

Hypobaric hypoxia causes deleterious effects on spermatogenesis in rats

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Abstract

The study was conducted to explore the effects of hypobaric hypoxia on spermatogenesis in rats. Adult male Wistar rats were randomly divided into four groups: three hypoxia-exposed groups and one normoxic control group. Rats in the normoxic control group were raised at an altitude of 300 m, while rats in the 5-, 15-, and 30-day hypoxic groups were raised in a hypobaric chamber simulating a high altitude of 5000 m for 5, 15, and 30 days respectively. Flow cytometry was used to detect the DNA content of testicular spermatogenic cells in rats. The apoptosis of germ cells in testis was analyzed by using TUNEL assay. Spermatogenesis was also evaluated by morphology. Flow cytometry analysis revealed that 5–30 days of hypobaric hypoxia exposure significantly reduced the percentage of tetraploid cell population in rat testis. After rats were exposed to hypobaric hypoxia for 30 days, the ratio of haploid and diploid cell populations in testis reduced significantly. Seminiferous tubules with apoptotic germ cell increased after exposure to hypoxia. Most apoptotic germ cells were spermatogonia and spermatocytes. Hypoxia also caused decrease of cellularity of seminiferous epithelium, degeneration and sloughing of seminiferous epithelial cells occasionally. The data suggest that hypobaric hypoxia inhibits the spermatogenesis in rats. Decrease of tetraploid spermatogenic cells (primary spermatocytes) induced by hypoxia is an important approach to suppress spermatogenesis. The apoptosis of primary spermatocytes and spermatogonia may contribute to the loss of tetraploid cell populations.

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Introduction

More than 20 million people worldwide live at altitudes above 3000 m today. Furthermore, every year several hundreds of thousands of people from lowland areas move to higher altitudes for work and travel. But high altitude hypoxia is a challenge for people residing in or visiting high altitudes. Among those physiological effects of hypoxia, it has been suggested that newcomers from low-lying areas have difficulties in fertility at high altitude, though the fertility rate in native residents at high altitudes is not lower than in populations at sea level (Gonzales 2007). Previous studies indicated that hypoxia reduced the fertility of male rats (Gosney 1984), rhesus monkeys (Saxena 1995), and men (Okumura *et al.* 2003, Verratti *et al.* 2008) from lowland by decreasing sperm count and sperm motility in semen. Morphological studies in animals revealed that hypoxia caused degeneration of germinal epithelium (Gosney 1984, Saxena 1995). These studies suggested that spermatogenesis was interfered with by hypoxia.

But little is known about which stages of spermatogenesis are mainly affected by hypoxia and how hypoxia

affects them. These questions should be answered. Furthermore, in the previous studies, spermatogenesis was evaluated only by employing histological analyses of testes. In contrast, the use of flow cytometry facilitates rapid, sensitive, objective measurements of reproductive effects due to environmental, occupational, and therapeutic exposure to toxicants, by quantitation of large numbers of testicular germ cells. As a consequence, it is widely accepted as an effective method of assessing spermatogenesis in a variety of species, including the rat (Spano & Evenson 1993).

Apoptosis, also referred to as programmed cell death, is a well-defined physiological process that eliminates individual cells from mammalian tissues. In adult animals, germ cell death is important for spermatogenesis and helps to regulate sperm output (Print & Loveland 2000, Kierszenbaum 2001). Elimination of germ cells via apoptosis occurs spontaneously under normal physiological conditions and is often heightened after chemical-induced testicular injury (Richburg 2000, Park *et al.* 2002, Assinder *et al.* 2007). Thus, it is likely that hypoxia reduces sperm production by promoting apoptosis of germ cells in testis. But it was not

investigated in previous studies. TUNEL staining of DNA fragments is routinely used for the detection of apoptotic cells in various tissues, and it is also a sensitive and valid method for identification of apoptotic cells within the seminiferous epithelium (Yu *et al.* 2001).

Therefore, the present investigation was undertaken to evaluate the effects of hypoxia on spermatogenesis in rat testis by flow cytometry, histological examination, and TUNEL assay.

Results

Histopathological examination

Light microscopy

The space between seminiferous tubules was enlarged in rats from the three hypoxic groups. In hypoxic testes, the basal lamina and subepithelial myoid layer appear more undulate. In most sections of hypoxic testes, spermatogonia appear to be losing their attachment to the basal lamina, and other seminiferous cells beyond the maturation step of spermatogonia appear to be losing their cell-to-cell attachments. Examination of testis in hypoxic rats also revealed that the luminal boundary is degraded and lacks the organized clusters of maturing germ cells, which normally characterize the luminal border of the seminiferous epithelium. The percentages of seminiferous tubules with sloughed seminiferous cells in 5-, 15-, and 30-day hypoxic rats were higher than that in control rats (7.2 ± 0.9 , 7.2 ± 0.8 , 4.6 ± 0.5 vs $0.8 \pm 0.4\%$ respectively; $P < 0.01$). In some sections, reduced cellularity and degeneration of germ cell were observed in seminiferous epithelium in rats from three hypoxic groups. Sertoli cell vacuolization was also observed in hypoxic rats. However, testes taken from the normobaric control rats did not show these morphological changes. The seminiferous epithelium was well organized, with normal germ cells and Sertoli cells at various stages in control rats (Fig. 1).

Electron microscopy

Control rat testis did not show any obvious ultrastructural change. In hypoxic rat testes, vacuolation and more lipid droplets were observed in the Sertoli cells. Many myelin-like structures were formed in the cytoplasm of Sertoli cells. Mitochondria distended with loss of cristae. Degenerated spermatogonia with condensed and margined chromatin were observed in rats exposed to hypoxia for 15 and 30 days. In testes from the 15-day hypoxic exposure rats, some degenerated spermatogonia lost attachment to the surrounding Sertoli cells and basal membrane. Empty spaces left by degenerated germ cells were also observed in rats exposed to hypoxia for 15 and 30 days. Nuclear envelope invaginations were found occasionally in primary spermatocytes in 30-day hypoxic exposure rats (Fig. 2).

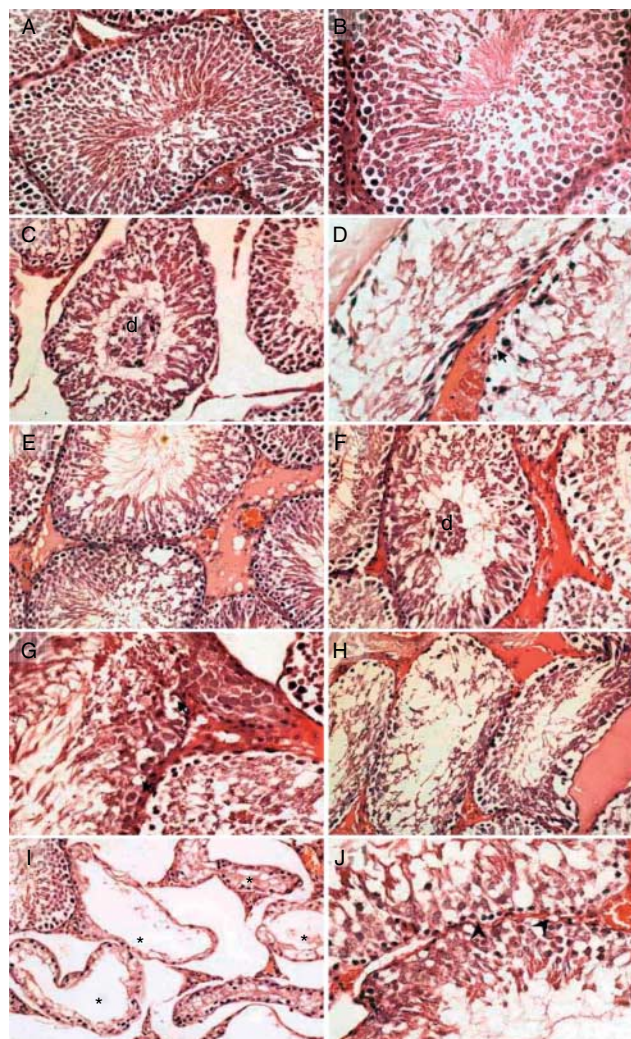
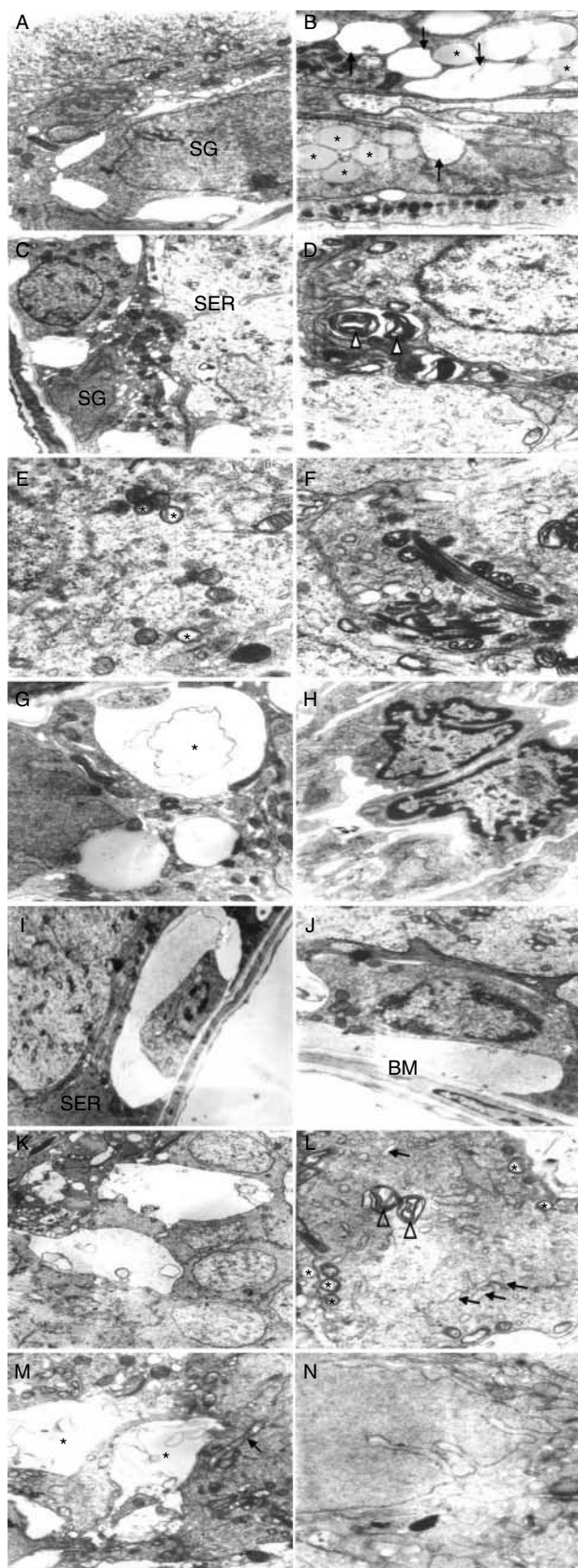


Figure 1 Light microscopy of H&E-stained rat testicular sections. (A and B) Testicular sections from control rats: A, $\times 400$; B, $\times 640$. (C and D) Testicular sections from rats exposed to hypobaric hypoxia for 5 days: C, degenerated germ cells sloughed out into the lumen were visible (d), $\times 400$; D, germ cell degeneration (arrow) and seminiferous epithelial vacuolation were found, $\times 640$. (E–G) Testicular sections from rats exposed to hypobaric hypoxia for 15 days: E, vacuolation was observed, $\times 400$; F, degenerated germ cells (d) and disorganized seminiferous epithelium were found, $\times 400$; G, degenerating germ cells (arrow) were found, $\times 640$. (H–J) Testicular sections from rats exposed to hypobaric hypoxia for 30 days: H, disorganized seminiferous epithelium and low levels of cellularity within seminiferous tubules were observed, $\times 400$; I, some degenerating tubules (asterisk) are shown. Spermatogenesis is absent, $\times 400$; J, folding of the basal membrane (tip of the arrows) and seminiferous epithelial vacuolation were observed, $\times 640$.

Flow cytometry analysis

The flow cytometric DNA content distribution of various germ cells in the control rats is well characterized by the presence of three main distinct peaks, representing spermatid (1C, including elongated spermatid and round spermatid), diploid spermatogonia (2C), and tetraploid primary spermatocyte (4C) populations.



Diploid population includes nongerm cell types like Sertoli cells, Leydig cells, etc. (<3% of the total germ cell population), and also includes secondary spermatocytes, whose life time is very short. The region before the haploid peak represents cells with subhaploid DNA content (debris that may be of apoptotic origin; Hacker-Klom *et al.* 1999).

Alteration in the relative germ cell percentages by hypoxia

The relative percentages of different testicular germ cell populations in control rats, 5-day hypoxic rats, 15-day hypoxic rats, and 30-day hypoxic rats are shown in Table 1.

Spermatogonia (2C)

The relative percentage of spermatogonia (2C) in 30-day hypoxic rats was significantly greater than that in control rats ($P < 0.01$). No significant change was observed in 5- and 15-day hypoxic rats as compared with that in control rats ($P > 0.05$).

Figure 2 Electron micrographs of seminiferous epithelium. (A) Seminiferous epithelium from control rats showing normal spermatogonia (SG) with homogeneous chromatin, $\times 7000$. (B) Seminiferous epithelium from rats exposed to hypoxia for 5 days, showing Sertoli cells with vacuoles (arrows) and lipid droplets (asterisks) within the cytoplasm, $\times 10\,000$. (C) Seminiferous epithelium from rats exposed to hypoxia for 5 days, showing degenerated spermatogonia (SG) about to be phagocytosed by a Sertoli cell (SER), $\times 4200$. (D) Seminiferous epithelium from rats exposed to hypoxia for 5 days, showing multilayered myelin-like figures in cytoplasm (arrowhead), $\times 10\,000$. (E) Seminiferous epithelium from rats exposed to hypoxia for 5 days, showing swollen mitochondria with reduced cristae (asterisks), $\times 14\,000$. (F) Seminiferous epithelium from rats exposed to hypoxia for 5 days, showing incomplete mitochondrial sheath surrounding sperm tail, $\times 14\,000$. (G) Seminiferous epithelium from rats exposed to hypoxia for 15 days, showing late apoptotic spermatogonia (asterisk), $\times 7000$. (H) Seminiferous epithelium from rats exposed to hypoxia for 15 days, showing degenerated germ cells with abnormal shape of nuclei and margined chromatin, $\times 10\,000$. (I) Seminiferous epithelium from rats exposed to hypoxia for 15 days, showing spermatogonia with margined and condensed chromatin detached from the surrounding Sertoli cells (SER). Shrinkage of cytoplasm and nucleus was observed, $\times 4200$. (J) Seminiferous epithelium from rats exposed to hypoxia for 15 days, showing spermatogonia with margined and condensed chromatin detached from basal membrane (BM), $\times 7000$. (K) Seminiferous epithelium from rats exposed to hypoxia for 15 days, showing a cluster of empty spaces left by degenerated germ cells, $\times 2100$. (L) Seminiferous epithelium from rats exposed to hypoxia for 30 days, showing multilayered myelin-like figures (arrowhead), degenerative mitochondria (asterisks), and enlarged endoplasmic reticulum (arrow), $\times 10\,000$. (M) Seminiferous epithelium from rats exposed to hypoxia for 30 days, showing empty spaces (asterisk) left by degenerated germ cell and abnormal giant mitochondria (arrow) in cytoplasm of surrounding Sertoli cell, $\times 7000$. (N) Seminiferous epithelium from rats exposed to hypoxia for 30 days, showing invaginations of nuclear envelope in primary spermatocyte, $\times 10\,000$.

Table 1 Alteration in the relative germ cell percentages by hypoxia (%; $n=6$).

	Sub-1C cells	1C cells	2C cells	4C cells
Control group	1.30±0.13	73.04±1.03	9.45±0.51	14.26±0.78
5-day hypoxic group	1.98±0.17	75.70±0.76*	9.12±0.59	11.67±0.28 [†]
15-day hypoxic group	2.18±0.33*	75.34±0.76*	10.47±0.50	10.56±0.59 [†]
30-day hypoxic group	3.08±0.38 [†]	71.20±0.30	12.19±0.35 [†]	10.39±0.51 [†]

* $P<0.05$; [†] $P<0.01$ compared with control group.

Primary spermatocytes (4C)

The relative percentage of primary spermatocytes (4C) in 5-, 15-, and 30-day hypoxic rats showed significant decline as compared with that in control rats ($P<0.01$).

Spermatids (1C)

Exposure of rats to hypoxia for 5 and 15 days resulted in a significant increase of relative percentages of spermatids compared with normoxic exposure ($P<0.05$). However, the relative percentage of spermatids in 30-day hypoxic rats did not change significantly ($P>0.05$, compared with control rats).

Sub-haploid cells

The relative percentage of sub-haploid cells was significantly more in rats from 15- to 30-day hypoxic groups than that in rats from control group ($P<0.05$ and $P<0.01$ respectively). No significant difference in relative percentage of sub-haploid cells was observed between 5-day hypoxic group and control group ($P>0.05$).

Alteration in the relative germ cell ratios by hypoxia

The ratios of different germ cell in 5-, 15-, 30-day hypoxic rats and control rats are presented in Table 2.

4C:2C ratio

A significant decline of 4C:2C ratio in 15- and 30-day hypoxic rats was observed as compared with that in control rats ($P<0.01$). There was no significant difference in the 4C:2C ratio between 5-day hypoxic rats and control rats ($P>0.05$).

1C:2C ratio

Exposure of rats to hypoxia for 30 days resulted in a significant decline in 1C:2C ratio compared with normoxic exposure ($P<0.01$). No obvious change of

Table 2 Alteration in the relative germ cell ratios by hypoxia ($n=6$).

	1C:2C	1C:4C	4C:2C
Control group	7.84±0.45	5.21±0.33	1.53±0.10
5-day hypoxic group	8.48±0.57	6.51±0.20*	1.30±0.08
15-day hypoxic group	7.28±0.36	7.24±0.40 [†]	1.03±0.09 [†]
30-day hypoxic group	5.86±0.15 [†]	6.93±0.34 [†]	0.86±0.05 [†]

* $P<0.05$; [†] $P<0.01$ compared with control group.

1C:2C ratio was found between control rats and either of 5- and 15-day hypoxic rats ($P>0.05$, compared with control rats).

1C:4C ratio

The 1C:4C ratio exhibited a significant increase in 5-day hypoxic rats ($P<0.05$), 15-day hypoxic rats, and 30-day hypoxic rats ($P<0.01$) as compared with that in control rats.

TUNEL

Most of the TUNEL-positive germ cells were detected to be primary spermatocytes and spermatogonia, while few apoptotic spermatids and nongerm cells were observed. Apoptosis in testis was quantitated using a scoring system based on the percentage of the seminiferous tubules with TUNEL-positive cells (Blanchard *et al.* 1996, Richburg 2000, Yamamoto *et al.* 2002). The percentages of seminiferous tubules with TUNEL-positive cells in 5-day hypoxic rats ($P<0.01$), 15-day hypoxic rats ($P<0.01$), and 30-day hypoxic rats ($P<0.05$) were more than that in control rats (Figs 3 and 4).

Discussion

This hypoxic rat model has been widely used by our lab and other labs to research the effects of high-altitude hypoxia on the body. The decompressing treatment did not obviously exacerbate the severity of the high-altitude effects on rats. No signs of high-altitude sicknesses such as cerebral or pulmonary edema were observed in these rats.

In this study, we found hypoxia caused damage to the germinal epithelium, folding of the basal membrane, degeneration and sloughing of spermatogenic cells in lumen of seminiferous tubule. We also observed that hypoxia caused lipid droplet deposition in Sertoli cells and spermatogonia degeneration with chromatin margination under electron microscopy. These changes of morphology in testes indicate that hypoxia impairs spermatogenesis in rats. Previous studies also revealed that hypoxia impaired spermatogenesis in testis by light microscopic analyses. The morphological changes in testis are attributed to the vulnerability of spermatogenesis to hypoxia. Men and male animals produce a great amount of sperm every day, indicating that

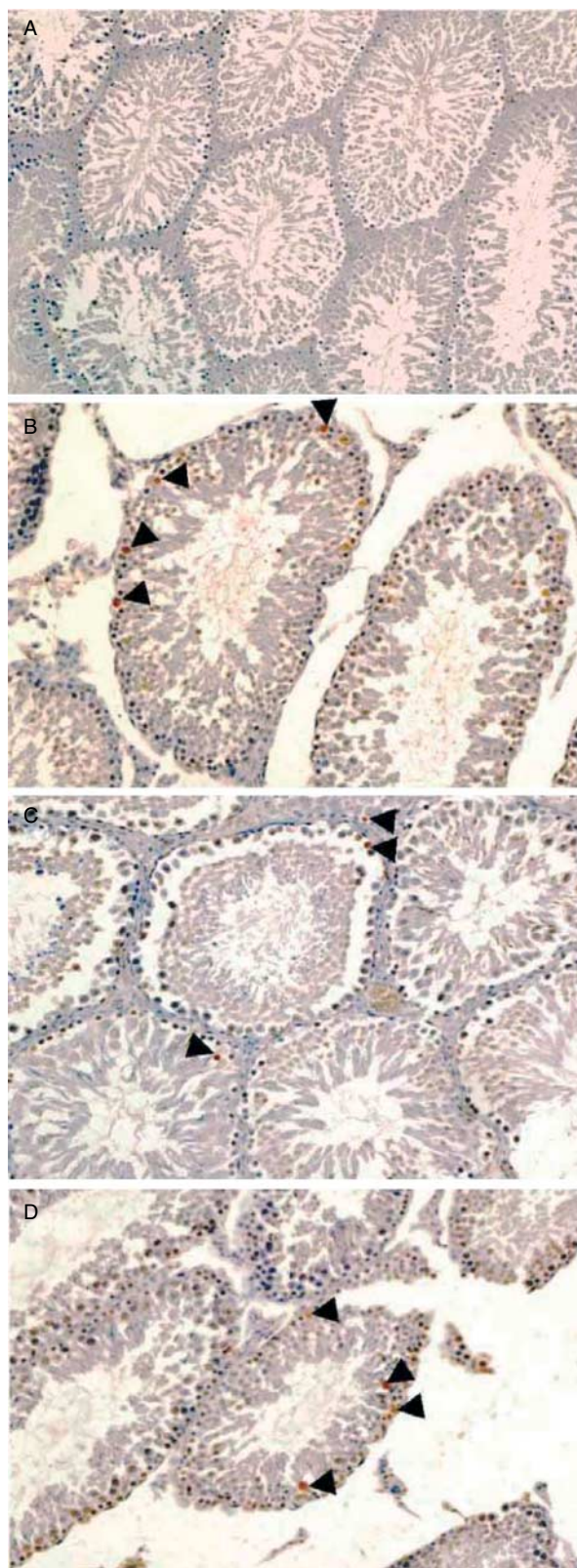


Figure 3 Immunohistochemical demonstration of apoptotic cells by the TUNEL method. (A) Control rats, magnification: $\times 200$; (B) 5-day hypoxic rats, magnification: $\times 320$; (C) 15-day hypoxic rats, magnification: $\times 320$; (D) 30-day hypoxic rats, magnification: $\times 320$.

spermatogenesis in the seminiferous tubules of the testis occurs under a high proliferation rate, which demands considerable oxygen consumption. However, blood vessels are located exclusively between the tubules, and oxygen reaches the lumen of the seminiferous tubules only by diffusion. The seminiferous epithelium was speculated to operate on the verge of hypoxia because the testicular PO_2 is relatively low, oxygen extraction is highly related to the metabolic demands of spermatogenesis, oxygen diffusion distance is comparatively long, and the testis has little capacity to increase total blood flow (Lysiak *et al.* 2000). Furthermore, exposure to hypoxia results in distribution of blood flow to the vital organs including brain and heart, and decrease in testicular blood flow. Koskinen *et al.* (2000) reported that breathing 10% $O_2/90\%$ N_2 resulted in a 24% decrease in testicular blood flow, but a 23% increase in cerebral blood flow. These characteristics may attribute to the morphological changes of spermatogenesis caused by hypoxia.

In this study, we also observed that hypoxia increases interstitial space of testis, which extends the oxygen diffusion distance and would impair oxygen delivery to germ cells. It makes germ cells more susceptible to damage, which was confirmed by our observation of degenerative germ cells in hypoxic rats under light and EM. A similar change was reported by Farias *et al.* (2005a).

Besides these, an increase in testicular temperature may be another factor involved in impairing spermatogenesis under hypobaric hypoxia (Farias *et al.* 2005b).

To explore which stages of spermatogenesis are mainly affected by hypoxia, flow cytometry was employed in this study. Flow cytometry can provide a rapid way to investigate germinal epithelium integrity and to perform a quantitative analysis of spermatogenesis, and it has been used in many laboratories to examine spermatogenesis in men and animals. In the present study, the relative percentage of 1C cells increased in 5- and 15-day hypoxic rats, which may be caused by inhibition of mature sperm release from spermatogenic epithelium. As a result, less sperm would enter the epididymal initial segment, and less sperm would reach the epididymal cauda 8 days later (8 days for the transit of spermatozoa from the epididymal initial segment to the proximal cauda in the rat). This was confirmed in our laboratory. We observed that epididymal caudal sperm reduced significantly after rats were exposed to hypobaric hypoxia for 15 and 30 days, while it did not significantly change after a 5-day exposure to hypobaric hypoxia. Gasco *et al.* (2003) also observed that the epididymal sperm count was significantly reduced at days 14, 28, and 35, but not reduced at days 3. These results suggest that hypoxia inhibits the release of sperm from the spermatogenic epithelium. However, the relative percentage of 1C cells in 30-day hypoxic rats did not significantly differ from the control. It was because hypoxia on the one hand decreased

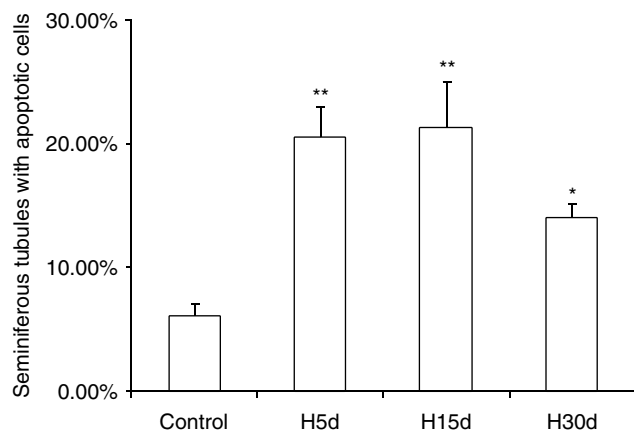


Figure 4 Effect of hypoxia on germ cell apoptosis in testis ($n=4$; * $P<0.05$; ** $P<0.01$ compared with control group).

spermatid number by restraining the spermatogenesis; on the other hand, it increased the amount of sperm in the testis by inhibition of sperm release from spermatogenic epithelium. The duration of spermatogenic transformation from spermatogonia to spermatid is 29.5 days in male rats (Adler 1996). Therefore, the inhibition of spermatogenesis is obvious only after a 30-day exposure to hypoxia, which was supported by our result that the total spermatogenic transformation (1C:2C cell ratio) dropped only in 30-day hypoxic rats. The inhibition of spermatogenesis neutralized the increase of the relative percentage of 1C cells caused by inhibition of sperm release from spermatogenic epithelium in 30-day hypoxic rats. The relative percentage of 2C cells increased significantly in 30-day hypoxic rats, when compared with that in control rats. Such an increase in relative percentage of 2C cells is related to the loss of primary spermatocytes and decrease of spermatid. The flow cytometry also provided convincing evidence that the suppressive effects of hypoxia on spermatogenesis were mainly caused by decreasing tetraploid spermatogenic cells. This was supported by the observations that the ratios of tetraploid to diploid cells were low and proportions of tetraploid cells were significantly decreased in rats exposed to hypoxia for 5, 15, and 30 days.

Increase of apoptosis in germ cells is an important mechanism, through which many reported that toxic substances inhibit spermatogenesis and decrease the production of mature sperm. Germ cell apoptosis induced by hypobaric hypoxia was not evaluated in previous studies. TUNEL assay is routinely used for the accurate detection of apoptotic cells in various tissues, which relies on abundance of 3' DNA ends generated by DNA fragmentation in apoptotic cells. In the present study, we evaluated germ cell apoptosis induced by hypoxia simulated to a high altitude of 5000 m by TUNEL assay. In the present study, several sporadically distributed apoptotic cells were observed in

control rats. This is in agreement with previous studies (Blanco-Rodriguez & Martinez-Garcia 1996, Zhang *et al.* 2001, Xiong *et al.* 2009), suggesting that apoptosis is an important mechanism in controlling numbers of germ cells. The percentage of seminiferous tubules containing apoptotic germ cells increased in hypoxic rats at 5, 15, and 30 days. Most apoptotic germ cells were spermatogonia and primary spermatocytes. As a result of the apoptosis of spermatogonia and primary spermatocytes, a decline in the tetraploid cell population was detected in hypoxic rats by flow cytometry.

Conclusions

Hypobaric hypoxia inhibits spermatogenesis in rats by decreasing the tetraploid spermatogenic cells (primary spermatocytes). The apoptosis of primary spermatocytes and spermatogonia, induced by hypoxia, may contribute to the loss of the tetraploid cell population.

Materials and Methods

Animals

Adult male Wistar rats were randomly divided into four groups: control group, 5-, 15-, and 30-day hypoxic groups. Hypoxic rats were raised in a hypobaric chamber, where atmospheric pressure was reduced to simulate high altitude of 5000 m. The partial pressure of nitrogen fell as total pressure declines on ascent, but nitrogen percentage did not change in atmosphere. Hypoxic rats were returned to normobaric condition for 40 min every day when food and water were provided, and cages were cleaned. The ascending speed and descending speed for the hypobaric chamber were limited to 4–5 m/s. Control rats were raised at an altitude of 300 m out of the hypobaric chamber. All the animals had free access to standard pellet food and water, and they were maintained under controlled light conditions (12 h light:12 h darkness). The experiments were approved by the Animal Care Committee of the Third Military Medical University. All efforts were made to minimize animal suffering and the number of animals used.

Histopathological examination

Light microscopy

Animals were killed by decapitation. Left testes were excised, fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections were cut at 5 μ m thickness and were stained with hematoxylin and eosin for light microscopic observations.

Electron microscopy

Testes were sliced and fixed in 3% glutaraldehyde. Ultrathin sections were stained in uranyl acetate and lead citrate, and they were observed in a Phillips TECNAI 10 transmission electron microscope.

Flow cytometry

The germ cells were released from seminiferous tubules in PBS, by mincing 200 mg right testicular tissue finely with scissors, and then digesting the tissue with 200 mg/l collagenase (Sigma) at 37 °C for 30 min. Sertoli cell aggregates and clumped debris were removed by filtering through a nylon mesh twice and were centrifuged for 10 min at 500 g. Pellet was washed twice and fixed in 70% chilled ethanol. The samples were stored at 4 °C. After germ cells were stained with 25 mg/l ethidium bromide (Sigma), the tubes were kept in dark at room temperature for 30 min before flow cytometry analysis. The samples were analyzed by a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) using an excitation wavelength of 488 nm.

TUNEL immunohistochemistry

Immunohistochemical apoptotic detection (TUNEL assay) was performed on 10% formalin-fixed 5 µm paraffin sections using *In Situ* Cell Death Detection Kit (POD, Roche). Briefly, after dewaxing and rehydrating, the tissue sections were incubated with 25 mg/l proteinase K for 20 min at 37 °C. Nonspecific labeling was blocked by pre-incubating the sections with 3% H₂O₂ in methanol for 10 min at room temperature. To each section, 50 µl of TUNEL reaction mixture were added and incubated for 60 min at 37 °C in a humidified atmosphere. After sections were rinsed, 50 µl of converter-POD were added on each section and incubated for 30 min in a humidified chamber at 37 °C. Sections were rinsed again and incubated with 100 µl DAB substrate for 10 min at room temperature. Color developing was stopped by washing the sections with PBS. Sections were counterstained with hematoxylin. For the negative control, TUNEL reaction mixture was replaced by the same volume of reaction buffer. The percentage of seminiferous tubules containing brown staining cell was analyzed.

Statistical analysis

Data were expressed as mean ± s.e.m. SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Differences in the mean values between the control group and each of three other groups were examined by one way ANOVA, followed by least significant difference multiple comparison test. Differences were considered significant when the *P* value was <0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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