased gradually as time went by. It was of statistical significance when comparing the actin expression of the 3M-hypobaric hypoxia model groups with that of the control group (Figure 3b). The smooth muscle actin expression and the muscular arteries of the 5 KM-4W group increased more significantly than those of the control group. The differences between the two groups were statistically significant ($p < 0.05$) (Figure 3b).

Ki67 Immunohistochemical Analysis

The Ki67 expressions of pulmonary arteriole smooth muscle cells of the 3KM-4W, 3KM-6W, 3KM-8W hypobaric hypoxia model groups (Figure 4a, B, C, D) increased more than that of the control group (Figure 4a, A). The Ki67 expressions gradually increased as time went by. The differences between the model groups and the control group were statistically significant ($p < 0.01$) (Figure 4b). Compared with the 3K-4W group (Figure 4a, B) and the control group (Fig-

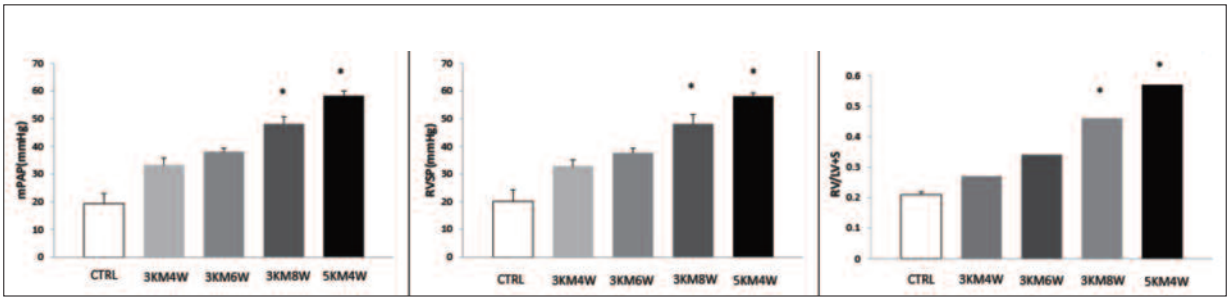

Figure 1. The differences of lung hemodynamic index in three rat groups. The bar graph represents the mPAP, RVSP, and RV/LV+S in various group rats, $*p < 0.05$, as compared with control group (n = 8, per group).

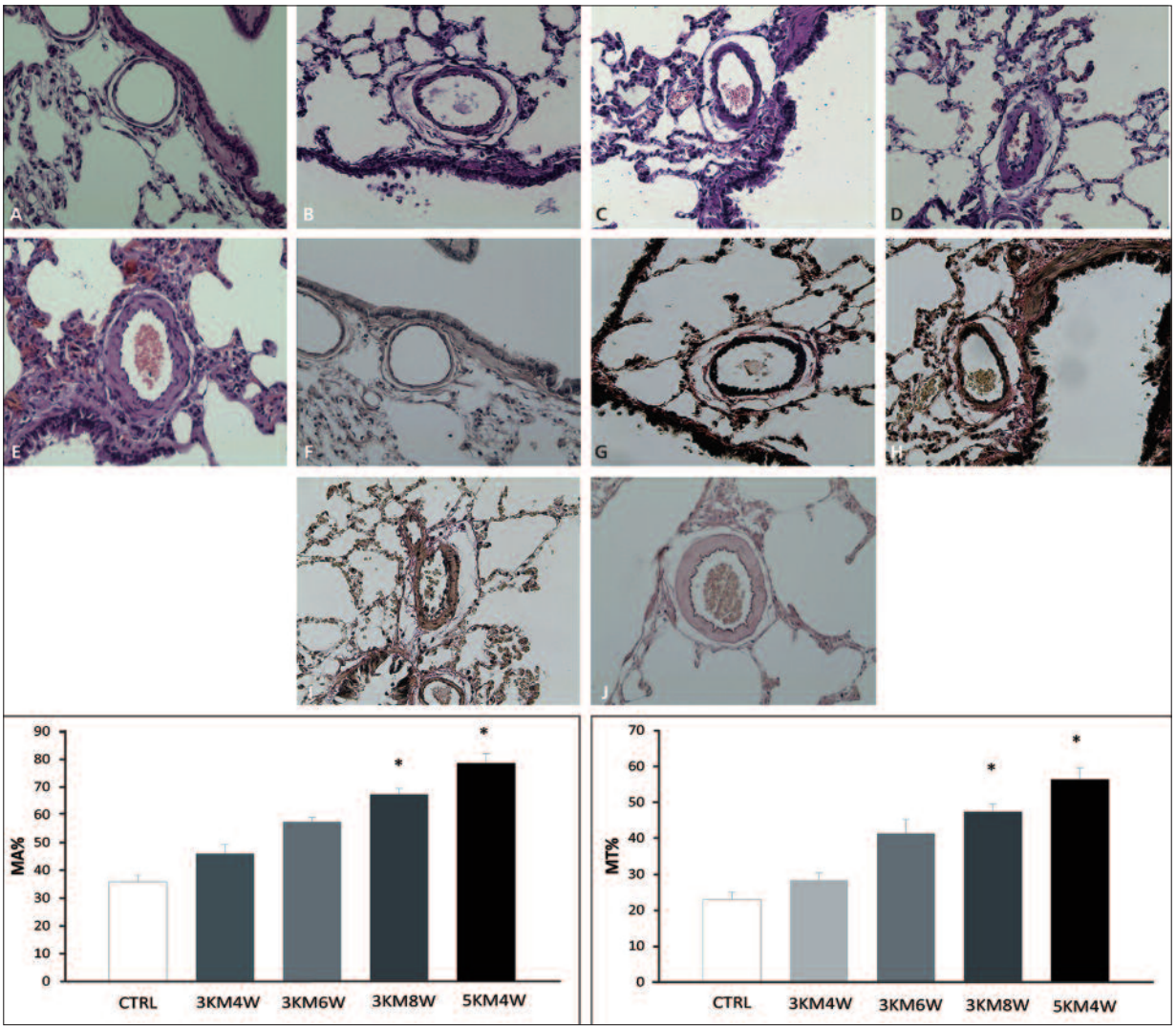

Figure 2. Pulmonary arteriole hematoxylin-eosin staining and elastic fibers staining of different rat groups. ***A, F,*** CTRL ×400; ***B,G,*** 3KM 4W ×400; ***C, H,*** 3KM 6W ×400; ***D, I,*** 3KM 8W; ***E, J,*** 5KM 4W. The graph (a) are shown demonstrating thickness of the arterial vascular medial induced by hypobaric and hypoxia, Note significant medial thickness ***(E, J)*** especially in 5KM 4W group (×400). The bar graph represents the medial/(medial + lumen) thickness ratios of the small pulmonary arteries in various group rats, $^{*}p < 0.01$ as compared with control group. $^{\#}p < 0.01$ as compared with 3KM 4Wgroup (n = 8, per group).

ure 4a, A), the Ki67 expression of pulmonary arteriole smooth muscle cells of the 5KM-4W group (Figure 4a, E) increased much more significantly, which was of great statistical significance ($p < 0.01$) (Figure 4b).

Discussion

High-altitude pulmonary hypertension is characterized by changes in pulmonary vascular functions and structure, including the dysfunction and remodeling of HPV and vascular endothelium, which can cause right ventricular hypertrophy and failure, and ultimately lead to death[12,13]. In the plateau area, hypoxia is the main cause of pulmonary hypertension. Contraction of pulmonary arteries and arterioles caused by acute hypoxia is called hypoxic pulmonary vasoconstriction (HPV), which can cause increased pulmonary vascular resistance, leading to increased pulmonary artery pressure and chronic persistent hypoxia. Gradually, HPV and pulmonary vascular remodeling may altogether lead to the persistent hypoxic pulmonary hypertension (HPH), which may lead to increased pressure and hypertrophy of the right

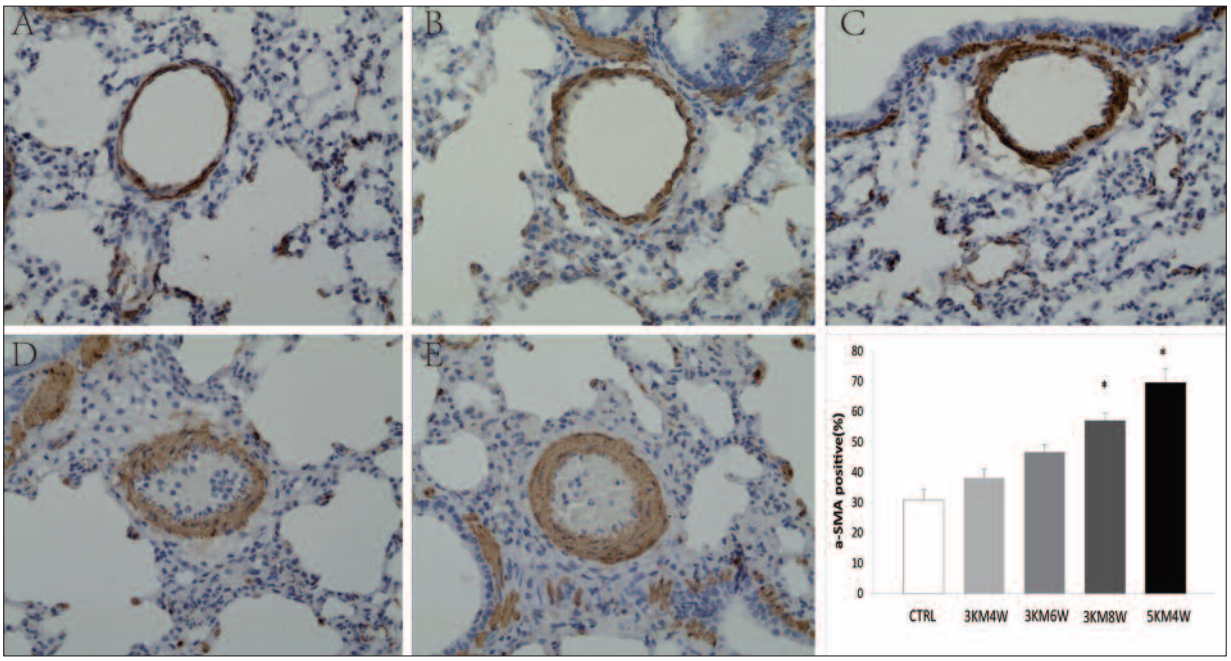

Figure 3. α-SMA Immunohistochemistry staining. ***A,*** CRTL ×400; ***B,*** 3KM 4W ×400; ***C,*** 3KM 6W ×400; ***D,*** 3KM 8W ×400; E 5KM 4W ×400. The graph (a) are shown demonstrating smooth muscle thickness of the arterial vascular medial smooth muscle induced by hypobaric and hypoxia, Note significant thickness especially in 5KM 4W group. The bar graph (b) represents the smooth muscle thickness ratios of the small pulmonary arteries in various group rats, $*p < 0.05$ as compared with control group (n = 8, per group).

ventricle and may even cause severe right heart failure. HPH is a critical phase of the progression of high altitude heart disease[14-16]. However, it is not clear that how long it will take and at which altitude for people living in the plateau area to have pulmonary vascular constriction and pulmonary hypertension. Since there are many limitations of studies on human beings, it is of great

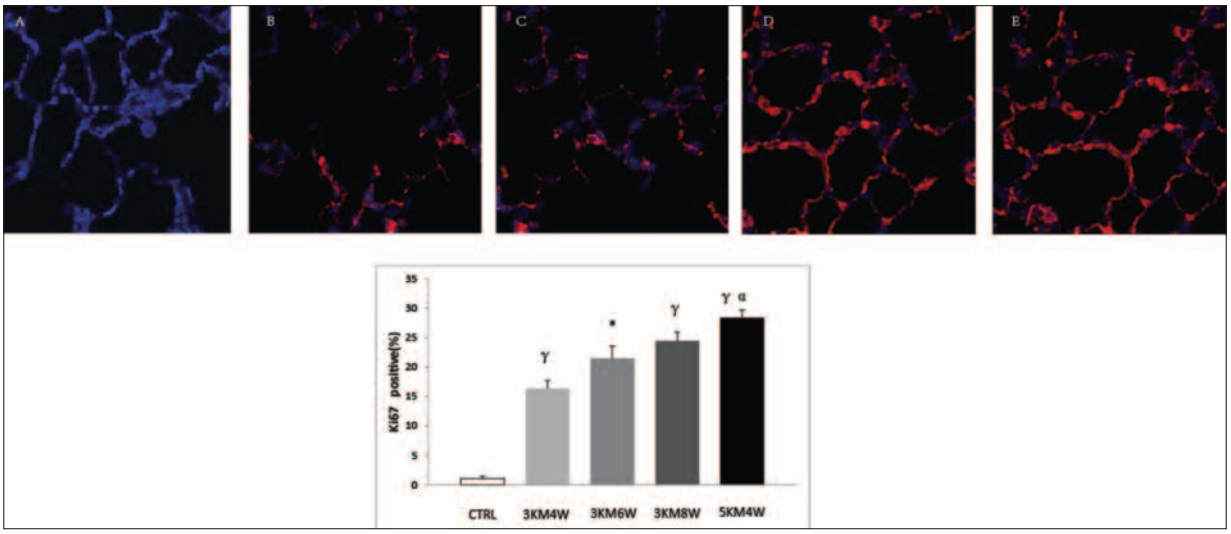

Figure 4. Ki67 staining. ***A,*** CTRL ×200; ***B,*** 3KM 4W ×200; ***C,*** 3KM 6W ×200; ***D,*** 3KM 8W ×200; ***E,*** 5KM 4W ×200. The Ki67 expression of each group AII increased, Note significant expression of Ki67 especially in 5KM 4W group. The bar graph (b) represents the Ki-67 positive ratios of the small pulmonary arteries in various group rats, $*p < 0.01$ various group rats compared with CRTL group, $^{\#}p < 0.01$ 5KM 4W group compared with 3KM 4W group (n = 8, per group).

significance to establish animal models with pulmonary hypertension in a simulated high-altitude environment, which can not only enable further study on the pathogenesis and development mechanisms of pulmonary hypertension, but also can allow us to make better clinical observations on drug treatment of this disease. Multiple animal models[17-20] had been established so far in order to explore the pathogenesis of hypobaric hypoxia-induced pulmonary hypertension. By observing the pulmonary hypertension rat models replicated in the simulated hypobaric hypoxia-high altitude chamber, we found that even though the pulmonary hypertension and right ventricular pressure did not increase significantly at the altitude of 3 km in the 4th week, the a-SMA and Ki67 expression began to increase, which suggested proliferation of pulmonary vascular smooth muscle cells, indicating that pulmonary vascular constriction and mild pulmonary vascular remodeling had already appeared. The contraction and remodeling became increasingly with the extension of time living in plateau area. Moderate pulmonary hypertension, right ventricular dysfunction and hypertrophy, moderate pulmonary vascular remodeling, orthotropic thrombosis and increased inflammatory cell infiltration could be seen in rat models at the altitude of 3 km in the 8th week. The study results mentioned above indicated that the animal models of pulmonary hypertension had been successfully established at the simulated altitude of 3 km in the 8th week. Compared with the 3KM-8W group, a significant increase in the mean pulmonary artery pressure and right ventricular pressure and an obvious pulmonary hemodynamic change could be seen in rats of the 5KM-4W group. The orthotropic thrombosis, significantly increased inflammatory cell infiltration and the muscularization of pulmonary arterioles within the acini could be detected in these rats. The thickness of the rats' smooth muscle layer between the inner and outer elastic fiber membranes, also called the middle layer of the micro-vascular wall increased more obviously. Further luminal stenosis, increased Ki67 and a-SMA expression and smooth muscle cell proliferation presented the characteristics of pulmonary arterial remodeling together with all descriptions mentioned above. It could be indicated that at the simulated altitude of 5 km, a better rat model of hypobaric hypoxia-induced pulmonary hypertension had been successfully established. The pulmonary vascular contraction and remodeling, right ventricular hypotrophy and the vascular smooth muscle proliferation of rats in the 5KM-4W group were much more evident than rats in the 3KM-8W group. Our study results showed that the development of hypobaric hypoxia-induced pulmonary hypertension could be greatly influenced by the altitude and time duration of hypoxia. The pulmonary vascular contraction and remodeling became more evident with the increase of altitude and hypoxia time. The pulmonary vascular resistance increased progressively, eventually leading to the pulmonary hypertension. The pulmonary vascular functioning and morphological characteristics of rat models exposed to a hypoxia environment at a stimulate-altitude of 5 km for 4 weeks were much more consistent with the pathophysiological characteristics of high-altitude pulmonary hypertension. This model is easy to be replicated with good reproducibility and provides evidence for clinical trial of drugs.

Conclusions

Rat models with pulmonary hypertension at different altitudes have been successfully established by automatic adjusting hypobaric hypoxia chamber in this study. Exposure to a low oxygen environment at a simulate-altitude of 3 km for 8 weeks caused the pathological remodeling of pulmonary vascular walls and pulmonary hypertension, and further led to a series of pathological changes like right ventricular hypertrophy. However, the pulmonary vascular functioning and morphological characteristics of rat models in a hypobaric hypoxia environment at a stimulate-altitude of 5 km for 4 weeks was more consistent with the pathophysiological characteristics of high altitude pulmonary hypertension. This model is easy to be replicated with good reproducibility and provides evidence for clinical trial of drugs.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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