

Articles

Role of vitamins E and C in mitigating hypoxia- and exhaustive exercise-induced aberrant stem cell factor expression and impaired reproductive function in male Wistar rats

Fahaid H. Al-Hashem, MBBS, PhD.

ABSTRACT

الأهداف: تقييم دور فيتاميني ج وهـ في حماية الخصى من الإصابات المحتملة من جهد السباحة العنيف وجهد نقص الأكسجة الناتج عن العيش بصفه دائمه في الأماكن المرتفعة.

الطريقة: أجريت الدراسة خلال الفترة من بين أكتوبر 2010م ونوفمبر 2010م وذلك على فئران تجارب ولدت وعاشت إما في منطقته منخفضة العلو عن مستوى البحر (الرياض) أو في منطقته مرتفعة العلو عن سطح البحر (أبها). تم تقسيم فئران التجارب في كل منطقته علو إلى 3 مجموعات العدد=6: المجموعة الأولى مجموعته ضابطة والمجموعة الثانية مجموعته تعرضت لجهد سباحة شديد بينما تمت معالجة المجموعة الثالثة بفيتامين ج وهـ وذلك قبل تعرضها لجهد السباحة الشديد. تم قياس مستوى هرمونات الخصويه في الدم وكذلك مستويات الكورتيزون. بعد نهاية التجارب تم قتل الفئران بطريقة رحيمة ومن ثم تم تشريح الخصى وقياس مستويات القلوثايون، التيبارس، سوبر أكسيد، وكذلك الكاتليز. كذلك تم قياس مستوى معايير خلايا الإخصاب الجذعية وعدد الحيوانات المنوية واخيرا تم أخذ عينة للفحص تحت المجهر.

النتائج: العيش في الأماكن المرتفعة قلل نسبة معامل الخلايا الجذعية للإخصاب، وعدد الحيوانات المنوية، مستويات هرمونات الخصويه بينما ارتفعت نسبة الكورتيزون وكذلك نسبة الأكسدة الضارة. السباحة العنيفة أعطت نفس النتائج السابقة عند فئران المرتفعات المنخفضة والعالية ولكن بصورة أكثر ضرا عند فئران المرتفعات العالية. أدى إعطاء الحيوانات مركب فيتاميني ج وهـ قبل بدء جهد السباحة إلى عودة المعايير السابقة إلى وضعها الطبيعي.

خاتمة: أن مركبات فيتاميني ج وهـ يمكن أن تحمي الخصى من الإصابات المحتملة الناتجة عن جهد الرياضة والمرتفعات العالية وبالتالي زيادة معدل الإخصاب.

Objectives: To evaluate and compare the potential role of vitamins E and C in protecting against acute swimming induced reproductive function damage at different altitudes.

Methods: The study was carried between October and November 2010. A total of 36 adult male Wistar rats weighing 250 ± 5 g, and aged 8 weeks were used in this study, which was carried at the Physiology laboratory of King Khalid University in Abha City (high altitude area [HA]), and at the College of Science, King Saud University in Riyadh city (low altitude area [LA]), Kingdom of Saudi Arabia. The rats of each area were divided into 3 groups (n=6 each): control, acute exercise stress, and vitamins E and C pretreated stress. At the end of the study, oxidative stress, reproductive function, histopathology, and expression of stem cell factor (SCF) were examined in all rats.

Results: Living under HA conditions decreased expression of SCF, sperm count, and serum levels of reproductive hormones, and significantly increased testicle tissue oxidative stress and lipid peroxidation. Exhaustive exercise-induced stress at both altitudes resulted in similar results with more deteriorating effects in rats of HA compared with LA. Supplementation of vitamins E and C prior to stress induction at both altitudes prevented all these structural and functional aberrations from happening.

Conclusion: High altitude or strenuous exercise, or both, may impair male reproductive function, while vitamins E and C in combination potentially mitigate these adverse effects.

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From the Department of Physiology, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Fahaid H. Al-Hashem, Department of Physiology, College of Medicine, King Khalid University, Abha 61421, Kingdom of Saudi Arabia. Fax. +966 (7) 2418364. E-mail: fahaid999@yahoo.com

Both high altitude (HA) exposure and submaximal exercise result in hypoxic stress in humans. The human neuroendocrine system is highly susceptible to hypoxic stress, and its activation following stress exposure may lead to a cascade of cardio-respiratory, endocrine, metabolic, nutritional, thermal, and psychological changes that help the human body to cope with the stressfully hostile environment.¹ It has been documented that lipid peroxidation and antioxidant system are involved in both HA- and strenuous exercise-induced hypoxic stress.²⁻⁵ However, the physical exercise-associated oxidative organ damage is more profound at HA⁴⁻⁶ in individuals who do not exercise regularly.⁷ In some cases, the cumulative effects of exercise under hypoxic exposure may persist for some time after return to sea level.⁸ In some other cases, acute mountain sickness (AMS) may occur in altitude training.⁹

Male reproductive hormone profile and function, related to oxidative stress in the testes, have been studied in animal and human studies in low altitude (LA) and HA environments.^{10,11} Results from these studies have shown structural, morphological, and metabolic abnormalities in the testes, which result in function impairment including germinal cell loss, spermatogenesis arrest, round spermatid mitochondrial oxidation,¹¹ and abnormal production of major male reproductive hormones including testosterone, follicular stimulating hormone (FSH), and luteinizing hormone (LH)^{12,13} at HA. However, most of these studies used experimentally manipulated altitude conditions and non-acclimatized subjects.¹⁰⁻¹³ As a result, the data need to be further validated in subjects native to test altitude environments and in natural altitude conditions.

Stem cell factor (SCF) is a protein produced by Sertoli cells and it plays a regulatory role in the proliferation, adhesion, and survival of spermatogonia.¹⁴ Nevertheless, testicular expression of SCF expression in rats with short or chronic exposure to HA and after strenuous exercise has not been well studied. Antioxidants, vitamins E and C, may have an ameliorative effect on hypoxic stress-induced male reproductive dysfunction. To date, however, there are limited studies that have monitored the oxidative stress status and reproductive function in native animals under natural hypoxic conditions provided by HA areas, with and without antioxidant

vitamins dietary supplementation under basal conditions and swimming-induced acute stress. Moreover, the involvement of SCF level of expression of altitude or exercise, or both, on regulation of reproductive function in animals and human has not been fully investigated. This study was therefore conducted to investigate the negative effects of HA- or exercise-induced or both hypoxic stress on oxidative status, SCF expression in the testes, and reproductive function as well as the potential of antioxidative vitamins E and C in ameliorating these negative effects in adult male rats native to HA and LA in real world altitude conditions.

Methods. Study areas. The study was carried out in 2 different altitude areas in the Kingdom of Saudi Arabia between October and November 2010. Abha city located in the Aseer Mountains 2,200 m above sea level was chosen as the HA area, whereas Riyadh, the capital of Saudi Arabia, located in the center of Saudi Arabia approximately 600 m above sea level was chosen as the low LA site of experiments. The basic geographical and environmental data for these 2 areas were collected from the national meteorological organizations of Saudi Arabia and are presented in Table 1.

Animals. Eighteen adult male Wistar rats, weighing 250±5 g and aged 8 weeks, from each of the 2 altitude areas described above were used. These animals were born and raised in the Experimental Animal Facilities, at the laboratory of the College of Medicine, King Khalid University in Abha City, and at the laboratory of the College of Science, King Saud University in Riyadh city, and were accordingly considered native to

Table 1 - General geographic and meteorological information from Riyadh (low altitude) and Abha (high altitude) in Saudi Arabia.

Variable	Riyadh	Abha
Coordinates (latitudes)	24.64083; 24° 38' 27 N	18.21639; 18° 12' 59 N
Coordinates (longitude)	46.77278; 46° 46' 22 E	42.50528; 042° 30' 19 E
Altitude (meters)	600	2200
Barometric pressure (mm Hg)	711	590
Atmospheric O ₂ tension (mm Hg)	145	120
Relative humidity (%)	15-50	20-30
Summer temperature (shade) (°C)	24-45	16-28
Winter temperature (shade) (°C)	10-25	5-15

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the HA or LA environment. Rats in both areas were the tenth generation offspring of the same lineage. The same housing conditions and diet were used for animals in both altitude areas. The study was approved by the Ethical Committee in the Department of Physiology at the King Khalid University Medical School, Abha, Saudi Arabia, and 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.¹⁵

Vitamins, dose selection, and route of administration.

Vitamins E (alpha-Tocopherol, natural [1 mg/ml]) purchased from BDH Chemicals Ltd., (London, UK) and vitamin C (ascorbic acid, powder) was purchased from Sigma (Dorset, UK). The dose and mode of administration of both vitamins were based on our previous findings using a similar protocol, which showed a highly protective effect of these doses against acute swimming-induced oxidative damage in the lungs,¹⁶ liver, kidneys,¹⁷ and thyroid function¹⁸ of rats of the same species in same altitude.

Experimental and study design. At each altitude, rats were divided equally into 3 groups (n=6): normal control where no stress and no vitamin supplementation were applied; acute stress where animals were stressed by forced swimming; and vitamin supplementation group where animals received a single intra-peritoneal dose of vitamin E (at 25 mg/kg) and an oral dose of vitamin C (at 20 mg/kg) one hour prior to stress induction. Acute exhaustive exercise-associated stress was achieved by forcing the animals to swim continuously for 2.5 hours in glass tanks (length 100 cm, width 40 cm, depth 60 cm) containing tap water (30 cm deep) at a constant temperature of 32°C.

Hormone measurement and hematocrit test.

Immediately after the end of the experimental procedure, 2 blood samples were collected directly from the heart of each animal into a plain tube and a heparinized tube. Blood in the plain tube was allowed to clot at room temperature, and serum was separated by centrifuging the tube at 4,000 rpm for 10 minutes. Serum levels of testosterone, cortisol, FSH, and LH were determined using commercially available ELISA kits from Seiko Instruments GmbH (Neu Isenburg, Germany) as instructed by the manufacturer. Blood in the heparinized tube was centrifuged in a micro-hematocrit centrifuge and hematocrit value was determined.

Tissue collection and processing. Animals were sacrificed by decapitation after blood collection. Both testes were immediately removed and placed in a Petri dish. After a brief wash in cold saline, fats,

connective tissues, and blood vessels were removed. As described below, parts of the testes were processed for histopathological examination and total RNA extraction, the parts were frozen at -80°C until use in other assays, and the epididymis was subjected to sperm count.

Sperm count. The cauda epididymis of both left and right testes was weighed and minced with a scalpel blade in the mid-to-distal direction in a petri dish in 1:20 (weight:volume) physiological saline (0.9% NaCl) solution. Suspension was kept at 37°C for 5 minutes, allowing dispersion of the sperm into the solution. After shaking gently for 20 times, a drop of the sperm suspension was placed in a hemocytometer and the total number of the sperm was counted under a Nikon microscope (Nikon Eclips E600) with a 40x objective lens as previously described.¹⁹ Each sample was counted thrice and the number of sperms/0.1 g tissue was calculated accordingly.

Thiobarbituric acid reactive substances assay. Frozen testes were homogenized in 0.1 M Tris-HCL buffer of pH 7.4 at a 1:4 weight to volume ratio, using a Potter-Elvehjem homogenizer at 4°C. The formation of thiobarbituric acid reactive substances (TBARS), as an indicator of lipid peroxidation, in the homogenate was assessed by the method described by Ohkawa et al.²⁰ In brief, a 4 mL reaction mixture containing 0.1 mL of tissue homogenate, 0.2 mL of sodium dodecyl sulfate, 1.5 mL of acetic acid with pH of 3.5 (20% acetic acid was pre-adjusted with 1 M NaOH to desired pH), 1.5 mL of aqueous solution of thiobarbituric acid (TBA) and 0.7 mL water was prepared. After heating at 95°C for one hour in a hot water bath and cooling, one mL distilled water and 5 mL n-butanol and pyridine (15:1) mixture were added and the mixture was shaken vigorously on a vortex mixer and then centrifuged at 3,000 rpm for 10 minutes. The absorbance of the upper organic layer was read at 532 nm.

Endogenous antioxidant activity assessment.

Superoxide dismutase (SOD) and reduced glutathione (GSH) levels in the testes tissue homogenate prepared above were measured using commercial kits from Randox Laboratories Ltd (London, UK). The GSH was expressed in mmol/L and SOD activity was expressed in U/mg tissue. One unit of SOD was defined as the amount that caused a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) under the assay condition. Catalase activity (CAT) was determined using a commercial kit K773-100 from Biovision Inc. (Milpitas, CA, USA). The CAT was expressed as U/mL; one unit of CAT was defined as the amount capable of

Table 2 - Primers used in polymerase chain reactions for soluble stem cell factor and β -actin.

Primers	Sense	Antisense	Annealing temperature	Product size (bp)
CSFs	TTCGCTTGTAATTGCCCTTTC	TTCAACTGCCCTTGTAAGACTTGA	57	250
β -actin	GACAGGATGCAGAAGGAGAT	TTGCTGATCCACATCTGCTG	61	146

decomposing 1.0 μmol of H_2O_2 per minute at pH 4.5 and 25°C.

Semiquantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from testicle tissue (30 mg) using the RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia) according to manufacturer's directions. First strand cDNAs were synthesized through reverse transcription (RT) using an oligo-dT primed cDNA synthesis kit from Roche Molecular Biochemicals (Auckland, New Zealand) as instructed by the manufacturer. The test gene SCF and the internal control gene β -actin were amplified by polymerase chain reaction (PCR) using 2 μl RT products from each sample in a 20 μl reaction containing [α - ^{32}P]dATP (0.75 mCi), Taq polymerase (0.01 U/ml), dNTPs (100 mM), MgCl_2 (1.5 mM), and buffer (50 mM Tris-HCl). The primer sequences and PCR conditions are presented in Table 2, which were based on previous studies.^{21,22} The PCR products (10 μl) were electrophoresed on 2% agarose gels containing 100 ng/ml ethidium bromide, and photographed with a Polaroid camera under ultraviolet illumination. Intensities of the SCF and actin bands were analyzed using the Image Master Software (SYDR-1990, Syngene, City/State, USA) and the SCF to β -actin band density ratio was calculated.

Histopathological assessment. Testes tissue collected at the time of animal sacrifice were fixed in 10%

neutral buffered formaldehyde solution (pH 7.0). Standard paraffin tissue section (4 μm) preparation and hematoxylin and eosin (H&E) staining were performed. Stained tissue sections were histopathologically assessed by an experienced animal pathologist blind to the specimens' identity under a light microscope and photographed.

Statistical analysis. Statistical analysis was performed by 2-way ANOVA using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) Version 6. To identify the sources of significant main effect, post hoc comparisons (Tukey's t test) were used. Data were expressed as mean \pm standard deviation (SD), and statistical significance was assigned at $p \leq 0.05$.

Results. Hematocrit and cortisol levels. Data on hematocrit values and serum levels of the primary stress hormone cortisol are presented in Figures 1A & 1B. Hematocrit values were significantly higher (14.8%, $p < 0.0001$) in rats native to HA than those native to LA. Exhaustive exercise resulted in an increase in hematocrit values by 7% ($p = 0.0063$), and 4.3% ($p = 0.001$) when compared to the basal values at HA and LA. Supplementation of vitamins E and C failed to mitigate the exhaustive exercise-induced increase in hematocrit values at either HA ($p = 0.9982$) or LA ($p = 0.7913$). The baseline serum levels of cortisol in control animals were similar at HA and LA ($p = 0.9973$).

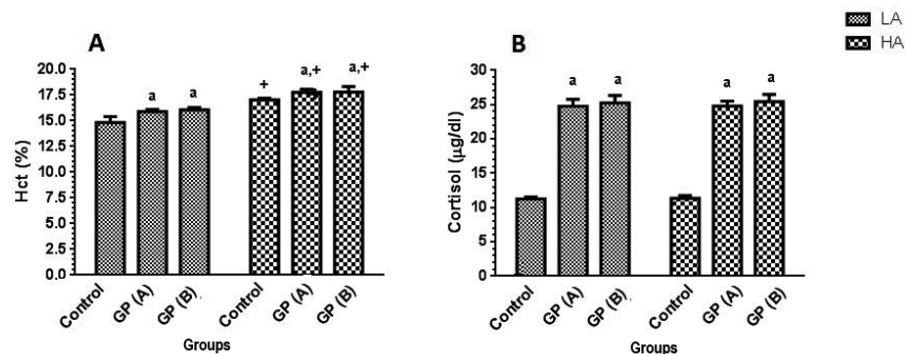


Figure 1 - Hematocrit percent (Hct%, A) and cortisol levels B) in the serum of all groups of rats at low altitude (LA) and high altitudes (HA). Control: resting state; stress group A [GP(A)]: rats under forced swimming exercise; and stress group B [(GP(B))]: rats received vitamin supplementation prior to forced swimming exercise. +significantly different when compared with the same similar groups at LA area. a - significantly different when compared with its altitude specific control group.

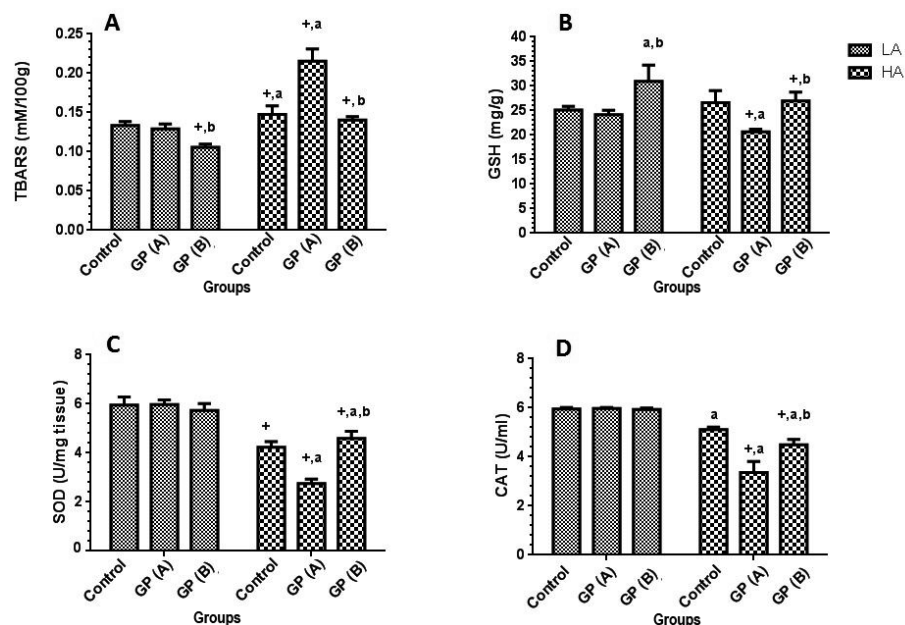


Figure 2 - Thiobarbituric acid reactive substances (TBARS, A), reduced glutathione (GSH, B), superoxide dismutase (SOD, C) and catalase (CAT, D) in the indicated groups of rats at low altitude (LA) and high altitudes (HA). Control: resting state; stress group A [GP(A)]: rats under forced swimming exercise; and stress group B [(GP(B))]: rats received vitamin supplementation prior to forced swimming exercise. +significantly different when compared with the same similar groups at the LA area. a - significantly different when compared with altitude specific control group. b - significantly different when compared with stress group (A) of the same altitude.

Acute stress following exhaustive exercise was associated with a significant elevation ($p < 0.0001$) in serum levels of cortisol at both LA (119.8%) and HA (118.6%) when compared to their basal levels. Administration of vitamins E and C prior to swimming exercise did not significantly affect cortisol levels at both LA ($p = 0.5466$) and HA ($p = 0.4031$).

Lipid peroxidation and endogenous antioxidant status. Rats native to HA had a significantly higher baseline level of testicular TBARS ($p = 0.0305$), significantly lower baseline activities of SOD and CAT ($p < 0.0001$), and a similar baseline level of GSH ($p = 0.466$) in the testes as compared to rats native to LA (Figure 2). Exhaustive exercise did not significantly affect testicle levels of TBARS ($p = 0.668$) and GSH ($p = 0.6847$), and activities of SOD ($p = 0.9869$) and CAT ($p = 0.9802$) at LA, but increased the level of TBARS by 45.7% ($p < 0.0001$) and decreased the level of GSH by 22.35% ($p < 0.0001$) and the activities of SOD 34.8% and CAT by 29.1% ($p < 0.0001$) at HA as compared with the resting control (Figure 2). Supplementation of vitamins E and C prior to stress induction decreased the level of TBARS by 21% ($p < 0.0002$) and increased the level of GSH by 28.16% ($p < 0.0001$), but had no significant effects on the activities of SOD ($p = 0.2148$) and CAT ($p = 0.9650$) at LA when compared with

the group of rats exposed to stress alone. The level of TBARS was significantly lower ($p < 0.0001$), and levels of GSH were significantly higher ($p < 0.0001$) in LA rats' testes administered vitamins prior to exercise when compared with their control levels. On the other hand, vitamin supplementation significantly decreased the level of TBARS ($p < 0.0001$) and increased the level of GSH ($p < 0.0001$) and activities of SOD and CAT ($p < 0.0001$) at HA, when compared with no vitamin supplementation. Vitamin treatment in these HA rats effectively brought all these parameters back to levels that were not significantly different from the baseline values in control animals ($p = 0.3429$ for TBARS, $p = 0.9517$ for GSH), the 2-way ANOVA showed that the levels of SOD in this group of rats were significantly higher ($p = 0.0442$), but the levels of CAT remained lower than the control basal level ($p < 0.0001$) (Figure 2).

Serum levels of major reproductive hormones and sperm count. Serum levels of testosterone decreased by 34.3%, LH by 23.9%, and FSH by 26% in control rats native to HA as compared with those native to LA; these decreases all reached a statistically significant levels ($p < 0.0001$, Figures 3A-3C). Exhaustive exercise significantly reduced levels of these 3 hormones as compared with the baseline levels in control animals ($p < 0.0001$) in both altitude environments. However,

the reduction magnitude was different for different hormones and at different altitudes; while forced swimming reduced serum levels of testosterone by 30.5%, LH by 37%, and FSH by 51.8% at LA, the reduction was 57.5% in testosterone, 53% at LH, and 66.2% in FSH at HA. Supplementation of vitamins E and C significantly improved profiles of these hormones at both HA and LA ($p < 0.001$ for all), by 59.8% in testosterone, 34.6% in LH, and 108.7% in FSH at LA, and by 148% in testosterone, 118.6% in LH, and 188.9% in FSH at HA. Vitamin supplementation effectively brought all these hormones to their baseline levels: $p = 0.6753$ for testosterone, and $p = 0.9843$ for FSH at LA, and $p = 0.4669$ for testosterone, $p = 0.829$ for LH, and $p = 0.911$ for FSH HA. Turkey's t test showed that the levels of LH in rats administered vitamins at LA remained significantly lower than their control baseline value ($p = 0.0003$). In control animals, sperm count was 30.9% lower in HA than in LA ($p < 0.0001$, Figure 3D). There was a significant reduction ($p < 0.0001$) in sperm count after exhaustive exercise-induced stress at both altitudes, but the reduction was more pronounced at HA than LA (43.9% versus 27.3%, $p < 0.0001$). Vitamin supplementation prior to forced swimming exercise ameliorated the acute stress-induced decrease in sperm count by 39.7% at LA and 75.3% at HA

($p < 0.0001$) when compared with their specific stress groups, thereby restoring sperm count to a level similar to the normal control level in both LA ($p = 0.9238$) and HA ($p = 0.9981$) environments (Figure 3D).

Stem cell factor mRNA abundance. As shown in Figure 4, SCF mRNA abundance was significantly lower in control rats native to HA than those native to LA ($p = 0.0018$), and was significantly decreased after acute forced swimming exercise ($p < 0.0001$) at both LA and HA (by 35% and 42.3%). Administration of vitamins E and C effectively and adequately restored SCF mRNA abundance to a level not significantly different from that in normal control animals in HA ($p = 0.7089$) rats, but significantly lower in LA ($p = 0.0383$) rats.

Histopathological findings. In the testes of normal control rats native to LA, completely differentiated seminiferous tubules were present, each possessing a definite basement membrane and a small lumen densely filled with sperm tails. Abundant and healthy spermatogenic cells including spermatogonia, primary spermatocytes, early spermatids, late spermatids, and Sertoli cells were clearly seen across the section (Figure 5A). In contrast, in the testes of normal control rats native to HA, seminiferous tubules had reduced diameters with less densely packed mature sperm and

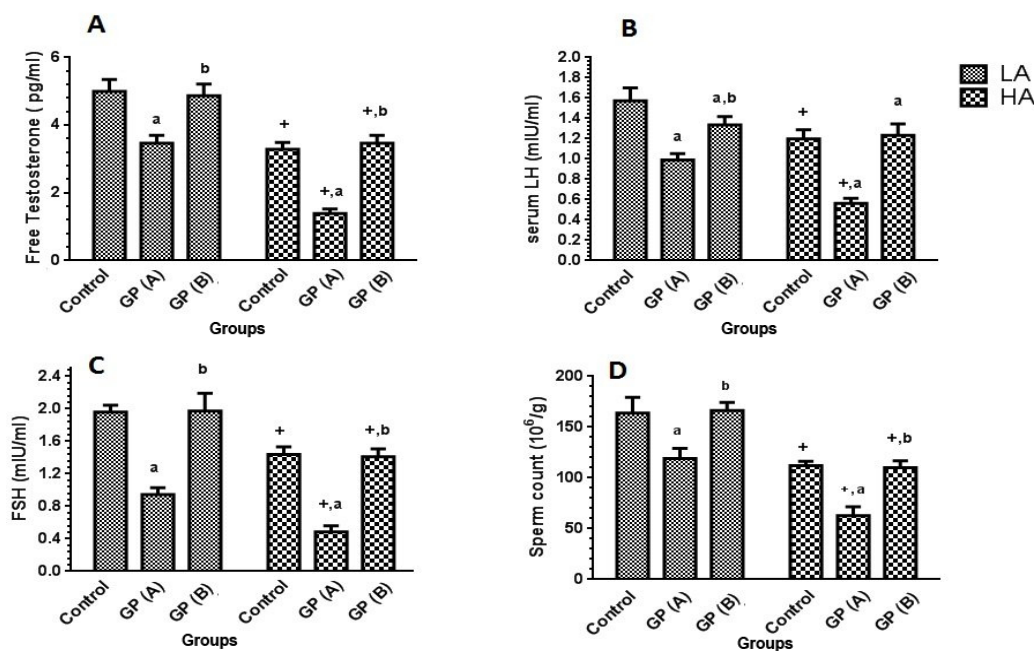


Figure 3 - Serum level of free testosterone (A), luteinizing hormone (LH, B), follicular stimulating hormone (C) and sperm count (D) in the indicated groups of rats at low altitude (LA) and high altitudes (HA). Control: resting state; stress group A [GP(A)]: rats under forced swimming exercise; and stress group B [GP(B)]: rats received vitamin supplementation prior to forced swimming exercise. +significantly different when compared with the same similar groups at the LA area. a - significantly different when compared with altitude specific control group. b - significantly different when compared with stress group (A) of the same altitude.

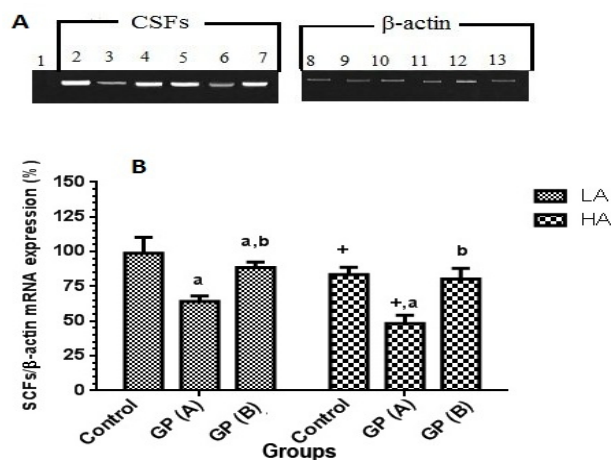


Figure 4 - Polymerase chain reaction analysis of soluble stem cell factors A) Semi-quantitative reverse transcriptase PCR analysis of testicular soluble stem cell factor (SCFs) shows the electrophoresis product of both mRNA of CSF and the housekeeping gene, β -actin. 1: negative control. 2 and 8: low altitude native rats. 3 and 9: low altitude stressed group (A). 4 and 10: low altitude vitamins treated stress group (B). 5 and 11: high altitude control. 6 and 12: high altitude stressed group (A). 7 and 13: high altitude vitamins treated stress group (B). B) shows gene expression of SCFs in the testes of all rats, which are graphed as percent of mRNA expressions against expression of the housekeeping gene for each group of rats. Control: resting state; stress group A [GP(A)]: rats under forced swimming exercise; and stress group B [(GP(B))]: rats received vitamin supplementation prior to forced swimming exercise. +significantly different when compared with the same similar groups at the LA area. a - significantly different when compared with altitude specific control group. b - significantly different when compared with stress group (A) of the same altitude.

sperm heads but seemingly normal spermatogenic cells of other stages in the lumen (Figure 5B). Forced swimming exercise did not alter the structure of seminiferous tubules, but reduced the number of mature sperm and increased the number of sperm heads in the lumen with little increased vacuolations in Sertoli cells at LA (Figure 5C). In contrast, at HA, exhaustive exercise adversely altered the structure of seminiferous tubules and spermatogenesis; seminiferous tubules and spermatogonia were slightly degenerated with resultant cellular debris present in the lumen, Sertoli cells were highly vacuolized, the number of spermatogenic cells was seen to be reduced with sperm bundles absent in most tubules and early and late stage germ cells absent but multinucleate giant cells seen in most tubule walls (Figure 5D). Treatment with vitamins C and E prior to acute swimming exercise effectively attenuated the exhaustive exercise-induced abnormalities in the seminiferous tube structure and spermatogenesis at both LA (Figure 5E) and HA (Figure 5F).

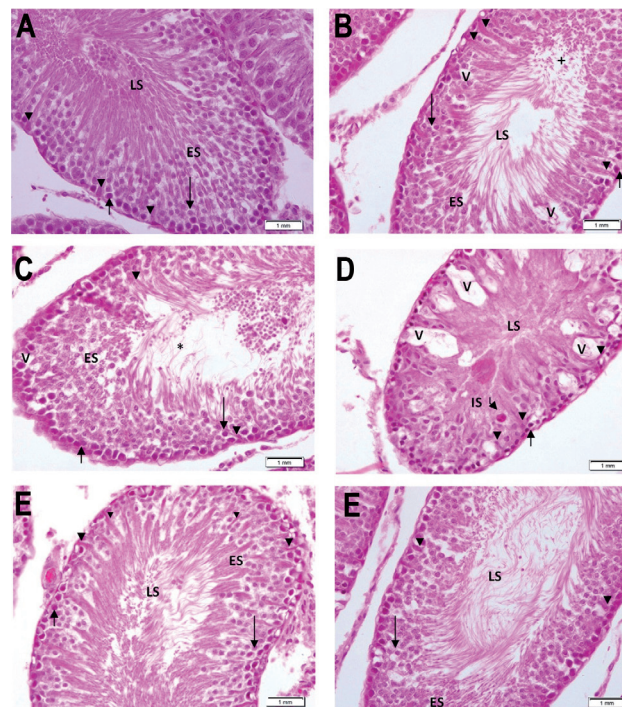


Figure 5 - A photomicrograph of a cross-section from the testis of a rat from all groups of rats: A) Low altitude native rats showing a normal seminiferous tubule with healthy spermatogenic cells; spermatogonia (short arrow), primary spermatocytes (long arrows), early spermatids (ES), late spermatids (LS) and Sertoli cells (arrow head). B) High altitude native rats showing a narrow preserved seminiferous tubule with different normal spermatogenic cells of all stages. The presence of sperm heads (+) and vacuolation (V) in Sertoli cells were seen. C) Low altitude exercise group rats showing decreased number of late spermatids (*) and well preserved seminiferous tubules with primary spermatocytes, early spermatids and very little vacuolated Sertoli cells (V). D) High altitude exercise group rats: showing damaged seminiferous tubules with highly decreased spermatogonia, presence of fibrinoid tissue (IS) with decreased number of primary spermatocytes and early spermatids and with absence of late spermatids. Note the presence of Sertoli cells (arrow heads) and the presence of few multinucleate giant cells (arrow head with short L) and elongated primary spermatocytes. E) Low altitude exercise group rats treated with vitamins showing normal seminiferous tubules with abundant spermatogenic cells; spermatogonia, primary spermatocytes, early spermatids, late spermatids and well preserved Sertoli cells. F) High altitude exercise group rats treated with vitamins showing normal seminiferous tubules with healthy spermatogenic cells; spermatogonia, primary spermatocytes, early spermatids, late spermatids and Sertoli cells. Hematoxylin & Eosin 40X.

Discussion. Cortisol is the primary stress hormone. In the present study, no significant difference was observed in baseline serum levels of cortisol between adult male rats native to LA and those native to HA. This observation agrees with the results from a previous

study.²³ One possible explanation for the lack of difference in cortisol levels at HA and LA is that these animals had adapted to their altitude environments and thus hypoxia had no significant stimulatory effects on adrenal cortex. It has been suggested that exercise may exacerbate cortisol release by increasing ventilation and blood flow to the adrenal gland via a reflex action of the carotid bodies and non chemoreceptor activation.^{24,17} In agreement with this notion, we observed that forced swimming at both altitudes resulted in a significant increase in serum levels of cortisol, indicating an exercise induction of intensive stress.

It has been well documented that hypoxia results in increased production of oxidative stress markers including reactive oxygen species (ROS) in the blood, urine, and various tissues of laboratory animals and humans in both natural and experimentally simulated altitude conditions,⁶⁻⁹ and that exercise at HA exacerbates this increased production.⁶ It has been also documented that acute hypoxic stress leads to activation of the tissue antioxidant defense system.⁶ In this study, we showed that lipid peroxidation was elevated in the testes of rats to native HA as compared with rats native to LA, and that exhaustive exercise exacerbated this elevation at HA. The exact mechanism underlying exercise-induced oxidative stress including ROS production has yet to be further defined but may involve dopamine and norepinephrine signaling pathways.²⁴ We have previously reported a role for antioxidant vitamins E and C in protection against pulmonary and hepatorenal exercise-induced oxidative stress in rats in both LA and HA conditions.^{16,17} The present study showed that supplementation of vitamins E and C effectively ameliorated exhaustive exercise-induced oxidation, particularly at HA, validating our previous report.

Limited studies have assessed the adverse effects of hypoxic stress on male reproduction but generated inconsistent results. In some of these studies, decreased serum levels of LH, FSH, and testosterone were reported in hypoxic conditions.^{13,25} In agreement with these studies, we observed in the present study that baseline serum levels of testosterone, FSH, and LH were significantly lower in rats native to HA than in those native to LA, and that exhaustive exercise further decreased levels of these hormones at both HA and LA but at a higher magnitude at HA. Our observations are in accordance with previous reports that prolonged physical activities significantly decreased secretion of FSH and LH.^{26,27} It is known that FSH and LH levels are under the control of gonadotropin-releasing hormones, which may be regulated by hypobaric hypoxia or various peptidergic neuron stimulants,^{28,29}

and that testosterone secretion is primarily regulated by the pituitary gonadotrophin LH. At present, the mechanism responsible for decreased testosterone secretion under exercise-induced hypoxic conditions is not fully understood, but a reduction in the sensitivity of the testis to LH or inhibition of Leydig cell testosterone biosynthesis has been proposed.^{26,29}

To evaluate effects of altitude and exercise with or without vitamin treatment on reproductive function, we also performed sperm count and histopathological assessment. We observed a significant decrease in sperm count at HA as compared to LA and in the exhaustive exercise-induced hypoxic stress condition as compared to the resting control state. We also observed an effective restoration of spermatogenesis after treatment of stressed rats with vitamins E and C in both altitude areas. In parallel to changes in spermatogenesis, HA and/or exercise-induced abnormalities and vitamin treatment-induced restoration were observed in the structure and morphology of the testes. Since most of these observations are novel, comparison references are scarce in the literature and thus further studies are needed to validate our findings.

Moreover, the mechanisms for the testicular abnormalities in structure and morphology as well as impaired spermatogenesis in hypoxic stress conditions at HA and/or following exhaustive exercise have yet to be further established. Garrido et al³⁰ and Koksai et al³¹ have proposed that hypoxic stress reduces the capacity of spermatozoa, which have a small amount of cytoplasm, to respond when confronted to an accumulation of ROS, resulting in death of spermatozoa and decreased sperm count. This proposed mechanism may explain our observation that acute forced swimming exercise at both altitudes exacerbated ROS and further decreased sperm count. However, it cannot explain why a decrease in sperm count and structural and morphologic abnormalities occurred but neither increased oxidative stress status nor lipid peroxidation was seen after exhaustive exercise in the LA situation. To look into other mechanisms, we evaluated the expression of SCF.

Stem cell factor is expressed by spermatogonia and Leydig cells³² and interacts with the c-kit receptor, a transmembrane tyrosine kinase.¹⁴ The c-kit receptor is immunolocalized to the acrosomal granules of spermatocytes and the acrosomes of spermatozoa.¹⁴ Deletions or mutations of the c-kit gene, or its ligand, SCF, have been reported to result in infertility in mice due to the loss of germ cells.³³ Decreased expression of SCF was observed in rats at HA and post exhaustive exercise in this study. This might be partially due to damage of Sertoli cells as indicated by increased vacuolization seen in the H&E-stained testicle tissue sections. Given

that FSH directly stimulates SCF synthesis,³⁴ down-regulated synthesis of the SCF protein as a result of decreased FSH secretion might be an alternative explanation. It has been previously reported that FSH and SCF can protect germ cells from apoptosis through expression inhibition of key apoptotic genes such as Bok,³⁵ a pro-apoptotic member of the Bcl-2 gene family. In this study, we did not assess Bok expression, but Bok mRNA was detected in spermatogonia, pachytene spermatocytes, and Sertoli cells in rat testes in a previous study.³⁶ Based on this together with our observation that the testicular pathology and decreased sperm count at HA, or following exhaustive exercise at both altitudes were proportional to serum levels of FSH and testicle expression of SCF, it is likely that HA and/or exhaustive exercise-induced hypoxic stress impairs the structure, morphology, and function of rat testes through FSH and SCF signaling- and Bcl-2 gene family-dependent mechanisms. One of the limitations of our study is that we did not measure the expression of these apoptotic genes in the spermatogonia, pachytene spermatocytes, and Sertoli cells of these rats' testes; to prove this hypothesis at this stage requires further research.

Supplementation of vitamins E and C prior to forced swimming exercise restored the normal serum levels of testosterone, LH, and FSH, normal sperm count, and normal testis histology suggesting an important beneficial role for these vitamins in fertility. Supporting this role, several previous studies in vitamin E deficient rats demonstrated a robust reduction in spermatogenesis and an increase in Leydig cell degeneration.^{37,38} Classically, vitamin E is considered as an anti-sterility vitamin, which is associated with normal function of the male reproductive system.^{39,40} Vitamin E action is capable of protecting against oxidative stress by reducing the level of malondialdehyde and improving the activity of the antioxidant defense system activity in testicular cells.⁴⁰ For the first time, we demonstrated an increased expression of SCF in the testes of rats at both altitudes after administration of vitamins E and C, suggesting another possible mechanism underlying the action of these vitamins in protection against hypoxic stress-induced male reproductive dysfunction. The increase in testicular expression of SCF after vitamin E and C administration might be due to increased secretion of FSH. Vitamin E is well known to increase the synthesis and secretion of FSH and LH from the anterior pituitary gland.⁴¹ Previous structural study with an electron microscopy demonstrated that the number of secretory granules in gonadotrophs in the anterior pituitary decreased in vitamin E deficient rats, which could be restored by vitamin E.⁴¹

In summary, we have demonstrated in this study that HA and exercise, each alone or in combination, may impose a hypoxic stress to adult male rats, which induces structural, morphological, and functional abnormalities in the testes possibly through disturbed levels of FSH and LH and down regulation of SCF expression, and that vitamins E and C in combination are able to effectively meliorate these abnormalities. Our observations suggest that supplementation of vitamins may offer effective protection against the negative impact of strenuous exercise-induced hypoxic stress, particularly in high altitude conditions, on male reproductive function. Future studies involving a correlation of these finding with apoptotic markers in rats testes, mating success, and birth weights under high altitude and/or acute swimming stresses are required.

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