

Increase in Testicular Temperature and Vascularization Induced by Hypobaric Hypoxia in Rats

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ABSTRACT: The exposure of male rats to continuous chronic hypobaric hypoxia (HH) and intermittent chronic hypobaric hypoxia induced evident changes in testicular morphology and spermatogenic cell metabolism. The mechanisms that underlie these changes under HH are not known. In this work, we have tested the hypothesis that in rats subjected to HH, the testis undergoes changes in vascularization leading to changes in temperature homeostasis. Male Wistar rats (247 ± 16 g) were maintained in normobaric or hypobaric (428 torr, equivalent to 4600 m a.s.l.) conditions. At days 0, 5, 15, and 30 postexposure, 12 rats were anesthetized with ketamine, and the intratesticular temperature was determined. These rats were subsequently sacrificed and the testicles were fixed in formaldehyde and processed for routine histological analysis. Our

results showed that the height of the seminiferous epithelium decreased significantly at day 5 posthypoxia and thereafter, indicating a decreased spermatogenesis. Intratesticular temperature increased (1.5°C) and remained high after 5 days of hypoxia exposure. Correlated with these changes, histometrical analysis of the number of blood vessels in the testicular interstitium was significantly increased by day 5 and afterwards. Morphological classification of interstitial blood vessels indicates a transition from capillaries to larger vessels as the hypoxia exposure progresses.

Key words: Reproduction, hyperthermia, angiogenesis, high altitude.

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Hypobaric hypoxia (HH) is experienced by an increasing number of sea-level natives exposed to high altitude because of tourism, border patrol, mining, or rural health and education activities (eg, Germack et al, 2002). It has been suggested that HH reduces fertility in humans. Nonetheless, epidemiological studies of high- and low-altitude populations have not been able to verify this proposal (see Vitzthum and Wiley, 2003, for a review). The exposure of male rats to continuous chronic HH and intermittent chronic HH induced evident changes in testicular morphology (Gonzales et al, 1990; Gasco et al, 2003; Farias et al, 2005), loss of germinal cells, and a strong metabolic stress in spermatogenic cells (Farias et al, 2005). The mechanisms that underlie these changes in testicular and spermatogenic cell physiology under HH are not known. Based on hormonal

changes observed in man and rats at high altitude, some authors have proposed that HH affects the hypothalamic/gonad axis (eg, Nelson et al, 1975; Sawhney et al, 1985; Gonzales, 2004). Although not discarding a possible effect of HH on the hypothalamic/gonad axis, and based on the known effects of hypoxia on mammalian body organs (eg, Harik, 1995; Stewart, 1997; Birot, 2004), we reasoned that since most tissues respond with local compensatory mechanisms to hypoxia, similar mechanisms could also be operating in the testis under this condition. An early response of cells and vasculature exposed to hypoxia is the stabilization and release of vascular endothelial growth factor (VEGF; Marti and Risau, 1998). This factor would trigger the subsequent remodeling and vascularization of the organ as a compensatory oxygen-delivery mechanism. In this work, we have tested the hypothesis that the testis, under HH, undergoes changes in vascularization and red blood cell concentration that lead to changes in temperature homeostasis in this organ, an event that would be correlated with the changes in spermatogenesis observed in this tissue. These oxygen delivery compensatory changes and the changes in testicular temperature homeostasis would provide a novel local mechanism responsible for the decrease in spermatogenic cell production.

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Table 1. Effect of hypobaric hypoxia on rat body mass*

Day	Nx (g)	CHH (g)
0	248 ± 16	248 ± 16
5	251 ± 9	213 ± 9†
15	275 ± 13	210 ± 9†
30	286 ± 16	203 ± 5†

* N = 10. Mean ± standard deviation (SD). Statistical analysis: analysis of variance followed by Tukey's analysis. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia.

† P < .05 vs Nx (control).

Materials and Methods

Experimental Design

Ten-week-old male Wistar rats (247 ± 16 g, n = 80) were separated in 2 groups: sea level normobaric control (Nx) and chronic hypobaric hypoxia (CHH) groups. Each group consisted of 40 individuals housed in cages with 4 individuals per cage in a 12:12-hour light:dark cycle. CHH animals were exposed to a 4600-m simulated altitude (428 torr; PO₂: 89.6 mm Hg) for a period of 5, 15, or 30 days. Pressure changes in the hypobaric chamber were achieved by steps of 150 m/min simulated altitude changes. The Nx animals were housed in the same room, next to the CHH animals (22°C ± 2°C, 15 g of food pellet per day, and 1 L of water per cage). All procedures were performed in agreement with the Principles of Laboratory Animal Care, advocated by the National Society of Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

Histological Procedures

To determine testicular vascularization and the effects of HH on spermatogenesis, the following protocols were used: At days 0, 5, 15, and 30, the animals exposed to CHH (428 torr) and to normobaric conditions (control rats) were anesthetized with ketamine (50 mg/kg). After testicular temperature determination (see below), 1 testicle from each animal was weighted and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 hours at room temperature. The testicles were embedded in paraffin after dehydration in ascending alcohol concentrations. Five-microgram sections were cut and mounted on glass slides. The sections were stained with hematoxylin-eosin (eg, Nalbandian et al, 2003). Four 5-μm tissue sections were obtained from rat testicles from the equatorial zone toward the testicular apex. The distance between the sections corresponded to 120 μm. For a better visualization of the blood vessels, we used a combination of tissue autofluorescence when illuminated with ultraviolet light and transmitted white-light images observed in a Nikon Diaphot microscope (Nikon Corp, Chiyoda-Ku, Tokyo).

Image Analysis

The interstitial spaces and seminiferous tubules were photographed using 40× objective with Nikon Coolpix 4300 camera (Nikon, Japan). The interstitial space and tubule dimensions were analyzed using the software Image Tool v3.0 (<http://ddsdx.uthscsa.edu/dig/itdesc.html>). From these images we also determined the number, diameter, and type of blood vessels pres-

Table 2. Effect of hypobaric hypoxia on testicular mass (in grams)*

Day	Nx (R)	CHH (R)	Nx (L)	CHH (L)
0	1.40 ± 0.07	1.40 ± 0.07	1.41 ± 0.04	1.41 ± 0.04
5	1.39 ± 0.06	1.38 ± 0.02	1.39 ± 0.03	1.38 ± 0.04
15	1.40 ± 0.03	1.38 ± 0.02	1.39 ± 0.02	1.39 ± 0.03
30	1.44 ± 0.04	1.07 ± 0.06†	1.44 ± 0.03	1.04 ± 0.6†

* N = 10. Mean ± standard deviation (SD). Statistical analysis: analysis of variance followed by Tukey's analysis. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia; R, right testis; and L, left testis.

† P < .05 vs Nx (control).

ent in the interstitial compartment and the height of the germinal epithelium. Our analysis was restricted to interstitial spaces between seminiferous tubules; we specifically avoided the zone adjacent to the tunica. The number of observations corresponded to 10 rats, 4 tissue sections per testis, and 10 fields of the microscope per tissue section for each group (Nx and CHH) on days 0, 5, 15, and 30. Formalin fixation could have produced some damage to interstitial vessels in our preparations of normoxic and hypoxic testicles. For this reason, the numbers of blood vessels obtained were used only for comparison between groups.

Testicular Temperature

Measurements of intratesticular temperature were performed using a thermocouple sensor (model 8502-12; Cole-Parmer, Vernon Hills, Ill). This sensor was introduced into the testicular parenchyma of the rats anesthetized with ketamine HCl (50 mg/kg rats) (eg, Saypol et al, 1981).

Hematocrit

Because blood viscosity and hematocrit are correlated in mammals (eg, Sun and Munn, 2004), to estimate a parameter related to changes in blood rheological properties, we determined the changes in blood hematocrit of rats subjected to HH. Hematocrit was determined by centrifugation of a capillary tube with heparinized blood in a microhematocrit centrifuge (IEC model MB; GSR Technical Sales, Canada), as described by Germack et al (2002). The blood samples were obtained by cardiac puncture of the left ventricle.

Statistical Analysis

The data obtained under CHH conditions were compared to those obtained under the Nx conditions using an analysis of variance test followed by Tukey's and Bonferroni analyses. Differences were accepted to be significant when P < .05. The data were analyzed using the GraphPad Prism software v2.01 (San Diego, Calif). The results are presented as mean ± standard deviation (SD).

Results

Body and Testicular Mass

In spite of the fact that food intake in Nx rats was adjusted to that of CHH animals, the body mass of CHH rats was

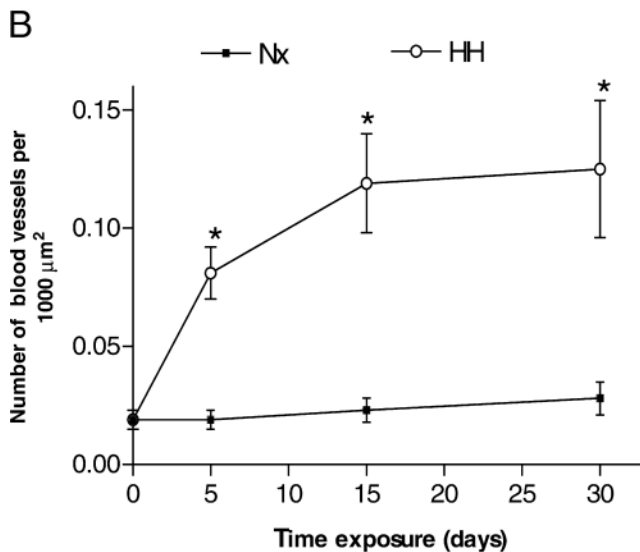
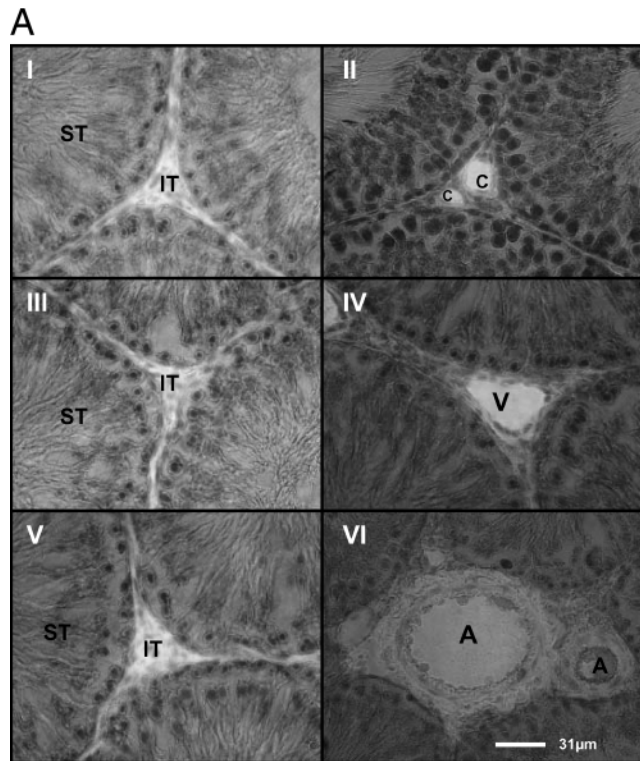


Figure 1. (A) Blood vessels in interstitial tissue of rat testis. I, III, and V: sea level normobaric control group at 5, 15, and 30 days. II and IV: chronic hypobaric hypoxia group at 5 and 15 days. VI, chronic hypobaric hypoxia group at 30 days. C indicates capillaries; V, veins; A, arterioles; IT, interstitial tissue; and ST, seminiferous tubule. (B) Number of blood vessels present in the interstitial compartment of the testis per 1000 µm². Mean ± SD. N = 10 for each group. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia. * P < .05 vs control.

lower compared to that of Nx rats during the exposure period (P < .05) (Table 1). There were no significant differences in left or right testicular mass between CHH and Nx groups during the first 15 days of exposure. However, on day 30 there was a substantial decrease in testic-

Table 3. Total diameter of blood vessels per 1000 µm² of the testis interstitial compartment*

Day	Nx (µm)	CHH (µm)
0	8.0 ± 3.0	8.0 ± 3.0
5	7.0 ± 1.0	23.0 ± 4.0†
15	7.0 ± 1.0	26.0 ± 7.0†
30	9.0 ± 2.0	27.0 ± 6.0†

* The number of observations corresponded to 10 rats × 4 histological sections × 10 fields of the microscope (400× per section) for every group (Nx and CHH) at days 0, 5, 15, and 30. Mean ± standard deviation (SD). Statistical analysis: analysis of variance followed by Tukey's analysis. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia.

† P < .05 vs Nx (control).

ular mass in the CHH vs the Nx group (P < .01) (Table 2).

Testicular Blood Vessels

The numbers of blood vessels per 1000 µm² of interstitial space as well as their diameters were significantly greater in the CHH vs the Nx group (P < .01) (Figure 1A and B; Table 3). A classification of the blood vessels was made based on their diameters and the presence or absence of the tunica intima, media, and adventitia, as well as on the presence or absence of an internal elastic lamina in the tunica intima (eg, Figure 1A). The blood vessels were classified as capillaries, veins, and small arterioles. Veins and small arterioles were progressively observed in the CHH group, whereas only capillaries were found in the Nx group (Table 4).

Intratesticular Temperature

An increase in the temperature of the testicular parenchyma was observed in rats of the CHH group during the first 5 days of CHH exposure. This temperature change remained constant up to 30 days postexposure. The intratesticular temperature was about 1.5°C greater in CHH rats compared to the Nx rats (P < .05) (Figure 2).

Hematocrit

The percentage of hematocrit was significantly greater in the CHH group compared to the Nx group (P < .05) during the 30-day period during which the study lasted. As early as day 5 of CHH exposure, there was a significant increase in the blood hematocrit in the CHH group (Table 5). Because this parameter is directly related to blood viscosity, this increment in blood hematocrit is likely to reflect a similar increase in blood viscosity in the CHH group.

Height of the Germinal Epithelium

The height of the spermatogenic epithelium in CHH rats presented a significant decrease in relation to Nx rats (P < .05) (Figure 3). These changes in the seminiferous ep-

Table 4. Classification and quantization of blood vessels per 1000 μm^2 of interstitial compartment*

Day	Capillaries (Nx)	Arterioles (Nx)	Venules (Nx)	Capillaries (CHH)	Arterioles (CHH)	Venules (CHH)
0	0.019 \pm 0.004	<0.003	<0.001	0.019 \pm 0.001	<0.003	<0.001
5	0.019 \pm 0.004	<0.003	<0.001	0.058 \pm 0.017††	0.010 \pm 0.006	0.011 \pm 0.030
15	0.023 \pm 0.005	<0.003	<0.001	0.068 \pm 0.004††	0.033 \pm 0.009§	0.013 \pm 0.010
30	0.028 \pm 0.007	<0.003	<0.001	0.076 \pm 0.020††	0.040 \pm 0.022§	0.017 \pm 0.010§

* The number of observations corresponded to 10 rats \times 4 histological sections \times 10 fields of the microscope (400 \times per section) for every group (Nx and CHH) at days 0, 5, 15, and 30. Mean \pm standard deviation (SD). Statistical analysis: analysis of variance followed by Tukey's and Bonferroni analyses. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia.

† $P < .05$ vs Nx (control).

†† $P < .05$ vs day 0 of CHH group.

§ $P < .05$ vs day 5 of CHH group within each blood vessel type.

ithelium strongly indicate that CHH rats presented a decreased proliferation of the male germinal epithelium compared to Nx rats.

Discussion

Male rats have been previously demonstrated to respond to HH with cardiovascular changes similar to those exhibited in humans in high-altitude conditions, validating this animal model to study the physiological adaptations to HH (Olson and Dempsey, 1978; Richalet et al, 1992; Germack et al, 2002). Exposure of male rats to HH has been shown to induce loss of body and testicular mass, as described here (Gosney, 1984; Costa, 1990; Mortola and Naso, 1997; Vats et al, 1999; Germack et al, 2002; Farias et al, 2005). The weight of rodent organs under hypoxia seemed to follow a pattern of changes that was not necessarily correlated with the changes in body mass but that was related to oxygen and blood flow homeostasis (increases in heart and lung mass) or to food intake adaptation (decreases in intestine and stomach mass) (eg,

Hammond et al, 2001). In organs that are not related to these functions, such as the testicle, the observed decrease in mass under HH was, hence, not predictable. The magnitude and time dependence of testicular mass changes are very relevant for interpreting the underlying physiological changes taking place in this tissue under hypoxia.

The exposure of rats to continuous and chronic HH produced deterioration of interstitial cells, increase of the interstitial space, damage to the germinal epithelium, and an increase in the seminiferous tubule lumen in the testis. Furthermore, loss of spermatogenic cells and a strong metabolic stress in spermatogenic cells was observed (Farias et al, 2005). The testicles also showed a remarkable blood accumulation. These results prompted us to reason that, as a consequence of the tissue hypoxia that affected cell metabolism, the testicles were also undergoing changes that affected blood supply and vasculature adaptations.

Our findings show that in HH, the testicles undergo dramatic changes in vascularization that were evident and robust 5 days after HH exposure and onward. Intratestic-

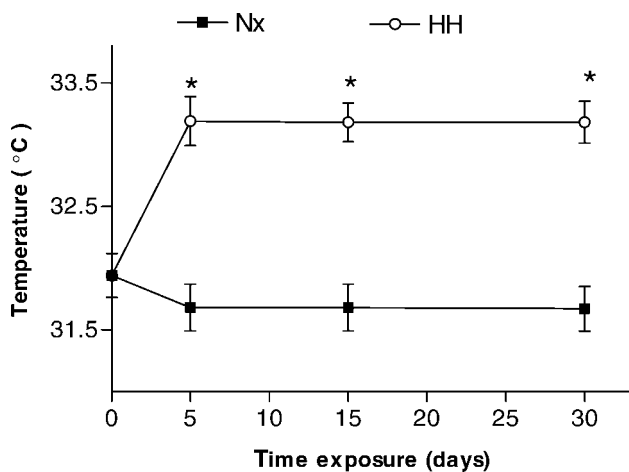


Figure 2. Intratesticular temperature. Mean \pm SD. N = 10 for each group. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia. * $P < .05$ vs control.

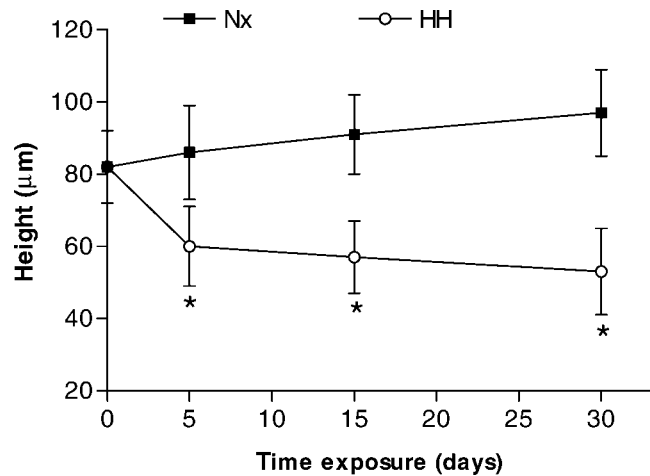


Figure 3. Effect of chronic hypobaric hypoxia (CHH) on the height of the germinal epithelium. Mean \pm SD. N = 10 for each group. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia. * $P < .01$ vs control.

Table 5. Effect of hypobaric hypoxia on hematocrit*

Day	Nx Hto (%)	CHH Hto (%)
0	38.0 ± 2.0	38.0 ± 2.0
5	38.0 ± 1.0	48.0 ± 5.0†
15	39.0 ± 2.0	49.0 ± 5.0†
30	38.0 ± 2.0	51.0 ± 3.0†

* N = 10. Mean ± standard deviation (SD). Statistical analysis: analysis of variance followed by Tukey's analysis. Nx indicates sea level (normobaric control); CHH, chronic hypobaric hypoxia; and Hto, hematocrit.

† P < .05 vs Nx (control).

ular temperature of male rats exposed to HH rose significantly 5 days after exposure and remained elevated. The increased vascularization and the changes in blood viscosity strongly indicate that the increase in intratesticular temperature can be related to an increased blood supply and extended blood transition time in the testis. In turn, this increase in testicular temperature is likely contributing to the inhibition of spermatogenesis observed in rats under HH conditions. In this respect, the response of the testis to HH would resemble other hyperthermia-related pathologies, such as varicocele and cryptorchidia (Pryor and Howards, 1987; Mieusset et al, 1993). Elevation of testicular temperature triggered apoptosis in dividing cell populations of the testis and a decreased output of maturing spermatids in rats (Rockett et al, 2001).

Most tissues respond to hypoxia with an increased expression and release of VEGF (Marti and Risau, 1998). This response is remarkable and closely related to the findings reported in this work, namely that overexpression of VEGF causes infertility in transgenic mice and rats, this effect being accompanied by an increased testicular vascularization (Korpelainen et al, 1998).

The effect of a reduced spermatogenesis under HH (see also Farias et al, 2005), accompanied by an increased vascularization and temperature in the testis, matches well with the idea that hypoxia induced remodeling and proliferation of blood vessels in the testis. These vascular changes, together with dynamic rheological changes in the blood, could induce the observed increase in testicular temperature. These blood and vasculature changes, combined with a temperature homeostasis response, could produce the diminished spermatogenesis observed under HH.

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