Int. J. Morphol.,
34(1):153-159, 2016.

Reversible Effect of High Altitude on Rat Testis Morphology and Spermatogenesis: Histological and Ultrastructural Study

Efecto Reversible de la Altitud Elevada sobre la Morfología y Espermatogénesis en el Testículo de Rata: Estudios Histológico y Ultraestructural

Asim M. Abdalla[*]; Tingari, M. D.[**]; Ali, K. Z.[*]; Rezigalla, A. A.[*]; Mubarak Al-shraim, [***] & Refaat, A. Eid, [***]

A**BDALLA, A. M.; TINGARI, M. D.; ALI, K. Z.; REZIGALLA, A. A.; AL-SHRAIM, M. & EID, R. A.** Reversible effect of high altitude on the rat testis morphology and spermatogenesis: Histological and ultrastructural study. *Int. J. Morphol., 34(1)*:153-159, 2016.

SUMMARY: The study was carried out at two different altitudes in the southern region of Saudi Arabia: Abha, 2,800 meters above sea level, the high altitude (HA) area and Jazan, 40 meters above sea level the low altitude (LA) area. Following exposure to high altitude, testes of rats revealed various types of atrophy and degeneration in the seminiferous tubules and in the interstitial tissue. There was detachment of the basal laminae of the tubules and a profound decrease in cellularity. When rats were brought back to their habitat (LA) and later examined, many tubules showed normal population of cells including spermatids and spermatozoa. Well-arranged epithelium was seen in most of the seminiferous tubules of these animals, normal interstitial space and no detachment of the basal lamina. Apparently complete recovery had been achieved ultrastructurally, in hypoxic group; some spermatogenic cells lost their normal architecture, being irregular in shape with some features of necrosis, such as shrinkage and pyknotic nuclei characterized by chromatin condensation. Significant decrease in epithelial height was noticed in these animals ($P < 0.05$). Also, the diameter of the tubules showed slight decrease with concomitant increase in interstitial spaces.

KEY WORDS: High altitude; Low altitude; Hypoxia; Semen; Reproductive hormones.

INTRODUCTION

At high altitude, haemoglobin caries less oxygen. This is because the partial pressure of oxygen decreases and the amount of oxygen available for diffusion into blood stream also decreases (Beall *et al*., 1994). High altitude hypoxia (hypobaric hypoxia, HH) is said to reduces fertility in humans (Farias *et al*., 2005a; Cikutovic *et al*., 2009; Hwang *et al*., 2009; Gat *et al*., 2010a, 2010b). However, epidemiological studies of high and low altitude populations did not support this hypothesis (Vitzthum & Wiley, 2003; Bartsch *et al*., 2004). In previous publications, it was shown that the exposure of male rats to continuous chronic hypobaric hypoxia and intermittent chronic hypobaric hypoxia induced evident changes in testicular morphology, arrest of spermatogenesis and other metabolic changes related to lipid peroxidation (Farias *et al*., 2005a). 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 altitude is not lower than in population at sea level (Gonzales, 2007). Recently, it has been established that in exposure times to HH beyond sixty days, resulted in morphological injury in the seminiferous tissue and associated with metabolic alterations in spermatogenic cells (Farias *et al*., 2005b). It is worth noting that the literature is silent with regard to simultaneous effect of high and low altitudes on spermatogenesis. Also, there is no reference describing such effects in the regions of the Kingdom of Saudi Arabia where there is large population habituating such HA areas. The present work aims to evaluate, the effects of chronic hypoxia (CH) on the structure (histological, histometric and ultrastructure) of rat testis and spermatogenesis, obtained by means of a constant exposure to a high altitude and returning to low altitude area to testify possibility of reversibility, or otherwise, of testicular tissue if any.

MATERIAL AND METHOD

Experimental design. Seventy-two adult male Wister rats (200-250 g) aged 7 w were used in this experimental study

[*] Department of Anatomy, College of Medicine, King Khalid University, Abha, Saudi Arabia.
[**] Department of Anatomy, Faculty of Veterinary Medicine, University of Khartoum, Khartoum, Sudan.
[***]Department of Pathology, College of Medicine, King Khalid University, Abha, Saudi Arabia.

with the approval of the Ethical Committee of King Khalid University (Abha, Saudi Arabia). The animals were divided on the basis of altitude exposure into control group (eight rats) kept at low altitude (LA) to the end of the study. Rats in the experimental groups (32 each) were transferred to Abha City and kept for different time intervals (8 d) and were classified as HA exposed rats (hypoxic groups, HGs) and reversal low altitude rats (RGS). All rats were sacrificed after 8 days intervals. The hypoxic rats were further divided into 8 subgroups, of 8 rats each; the first four subgroups (32 rats HG.1, 2, 3 and 4) spent 8, 16, 24 and 32 d respectively at high altitude. The last four subgroups (reversal groups, 32 rats) were returned to low altitude (LA) after exposure period of 32 d at high altitude (HA). RG.1, 2, 3 and 4 and subsequently sacrificed after the same periods of interval (8 d) as the hypoxic rats to testify the reversibility of testicular structure if any.

Methods. Tissue collection. All rats were sacrificed by an overdose of anaesthetic phenobarbital (65 mg/kg). Both testes were removed; adipose tissue, connective tissues and blood Vessels were dissected out and then weighed.

Histology and histometry. The testes were fixed in fresh aqueous Bouin's solution, and processed for the preparation of routine paraffin sections stained with haematoxylin and eosin (H&E) and Masson's trichrome, for light microscopic observations. Morphometric study was performed on tissue from both the control and experimental animals to detect the diameter of seminiferous tubule, (DST) height of germinal epithelium (HE) and thickness of interstitial tissue (TI). An Olympus microscope (Tokyo) with ocular micrometre lens X6 and an objective lens x40 was used for the measurements. For all measurements, the averages were calculated out of five readings (Thienpot *et al*., 1986).

Ultrastructure. Small testicular samples were rapidly fixed in 0.1 M cacodylate buffered 3 % gluteraldehyde and processed for ultrastructural observation and examined under the transmission electron microscope (JEOL, 1200EXII, Tokyo, Japan) at an accelerating voltage of 80 kV (Bancroft & Gamble, 2003).

Statistical analysis. Results were expressed as Mean±SD. analysed by using ANOVA followed by Tukey's t test. And the result was considered significant if $P < 0.05$.

RESULTS

Histology. Control rats revealed normal features of seminiferous tubules. Each tubule was surrounded by boundary tissue. The seminiferous epithelium was formed of spermatogenic and sustentocytes (Sertoli cells). The main structural components of the interstitial tissue were the interstitial endocrine cells (Leydig cells), together with other connective tissue elements and blood vessels.

High altitude rats. A mild shrinkage of the seminiferous tubules was observed in hypoxic group one (8 d) which led to an increase of the interstitial space. Also, there was slight drop in the height of the epithelium. Some vacuoles appeared within the seminiferous epithelium as well as in the interstitial tissue. A slight reduction in the early and mature spermatids was evident in some of the seminiferous tubules. Remarkable damage was observed from 16 d exposure to high altitude onwards, as evidenced by a severe decrease in the cellularity and drop in the epithelial height. Early stages of spermatids were rare or altogether absent. The seminiferous tubules appeared to lose their usual configuration, atrophic and almost lost. The basal laminae became folded and detached. Degeneration of the germ cells was observed in many tubules. The spermatogenic component of tubule showed more vacuolation and the cells became disorderly arranged. There was an increase in the luminal diameter of seminiferous tubules accompanied by a decrease in the concentration of spermatozoa. Relatively large blood vessels as well as fibrin deposition were noticed (Masson's trichrome positive) in the interstitial spaces reflecting interstitial haemorrhage and fibrosis as described by Bancroft & Gamble.

Reversal low altitude rats. Indications of complete recovery were clearly noted, some of seminiferous tubules showed recovery as evidenced by rounded seminiferous tubules, well organized seminiferous epithelium and increased cellularity. The concentration of luminal spermatozoa was increased as well but, there were a few detached spermatozoal heads. Vacuoles within the interstitial tissue were not as remarkable as those seen in hypoxic rats. Clear arrangement of the epithelial layer was observed in most tubules of this group. Many tubules showed normal population of cells including spermatids and spermatozoa. Well-arranged epithelium was seen in most of the seminiferous tubules of these groups together with normal interstitial space and no detachment of the basal lamina. Vacuoles were rarely seen.

Ultrastructure. Control group: Native low altitude rats. The testis of the control rats showed the normal ultrastructural features. The seminiferous tubules showed that the seminiferous epithelium consisting of sustentocytes (Sertoli cells) and spermatogenic cells with cellular characteristics typical of those seen during active spermatogenesis. The interstitial endocrine cells were normal and contained large spherical or oval nuclei surrounded by clear nuclear membrane and possessed distinct nucleoli (Figs. 1D and 2B).

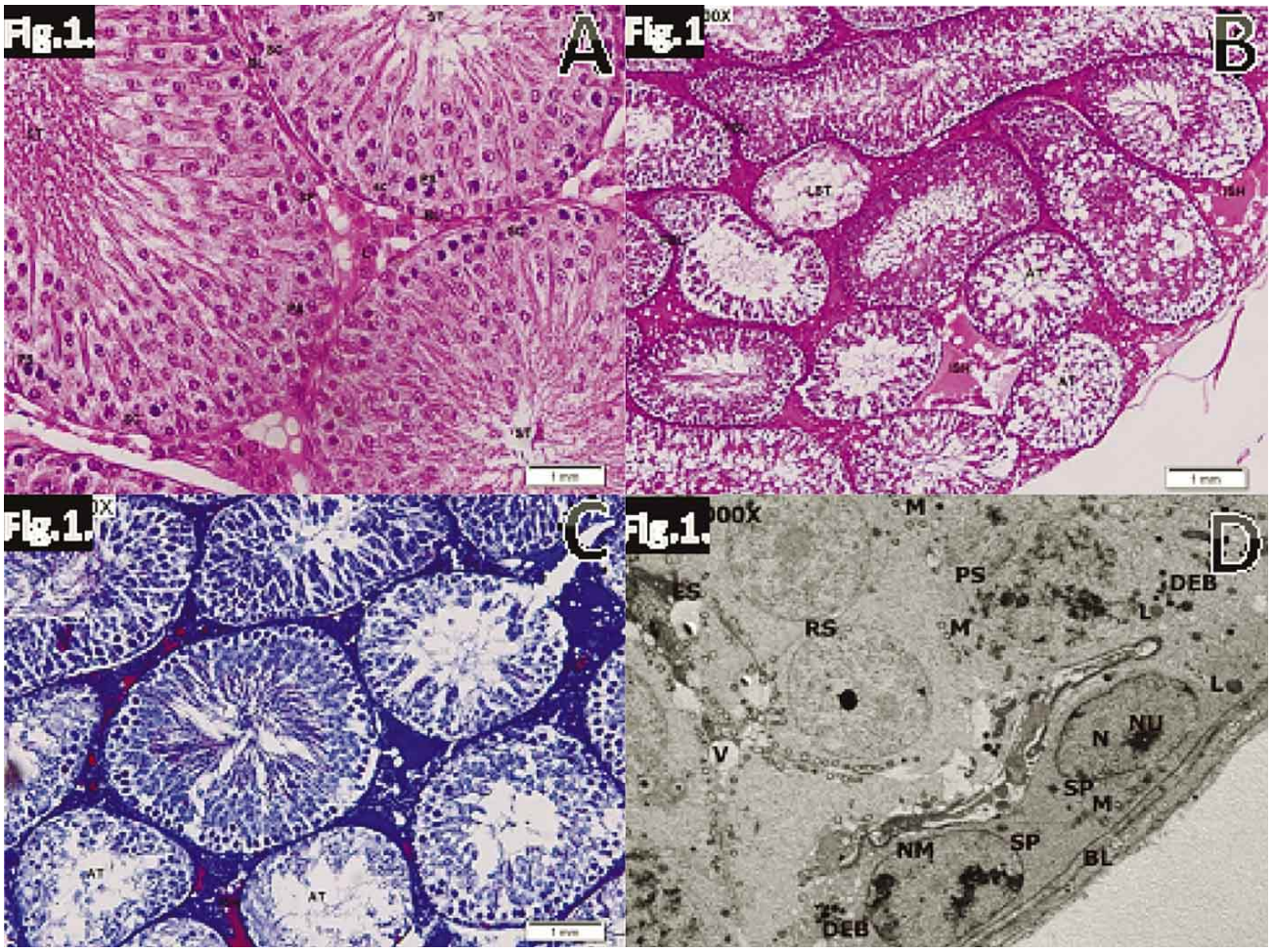

Fig. 1. A) A photomicrograph of seminiferous tubules of the testis of control rats showing basal lamina (BL), spermatogonia (SP), sustentocytes (Sertoli cells) (SC), primary spermatocytes (PS) and interstitial endocrine cells (Leydig) (L). (H&E X 400). B) A photomicrograph of seminiferous tubules of the testis of the hypoxic rats (16 days of exposure to HA), showing interstitial haemorrhage (ISH), vacuoles, lost (LST) and atrophic (AT) seminiferous tubules. (H&E X 200). C) A photomicrograph of seminiferous tubules of the testis of the hypoxic rats (16 days of exposure to HA), showing Fibrin (plasma protein) in the interstitial tissue which is commonly as a result of tissue damage in acute inflammatory reaction leading to leakage of fluid and plasma proteins out of the damaged vessels. The plasma fibrinogen polymerizes to form insoluble fibrin outside the vessels. (Bancroft 2003). (Masson's trichrome X 400). D) An electron photomicrograph of the control rat's testis showing spermatogonia (SP) resting on basal lamina (BL) with large nucleus (N) with clear nuclear membrane (NM) and nucleolus (NU). Primary spermatocyte (Ps) contains large nucleus (N) with electron dense chromatin masses displaying pachytene stage. Its cytoplasm shows mitochondria (M), electron dense bodies (EDB) and lipid droplets (L). Rounded (RS) and elongated (ES) spermatids can be observed (TEM 5000 X).

Ultrastructure of hypoxic high altitude rats. The experimental hypoxic rat testes showed various ultrastructural alterations of testicular tissue. Irregularity, thickening and detachment of basal lamina of seminiferous tubules were noticed in many sections. In hypoxic group 2 and 3, some spermatogenic cells lost their normal architecture, being irregular in shape with some features of necrosis, such as shrinkage and pyknotic nuclei characterized by chromatin condensation. Accordingly, this resulted in the formation of spaces within these necrotic cells. Mitochondria appeared distended. Degenerated spermatogonia with condensed chromatin were observed in HG2 and HG3 (Fig. 2A).

Ultrastructure of reversal low altitude rats. The first two reversal groups (8 and 16 d at low altitude) were nearly similar to the last hypoxic group of rats in ultrastructure, but the mitochondria appeared less distended. Sustentocytes and the spermatogonia showed few vacuolation and lipid droplets (Figs. 2C and 2D). The testes of the last two reversal groups (24 and 32 d at low altitude) showed nearly normal

ultrastructure of sustentocytes, spermatogenic cells as well as interstitial endocrine cells compared to the control group of rats.

Histometric analysis. Histometric analysis of testicular tissues stained by H&E (Table I) showed no significant differences in the diameter of seminiferous tubules of all groups of rats exposed to HA environment at all-time intervals compared to control LA group. Also, returning the hypoxia exposed rats to LA environments showed similar non-significant results. Nonetheless, epithelial height was significantly reduced ($P < 0.05$) only after 2 and 3 weeks of exposure to HA (HG2 and HG3). The epithelial heights in these groups of rats were about 18 % lower than that measured in the control LA group. HG4 and all reversal groups (RG1-RG4) showed normal epithelial heights which were not significantly different from those of the control LA group.

The ANOVA test revealed that the epithelial heights measured in HG4, RG1, RG2, RG3 and RG4 were not

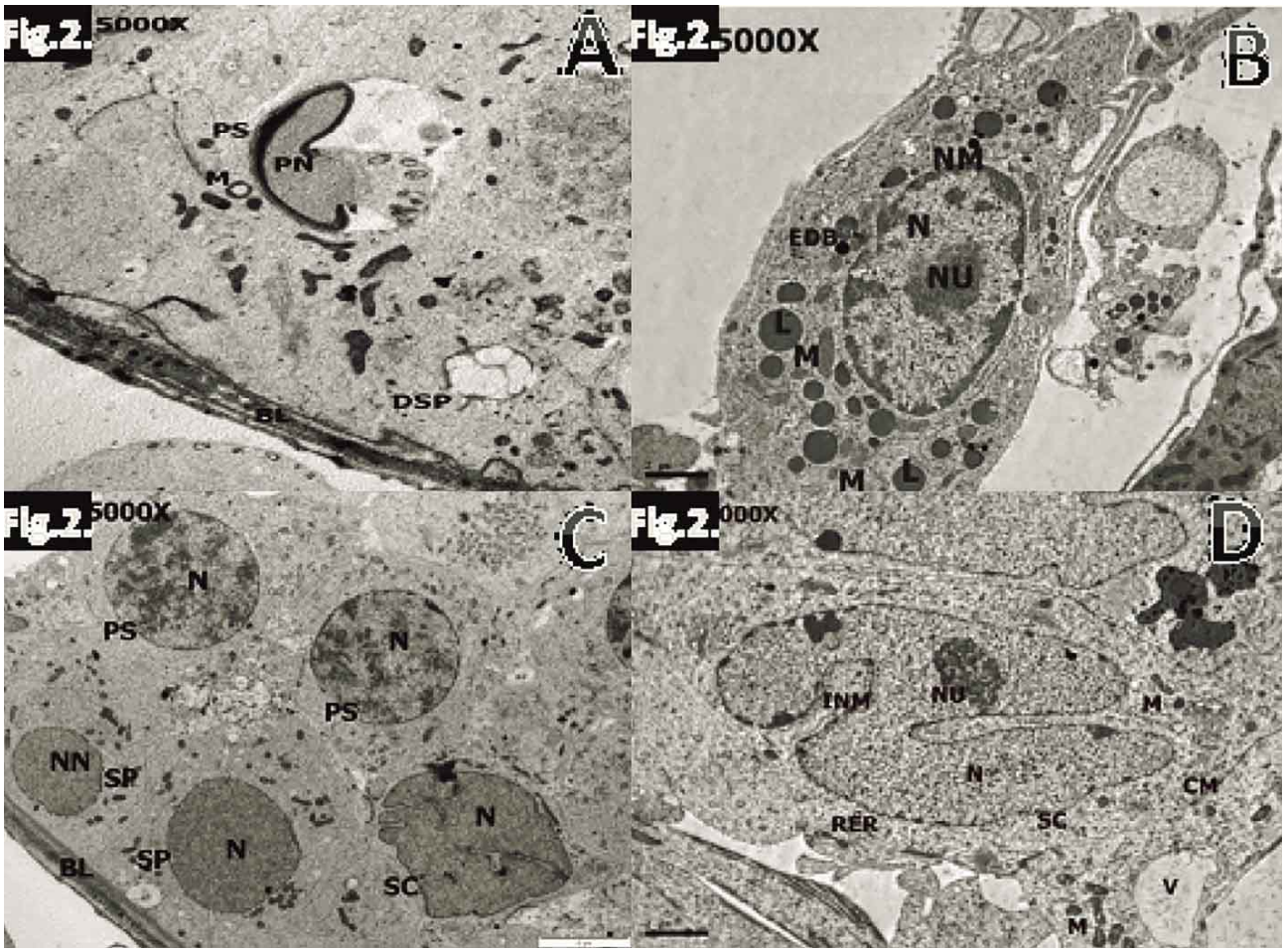

Fig. 2. A) An electron photomicrograph of the hypoxic group three rat's testis showing spermatogonia (SP) resting on basal lamina (BL) with large atrophic pyknotic vacuolated nucleus (NPN) (V) (TEM 5000 X). B) An electron photomicrograph of an ultrathin section of the control rat's testis showing interstitial endocrine cells (Leydig) with large oval indented nucleus (N) with condensed heterochromatin and prominent nucleolus (NU). Cytoplasm appears with mitochondria (M), electron dense bodies (EDB) and many lipid droplets (TEM 5000 X). C) An electron photomicrograph of an ultrathin section of the reversal group four (32 days to low altitude after exposure to the same period at high altitude) rat's testis showing spermatogonia (SP) with their nuclei (N) resting on the basal lamina (BL) primary spermatocytes (PS) with large nuclei (N). Also sustentocytes (Sertoli cells) (SC) with indented nucleus is resting in the basal lamina. (TEM 5000 X). D) An electron photomicrograph of the reversal group one (8 days exposure to low altitude after spent 32 days at high altitude) rat's testis showing sustentocytes (Sertoli cells) (SC) with a large euchromatic nucleus (N) with prominent nucleolus(NU) surrounded by clear indented nuclear membrane (INM). The cytoplasm contains mitochondria (M) rough endoplasmic reticulum (RER) and cell membrane (CM). (TEM 5000 X).

Table I. Histometric analysis of the testicular tissues stained by H&E in the control & experimental rats.

	ST diameter (μm)	Epithelial height (μm)	Interstitial space (μm)
Control	288.3±13.70	92.77±4.93	103.7±12.50
HG1	283.4±23.58	86.96±9.105	136.7±21.25*
HG2	273.0±24.13	75.92±5.0^{*a}	154.1±12.63*
HG3	264.0±21.03	76.88±9.17^{*a}	172.6±16.57^{*ab}
HG4	289.7±24.63	91.81±8.96^{bc}	131.8±17.55^{*abc}
RG1	293.5±24.73	88.23±10.38^{bc}	131.8±15.48^{*abc}
RG2	280.1±13.10	91.23±5.11^{bc}	127.8±7.449^{*abc}
RG3	281.9±11.49	94.06±10.90^{bc}	117.9±4.697^{abcd}
RG4	291.0±17.37	89.60±4.70^{bc}	116.3±7.467^{abcd}

significantly different from one another but significantly higher than those measured in HG2 and HG3. In contrast, gradual increases in the interstitial space were seen in the testis of hypoxic groups of rats at weeks 1, 2 and 3 of hypoxic episodes. The maximum measured interstitial space was seen in HG3, which showed 76.5 % increase. The interstitial spaces in HG4, RG1 and RG2 revealed gradual significant decreases as compared to control rats.

DISCUSSION

The present study is a unique experimental rat model of adaptation following their exposure to natural high altitude (HA) when transferred from their natural low altitude (LA) habitat. The study has demonstrated that HA exposure (2800-3150 m.a.s.l.) is linked with adapted reversible changes in the reproductive function of animals. Such changes included decreased weight and altered testicular histology during the first three intervals of HA exposure. To the best of our knowledge, this is the first report in the literature investigating the time-course complete recovery at the levels of these reproductive parameters in HA hypoxic animals after their return to LA habitat. Furthermore, the study avails ready information on the effects addressed in the different areas of altitude in the Kingdom of Saudi Arabia. Most investigations were carried out in high altitude laboratories or hypobaric chambers and did not simulate natural conditions (Saxena, 1995; Farias *et al*., 2010). The current study is again unique, as it addresses the effect of natural hypoxia on the morphology of rats exposed to natural areas of HA and then LA in the same country. Also, all rats were housed under the same laboratory conditions and were fed on the same diet. Studies of acute simulated HH at moderately and extremely high altitude (HA) resulted in damage of all testicular cells, including spermatogenic and somatic elements *i.e*. sustentocytes and interstitial endocrine cells (Shevantaeva & Kosyuga, 2006). Similar effects were also reported in the studies carried out on rodents including highly vacuolated sustentocytes, decreased germ cell numbers, sperm count and motility, increased abnormal sperm and pyknotic germ cell, expansion of testicular blood vessels and reduction in number of interstitial endocrine cells (Farias *et. al*, 2010, 2012; Bustos-Obregón *et al*., 2006; Vargas *et al*., 2011). Morphological studies have revealed that chronic hypoxia causes significant decreases in testicular and epididymal masses (Farias *et al*., 2005a; Liao *et al*., 2010). The current study has shown that partial recovery in the levels of these parameters began to reverse during the 4th week of HA exposure. Also, the amelioration was achieved in these parameters and started gradually to normalize after returning to LA area with nearly complete recovery being achieved after 3 weeks of return to LA area. Supporting these findings is the report of Verratti *et al*. (2008) who demonstrated that, chronic hypoxia, induces a state of reversible oligozoospermia in healthy men. In this study, hypoxia caused degeneration and sloughing of spermatogenic cells, with and folding of the basal membrane. Ultrastructural observations confirmed spermatogonial degeneration with chromatin margination and deposition of lipid in sustentocytes. Such changes are indicative of the effect of hypoxia impairing spermatogenesis. Earlier light microscopic studies have shown that hypoxia impairs spermatogenesis (Farias *et al*., 2005a). The morphological changes in testis were attributed to the vulnerability of spermatogenesis to hypoxia. 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 partial pressure of oxygen (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 redistribution 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 ce-

rebral blood flow. These characteristics may contribute to the morphological changes of spermatogenesis caused by hypoxia. In this study, it was observed that hypoxia decreased the diameter of seminiferous tubules and the epithelial height but increased the testicular interstitial space with a result of extending the oxygen diffusion distance and hence impairing the oxygen delivery to germ cells, thus became susceptible to damage. This was also confirmed by observation of degenerative germ cells in hypoxic rats under both light and electron microscopy. A similar change has been reported by Farias *et al*. (2005a).

ACKNOWLEDGEMENTS

We wish to acknowledge the help of Prof. Awad Elmekki, Dr. Mohammed Babiker and Mr. Elhaj Abbass.

ABDALLA, A. M.; TINGARI, M. D.; ALI, K. Z.; REZIGALLA, A. A.; AL-SHRAIM, M. & EID, R. A. Efecto reversible de la altitud elevada sobre la morfología y espermatogénesis en el testículo de rata: Estudios histológico y ultraestructural. *Int. J. Morphol., 34(1)*:153-159, 2016.

RESUMEN: El estudio se realizó en dos ciudades con alturas diferentes en la región sur de Arabia Saudita: Abha, a 2.800 metros sobre el nivel del mar, una zona de gran altura (GA) y Jazan, a 40 metros sobre el nivel del mar, área de baja altitud (BA). Después de la exposición a una gran altura, los testículos de ratas revelaron varios tipos de atrofia y degeneración en los túbulos seminíferos y en el tejido intersticial. Hubo desprendimiento de la lámina basal de los túbulos y una disminución profunda en la celularidad. Cuando las ratas fueron devueltas a su hábitat (BA) y posteriormente examinadas, muchos túbulos mostraron un número normal de células, incluyendo espermáticas y espermatozoides. El epitelio se observó normal en la mayoría de los túbulos seminíferos de estos animales, con el espacio intersticial normal y sin desprendimiento de la lámina basal. Al parecer, se logró una recuperación ultraestructural completa en el grupo de hipoxia; algunas células de espermatogénesis perdieron su arquitectura normal, siendo de forma irregular con algunas características de necrosis, como la contracción y núcleos picnóticos caracterizados por condensación de la cromatina. Se observó disminución significativa en la altura del epitelio en estos animales (P <0,05). Además, el diámetro de los túbulos mostró una ligera disminución con aumento concomitante en los espacios intersticiales.

PALABRAS CLAVE: Gran altura; Altura baja; Hipoxia; Semen; Hormonas reproductivas.

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Correspondence to:
Dr. Asim Mohammed Abdalla
Department of Anatomy
College of Medicine, King Khalid University,
P.O. Box 641
Abha
SAUDI ARABIA

Email: asim2011o@gmail.com

Received: 13-09-2015
Accepted: 24-12-2015