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Response to chronic intermittent hypoxia in blood system of Mandarin vole (*Lasiopodomys mandarinus*)

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ABSTRACT

Mandarin voles (*Lasiopodomys mandarinus*) spend almost all their lives underground and must have evolved remarkable physiological adaptations to subterranean hypoxic stress. To understand the response to hypoxia in blood system of this rodent in a region with lower altitude, we tested and compared the responding characteristics between Mandarin vole and Kunming mouse (*Mus musculus*) under chronic normobaric hypoxic treatment (10.0% O_2, 4 w). The results showed that: 1) as for responses to chronic hypoxia, HIF-1α, EPO and VEGF exhibited similar patterns in two species. The expression of HIF-1α and VEGF significantly increased while EPO decreased significantly, and HIF-1α showed a greater increase at 10.0% oxygen level in Mandarin voles; 2) both rodents responded to chronic hypoxia mainly by increasing MCH, though KM mouse responded more acutely; 3) the change in MCHC in Mandarin vole was ignorable though it is significantly higher than that in KM mouse whose MCHC changed extensively and 4) both before and after hypoxic treatment, the capillary density in Mandarin vole was significantly higher than that in KM mouse, and it increased sharply in KM mouse after treatment. Our results indicated that, compared to KM mice, Mandarin voles did respond effectively to hypoxia stress after long-term adaptation to subterranean life environment.

1. Introduction

Oxygen is essential to life for most organisms, and the concentration of oxygen in the surroundings, consequently the availability of oxygen, usually changed with temperature, humidity, atmospheric pressure, altitude, etc. Among the fluctuations of oxygen level, hypoxia, a low oxygen level in tissue, is a common stress affecting an organism's homeostasis, and it was regarded as a great threat to humans and most mammals who show weak tolerance to hypoxia (White and Zhang, 2003; van der Meer et al., 2005). Nonetheless, certain species that chronically inhabited hypoxic ecological niches have developed unique and effective strategies and physiological mechanisms to survive under hypoxia conditions (Shams et al., 2005). Different adaptive changes both functional and anatomical have been found in rats and other laboratory animals during acclimatization to the hypoxia condition, including cardiac, pulmonary and endocrine gland changes which were observed in animals exposed to simulated hypoxia conditions (Chaiban et al., 2008; Ma et al., 2008; Nevo et al., 2001).

Those subterranean rodents which spend almost all their lives underground and are exposed to fluctuated O_2 and CO_2 levels in their burrows represent an example of hypoxia-adapted animals. Concentrations of O_2 and CO_2 usually varied with rainfall and agro-type, and the oxygen concentration inside burrows even reached 7% of normal after rain (Kuhnen, 1986; Shams et al., 2005). Subterranean mammals have evolved a unique set of structural and functional modifications in their cardiovascular and respiratory systems to ensure themselves successfully inhabiting the extreme environment for prolonged periods (Avivi et al., 1999). Physiological alterations include higher myocardial performance (Edoute et al., 1988), increase in lung diffusion capacity (Widmer et al., 1997), and larger density of blood vessels that correlated with the expression pattern of vascular endothelial growth factor (VEGF) (Widmer et al., 1997; Avivi et al., 2005). Subterranean voles also evolved anatomical adaptations including decreased diffusion distance of oxygen to the mitochondria, and species-based differences in hemoglobin and erythrocyte number that may augment oxygen delivery at low oxygen level (Arieli, 1990; Yang et al., 2006). Consequently, the oxygen transportation in vessel and diffusion between tissues were promoted.

Cardiovascular system, functionally delivering oxygen to tissues, would unsurprisingly have animals respond to hypoxia stress, and the responses mainly concerning erythropoiesis and vascularization which were regulated by erythropoietin (EPO) and VEGF, respectively (Avivi et al., 2005; Shams et al., 2004). The blood properties, such as increased erythrocyte number (Arieli et al., 1986), reduced mean corpuscular volume (MCV) (Arieli et al., 1986), and changed 2,3-diphosphoglycerate:hemoglobin ratio (Ar et al., 1977), would facilitate O_2 transportation. Diffusion distance for oxygen to mitochondria would be shortened with increase in capillary density (Avivi et al., 1999). The hypoxic response is mainly regulated by hypoxia-inducible

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factor 1α (HIF-1α), a subunit of HIF-1. Under hypoxia conditions, HIF-1α could regulate O_2 homeostasis in mammals at both transcription and post-transcription stages (Avivi et al., 1999), and initiate immediately a response to counteract the potential deleterious effects of hypoxia in molecular level (Jewell et al., 2001; Manalo et al., 2005). As a key factor involved in erythropoiesis and expressed mainly in fetus liver and adult kidney (Zanjani et al., 1977), EPO would regulate the level of circulating red blood cells (RBC), and also angiogenesis stimulates neovascularization (Jaquet et al., 2002; Ribatti et al., 1999). What's more, being a potent angiogenic factor, VEGF is critical during physiological production of new blood vessels (Risau, 1997), while the controlling factor in VEGF expression is oxygen concentration (Shweiki et al., 1992).

Mandarin vole (*Lasiopodomys mandarinus*), a small-sized subterranean species of rodent pest, inhabited underground environment almost all its life span in cropland in most area of north China (Zhang and Wang, 1998). Although much is known on its population ecology including mating system (Tai et al., 2000), life history characters (Zhang et al., 1997), population ecology (Wang and Zhang, 1995), social behavior and heredity (Wang et al., 2003; Jia et al., 2008), relatively little is reported about the adaptation and mechanisms to prolonged or chronic hypoxia in underground environment. Previous studies on this animal showed that there were sexual differences in blood physiology and biochemistry, the RBC and hemoglobin (HGB) in females were significantly higher than that in males, which led to a higher oxygen-carrying capacity in female under hypoxic conditions (He et al., 2001). In this research, we intend to investigate, under the chronic normobaric hypoxia condition, the blood properties and capillary density of cardiac muscle in Mandarin voles, to reveal the responding mechanisms to normobaric hypoxia of this subterranean rodent species occurring in plain region with lower altitude, and provide basic data for hypoxia acclimatization.

2. Materials and methods

2.1. Animals

Mandarin voles (*L. mandarinus*) were captured live from cropland in Xinzheng (34°52′N, 113°85′E), Henan, China, and housed individually in polycarbonate cages (37 cm × 26 cm × 17 cm) for 2–4 months following a 12:12 light cycle (lights on: 0800–2000 h) and maintained at a temperature of 20–24 °C. Plenty of cottons were provided for nesting material. Animals were fed with carrots and a mixture of mice/rabbit feed (products of Laboratory Animal Center of Henan Province, China), and water was accessed *ad libitum*. All procedures of this research were approved by the Animal Care and Use Committee of Zhengzhou University and were in accordance with the Guide for the Care and Use of Laboratory Animals of China.

A total of 15 healthy female adult Mandarin voles, similar in body mass (30–40 g) were selected for the experiment. Ten voles were selected randomly to follow the chronic hypoxic treatment, and the others were for control. Fifteen female Kunming (KM) mice (*Mus musculus*) (30–35 g) (Laboratory Animal Center of Henan Province, China) were selected and grouped following Mandarin voles.

2.2. Experimental procedure

2.2.1. Hypoxic treatment

DS-II hyperbaric cabin for animal experiment (Huaxin Hyperbaric Cabin LTD., Weifang, China) was adapted in this research.

In April 2009, experimental animals in each cage were transferred into cleaned Oxygen Cabin for 4 h per day (8:00–12:00 h), and lasted for 4 weeks (Shams et al., 2004), under the oxygen concentration of 10% relative to normoxia. This was referred to chronic intermittent normobaric hypoxic treatment. The oxygen level was kept stable by balancing the flow rate of oxygen and nitrogen, and was monitored via an oxymeter. The normobaric was controlled by balancing the outflow of gas and monitoring via a micromanometer. To eliminate the potential influence of CO_2 given out by animals themselves, a bottle of sodium hydroxide was placed in the cabin to absorb the CO_2.

After the trial each day, we took the subjects away from the Cabin, and cleaned the inside wall and floor of the cabin by 75% alcohol.

The control groups of Mandarin vole and KM mouse breathed the room air and lasted for 4 weeks before blood sampling at the same time as the hypoxic treatment.

2.2.2. Tissue and blood sampling

Once the hypoxia treatment was completed, experimental animals were quickly taken out from the Cabin and anesthetized by i.p. injection of 20% urethane (5 mL/1000 g body mass). The eye of the tested subject was extirpated to collect blood with an anticoagulant tube for the hemogram measurement. The kidney was taken out immediately after dissection, and immersed in liquid nitrogen and stored at −80 °C. Cardiac muscle (quadrate) for capillary density analysis was placed in 4% paraformaldehyde for 12 h at 4 °C.

2.2.3. Measurement of blood indices

An Xs-800i Automatic Hematology Analyzer (Sysmex, Tokyo, Japan) was used to measure the blood indices including hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCHC).

2.2.4. Detection of expression of HIF-1α, EPO and VEGF

Expressions of HIF-1α, EPO and VEGF in the kidney were detected by using an enzyme-linked immunosorbent assay (ELISA) KIT (ADL, San Antonio, USA). The process follows the manufacturer's instruction, and this method has been proved successful and repeatable by previous studies (Portero-Otin et al., 1999; Buss et al., 1998).

2.2.5. Measurement of cardiac muscle microvessel

Cardiac muscle microvascularity was determined by quantifying the density of capillary structures (Widmer et al., 1997). Capillary structures were identified by staining with Toluidine Blue (Mathieu-Costello, 2001). Sections from paraformaldehyde-fixed paraffin-embedded cardiac muscle blocks were cut at 5 μm, deparaffinized and dehydrated with xylene and graded alcohol. Three samples in hypoxic Mandarin vole groups and two in hypoxic KM mouse groups were canceled owing to failed operation during cutting sections and staining. The number of vessel profiles was counted at 400× magnification by a Nikon microscope (eclipse e200, Nikon, Japan). Evaluation of vascularity was performed in microscopic fields where the myofibrils were sectioned transversely, and the cross sections and other characteristics were not counted (Avivi et al., 1999). The diameter of the field of vision at this magnification is 0.50 mm. Total five fields were counted for each tissue sample and the average number of counts was recorded as vessels per field at higher magnification. All this work was done by one person to eliminate errors as far as possible.

2.3. Data analysis

All data were expressed as Mean ± SE, and statistical analysis was performed by employing SPSS for Windows (version 13.0). Student's *t*-test was used for comparisons between data sets. Two-way ANOVA was used to evaluate the differences between groups. One grouping factor was species (Mandarin vole and KM mouse), and the other grouping factor was the hypoxic treatment (10.0% and normoxia). All tests were two tailed and $P<0.05$ was considered significant.

3. Results

3.1. Measurement on expression of HIF-1α

We measured the expression of HIF-1α in kidney in two rodent species and the results were presented in Fig. 1. The expression of HIF-1α significantly increased under chronic hypoxic treatment compared to the normal hypoxia in both Mandarin voles ($t=-6.803$, $P<0.01$) and KM mice ($t=-6.581$, $P<0.01$) (Fig. 1). At normal hypoxia level, however, HIF-1α was expressed remarkably lower in Mandarin vole than that in KM mice ($t=3.176$, $P<0.05$) (Fig. 1).

No significant interaction between species and hypoxic treatment existed in this research ($F_{(1,26)}=2.289$, $P>0.01$) (Table 1). However, the species ($F_{(1,26)}=9.889$, $P<0.01$) and oxygen level ($F_{(1,26)}=88.140$, $P<0.01$) significantly influenced the expression of HIF-1α (Table 1).

3.2. Variations in EPO expression

The expression of EPO in the kidney was detected in two rodents and the result was given in Fig. 2. The response patterns to hypoxia were similar in our experimental animals, and EPO values significantly decreased in Mandarin voles ($t=7.296$, $P<0.01$) and KM mice ($t=5.287$, $P<0.01$) before and after hypoxia process, respectively. Surprisingly, no significant difference was found between two rodents at both normoxia and hypoxia groups (Fig. 2).

There was a significant effect of hypoxic treatment ($F_{(1,26)}=70.747$, $P<0.01$), but an insignificant difference existed between two rodents. Nor did we find a remarkable interaction between oxygen level and species (Table 1).

3.3. Variation in hemogram

The effects of chronic normobaric hypoxia on hemogram in two tested animals were shown in Fig. 3 and Table 1. The results revealed that, at normoxia level, the HCT ($t=-4.107$, $P<0.01$) (Fig. 3, A), MCV ($t=-5.526$, $P<0.01$) (Fig. 3, B) and MCH ($t=-2.971$, $P<0.01$) (Fig. 3, C) in Mandarin vole were significantly lower, while the MCHC was higher ($t=5.434$, $P<0.01$) (Fig. 3, D), than that in KM mouse. For Mandarin vole, after chronic hypoxic treatment, MCH ($t=-2.779$, $P<0.05$) increased significantly compared to the control group, as HCT and MCV insignificantly increased. In KM mouse, HCT ($t=-3.498$, $P<0.01$), MCV ($t=-2,976$, $P<0.01$) and MCH ($t=-4.089$, $P<0.01$) were all significantly increased after treatment. It should be emphasized that, at 10.0% oxygen level, HCT ($t=-4.158$, $P<0.01$), MCV ($t=-7.068$, $P<0.01$) and MCH ($t=-3.090$, $P<0.01$) were significantly lower, and the MCHC was significant higher ($t=10.162$, $P<0.01$) in Mandarin voles than that in KM mice (Fig. 3).

Two-way ANOVA revealed that there was no marked interaction between species and oxygen levels (Table 1). However, the factor of species significantly affected HCT ($F_{(1,26)}=37.076$, $P<0.01$), MCV ($F_{(1,26)}=82.162$, $P<0.01$), MCH ($F_{(1,26)}=17.122$, $P<0.01$), and MCHC ($F_{(1,26)}=93.656$, $P<0.01$); and oxygen levels, the other factor, was also found significantly influencing HCT ($F_{(1,26)}=9.348$, $P<0.01$), MCV ($F_{(1,26)}=12.024$, $P<0.01$) and MCH ($F_{(1,26)}=19.788$, $P<0.01$) (Table 1).

Table 1
Two-way ANOVA analysis of HIF-1α, EPO, HCT, MCV, MCH, MCHC, VEGF and capillary density in Mandarin vole and KM mouse under chronic hypoxia treatments.

	HIF-1α	EPO	HCT	MCV	MCH	MCHC	VEGF	Capillary density
Species	*	ns	*	*	*	*	ns	*
Oxygen concentration	*	*	*	*	*	ns	*	*
Species Oxygen concentration	ns	ns	ns	ns	ns	ns	ns	*

*$P\leq0.01$; ns (no significant) $P\geq0.01$.

3.4. Variations in VEGF expression

Fig. 4 presented the detected result in expression of VEGF in kidneys of two rodents. The response patterns of VEGF to hypoxia were similar in Mandarin vole and KM mouse. Compared to normoxia treatment, VEGF expression significantly increased at 10.0% oxygen level in both Mandarin vole ($t=-6.582$, $P<0.01$) and KM mouse ($t=-5.223$, $P<0.01$) (Fig. 4).

The results from two-way ANOVA revealed that, the interaction between treatment and species was not significant. Species factor did not remarkably affect the expression in VEGF, whereas the influence on VEGF expressions were distinguished ($F_{(1,26)}=70.634$, $P<0.01$) (Table 1).

3.5. Variations of capillary density

The detected values of capillary density in cardiac muscle in two rodents were showed in Fig. 5. The capillary density in Mandarin vole was significantly higher than that in KM mouse both at normoxia ($t=-5.519$, $P<0.01$) and 10.0% oxygen level ($t=-2.411$, $P<0.05$). Furthermore, compared to normoxia level, the value of capillary density in Mandarin vole increased slightly under hypoxic treatment; in KM mice, however, it increased remarkably ($t=-6.608$, $P<0.01$) (Fig. 5).

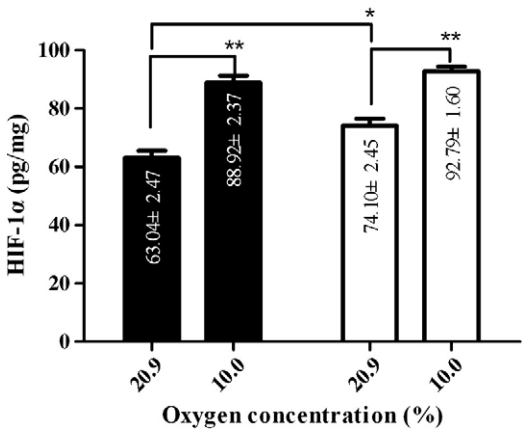

Fig. 1. Comparisons of the HIF-1α values between the 10.0% and 20.9% oxygen levels in Mandarin vole (■) and KM mouse (□). *: $P<0.05$, **: $P<0.01$.

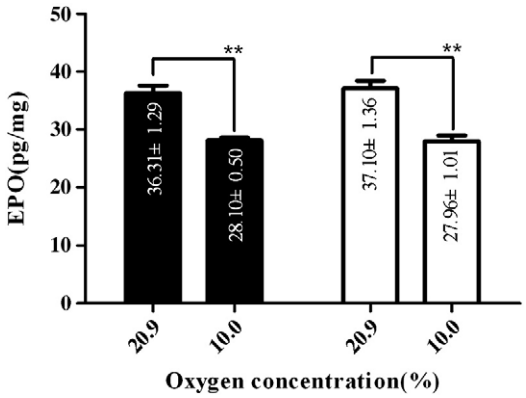

Fig. 2. Comparisons of the EPO values between the 10.0% and 20.9% oxygen levels in Mandarin vole (■) and KM mouse (□). **: $P<0.01$.

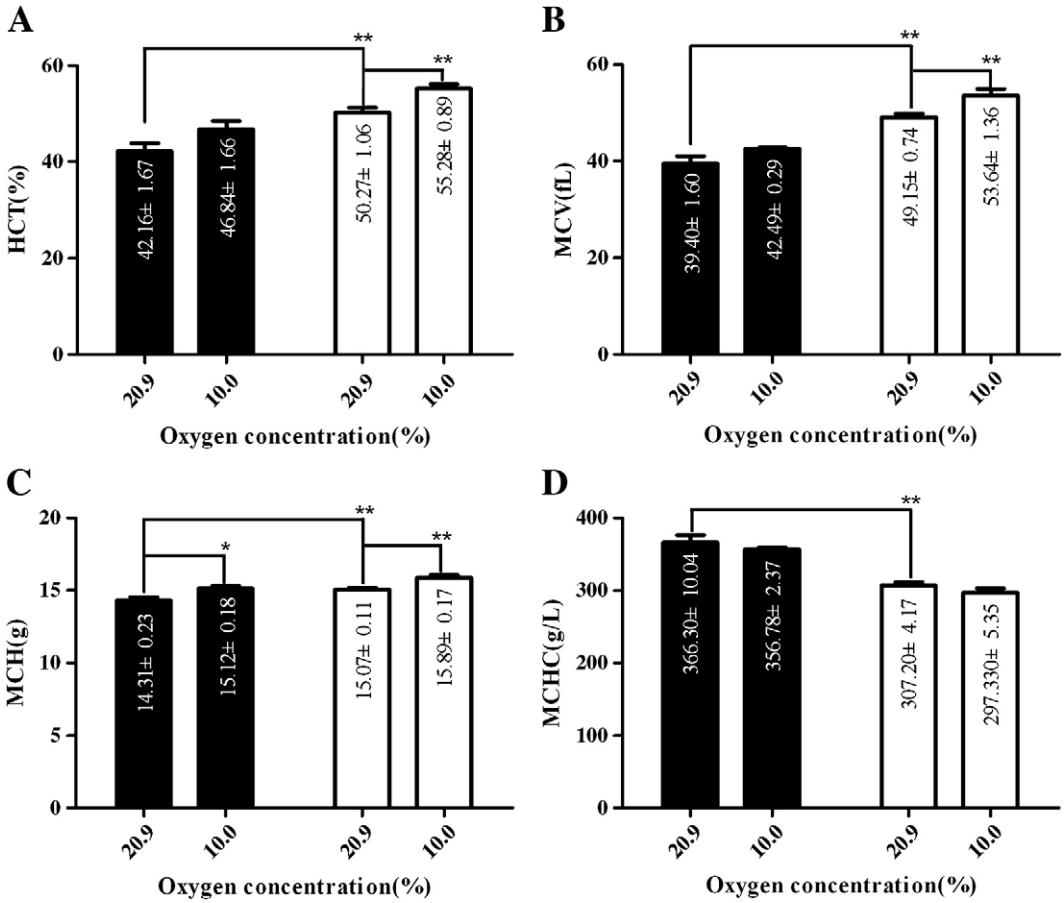

Fig. 3. Values of hematocrit (HCT) (A), mean corpuscular volume (MCV) (B), mean corpuscular hemoglobin (MCH) (C), and mean corpuscular hemoglobin concentration (MCHC) (D) at the 20.9% and 10.0% oxygen concentration levels between Mandarin vole (■) and KM mouse (□). *: $P<0.05$, **: $P<0.01$.

The result from two-way ANOVA indicated that, oxygen levels played a significant effect on capillary density ($F_{(1,21)}=34.289$, $P<0.01$), and the impact derived from species was also significant ($F_{(1,21)}=10.726$, $P<0.01$). Furthermore, capillary density was significantly affected by the interaction between species and treatment ($F_{(1,21)}=7.908$, $P<0.01$) (Table 1).

4. Discussion

Long-term hypoxic exposure leads to related physiological changes in mammals (Johnston et al., 2002), subterranean voles were thus considered an ideal model for hypoxic research (Kerem et al., 1973; Nevo, 1991), and the strategies to hypoxia in subterranean voles differed from those high-altitude mammals (Burlington and Maher, 1968) and diving mammals (Kerem et al., 1973), which would also respond to the special condition effectively and quickly.

Among general adaptive strategies to hypoxia stress in subterranean voles, blood properties and blood vessel density were usually used to reveal the mechanism of response, because the variation in blood properties can directly facilitate O_2 transportation for subterranean voles in burrowing atmospheres (Arieli et al., 1986; Arieli, 1990; Shams et al., 2005). However, the enhancement in HCT and hemoglobin concentration will increase viscosity and resistance

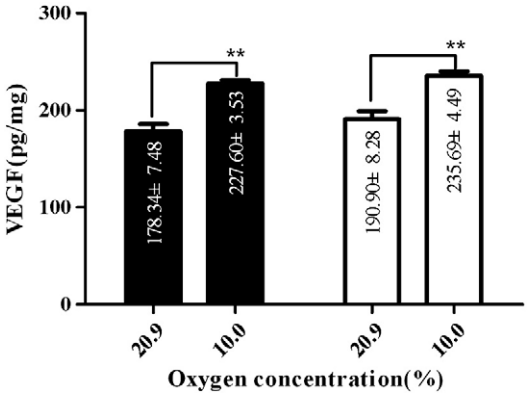

Fig. 4. Comparisons of the VEGF values between the 10.0% and 20.9% oxygen levels in Mandarin vole (■) and KM mouse (□). **: $P<0.01$.

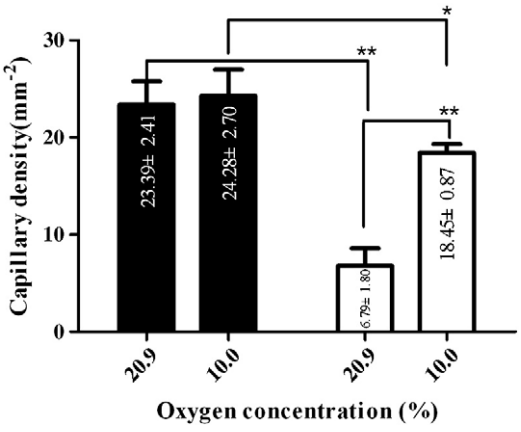

Fig. 5. Comparisons of the capillary density between the 10.0% and 20.9% oxygen levels in Mandarin vole (■) and KM mouse (□). *: $P<0.05$, **: $P<0.01$.

during blood circulation, and consequently result in pulmonary hypertension and polycythemia (Tucker et al., 1977; Zhang et al., 1982). These voles would evolutionarily avoid the impacts by reducing the MCV (Ar et al., 1977; Arieli et al., 1986) and the blood viscosity (Lacey et al., 2000; Ooi et al., 2000; Shams et al., 2005), and by modulating the rate between 2,3-diphosphoglycerate and hemoglobin (Ar et al., 1977; Arieli et al., 1986). Another important component of the evolved adaptations to hypoxia stress was increased capillary density which would shorten diffusion distance for oxygen to the mitochondria (Avivi et al., 1999).

Mandarin voles, though occurring in lower altitude area, live their lifetimes underground, they thus suffered a normobaric hypoxia in closed burrow systems, especially during rain and flood season, which would not recharged O_2 breathing (Arieli, 1990), and limited vertical ascent (Burlington and Maher, 1968). These voles must have evolved necessary adaptations to cope with the subterranean hypoxic stress.

4.1. Expression of HIF-1α, EPO and VEGF

HIF-1 exerts a key role in cellular responses to hypoxia, including the regulation of angiogenesis, energy metabolism and apoptosis (Avivi et al., 2005). HIF-1α would be rapidly degraded by proteasome under normoxic condition, and otherwise keep stable in hypoxia (Cockman et al., 2000; Jaakkola et al., 2001). We thus directly detected expression of HIF-1α at protein level in this experiment, other than the mRNA level in previous researches (Shams et al., 2004).

Although the expressions of EPO and VEGF are regulated by HIF-1α, we could not linearly confine the effect of HIF-1α expression on that of the EPO and VEGF expressions (Avivi et al., 2005; Shams et al., 2004). Under hypoxic conditions, EPO is mainly used for relative shorter processes in maintaining the erythrocyte level and urgent increases of erythrocyte production which are represented in hemogram, whereas VEGF is necessary for longer processes, including angiogenesis and neovascularization which lead to changes in capillary density. It needs to be noted that, the EPO-dependent erythropoiesis will take a few weeks to elevate the hematocrit (Koury et al., 1989). Our results showed that, the expression of HIF-1α significantly increased both in Mandarin vole and KM mouse without any significant differences between them. However, the expressive value of HIF-1α at normoxia was less in Mandarin voles than that in KM mice. That is to say, Mandarin voles showed an extensive response in HIF-1α to chronic hypoxia which may be due to the long-term underground life. At 10.0% oxygen level, compared to normoxia in both species, expression of EPO decreased significantly which was possibly because EPO combined with the accepter quickly for initiating hypoxic response, while VEGF increased remarkably since it was accumulated for later utilization in increasing capillary density. We thus think that EPO and VEGF are vital factors in chronic hypoxia adaptation in both rodents, and that EPO acts rapidly than VEGF, though other factors might exert a role in this response (Gassmann et al., 2003).

4.2. Hemogram and capillary density

Our results demonstrated that Mandarin voles and KM mice responded to chronic hypoxia in hemogram mainly by increasing MCH, and KM mice exhibited a rapid response. The HCT, MCV and MCH in Mandarin vole were all significantly higher than that in KM mouse, and the abovementioned three indices in Mandarin vole appeared to be more moderate than those in KM mouse under chronic hypoxia condition. These adaptive changes in Mandarin vole would decrease the viscosity and resistance of blood circulation, consequently, the oxygen delivery capacity would be increased to maintain higher hypoxic tolerance. Furthermore, these changes would effectively prevent pulmonary hypertension and polycythemia (Tucker et al., 1977; Zhang et al., 1982). The MCHC in Mandarin voles was also significantly higher than that in KM mice, which would produce higher oxygen-carrying capacity.

The capillary density in Mandarin voles, under normoxia environment, was significantly higher than that in KM mice. This also makes sense under chronic hypoxic treatment. Interspecific comparison showed that, after hypoxic treatment, the capillary density in Mandarin vole changed insignificantly, while increased about 2.7 times in KM mice.

In conclusion, our results suggested that, after long-term adaptation to underground environment, the subterranean Mandarin vole exhibited effective adaptation in blood system to hypoxic threat than KM mouse that is derived from a ground-inhabited ancestor. The molecular and evolutionary mechanisms in hypoxia adaptation in Mandarin vole need some further researches.

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