Ram semen deterioration by short-term exposure to high altitude is prevented by improvement of antioxidant status

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Ovine reproduction efficiency in herds at high altitude (ha) is lower than that at low altitude (la). In ewes, ha effects are due to hypoxia and oxidative stress. Our aim was to establish the effect of antioxidant vitamin supplementation on semen traits and antioxidant status of rams exposed to short or long time ha. A total of 32 rams native to la (~500 m) were used, 16 were kept at la and the other 16 were brought to ha (~3600 m), where they were placed in the same flock as the ha native rams (n = 16). Half of the animals in each group were supplemented daily with vitamins C 600 mg and E 450 IU per os, during the entire experimental period, starting the 4th day after animal’s arrival at ha (day 0). At days 0, 30 and 60 of treatment, blood and semen samples were collected for evaluation of antioxidant status and semen standard characteristics. Data were compared within each experimental time by analysis of variance using a general linear model. Elevated concentrations of oxidative stress biomarkers were present in blood from animals maintained at ha. Ejaculates from ha exposed rams showed decreased sperm concentration, progressive motility and viability, in addition to decreased antioxidant status in seminal fluid. A total of 30 days of oral supplementation with vitamins C and E prevented some ha negative effects on semen characteristics, mainly in recently ha exposed rams. It is concluded that exposure of rams to ha negatively affects semen quality, where oxidative stress plays a predominant role. These effects are mainly prevented by oral supplementation of vitamins C and E, which constitutes a simple and cheap alternative to improve semen quality of rams when they are moved to ha.

Keywords: ram semen, high altitude, oxidative stress, antioxidant vitamins

Implications

The results of this study shows that exposition of rams to high altitude by short or long term induces blood and seminal oxidative stress, and deteriorate some sperm characteristics. These changes may be partly responsible for the low fertility of ovine herds maintained at high altitude. Oral supplementation with vitamins C and E prevents oxidative stress and improves sperm characteristics, especially in rams exposed to high altitude for a short time. This supplementation can be a simple and inexpensive tool to improve the ovine fertility at high altitude.

Introduction

Highland sheep farming is extremely important in the Andean and Qinghai-Tibetan plateaus, where an estimated 25 million people economically depend on sheep breeding (Huddleston et al., 2003). However, sheep breeding at high altitude (ha) is hampered by a reduced reproductive efficiency when compared with their low altitude (la) counterparts (Parraguez et al., 2006).

Most of the studies at ha have been focused in female reproductive efficiency and have shown that low fertility in ewes results from inadequate function of the reproductive axis, as a consequence of hypoxia, oxidative stress or both (Parraguez et al., 2013). In spite of the greater relative importance of males in sheep flocks (one male mates 20 to 25 females), there is a scarcity of similar studies in rams. There appears to be only one report from Peruvian researchers, published in the 1940s, that addressed severe disturbances in the ejaculates and sexual libido of rams taken to ha (Monge et al., 1945). Studies in humans (Okumura et al., 2003) and rats (Farias et al., 2008) have shown clear evidence of deleterious effects on the male reproductive axis after ha exposure, including deterioration in both testicular and seminal parameters. Interestingly, in human populations

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living at ha over multiple generations, these negative male reproductive changes are absent, indicating an adaptation to that environmental condition (Gonzales, 2007).

Previous studies in ewes have demonstrated that most of the deleterious effects of ha on reproductive efficiency, either at short- or long-term exposure, are associated with oxidative stress secondary to hypoxia (Parraguez et al., 2006, 2011 and 2013). Oxidative stress results from unbalanced production of reactive oxygen species (ROS) and the protective effect of the endogenous antioxidant system, where superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are protagonist enzymes. Deleterious effects of ROS are mainly related to changes in lipids, protein and DNA, resulting in damage to or cell death. Spermatozoa are highly vulnerable to ROS effects, leading to fertility disorders (Walczak-Jedrzejowska et al., 2013).

There is currently no data regarding redox balance in rams at ha, but recently we have demonstrated that increases in seminal antioxidant status in rams at la can be obtained by supplementation with antioxidant vitamins, leading to increased sperm concentration, motility and viability (Cofré et al., 2016). Likewise, data from human and rodent models point to the main role of oxidative status in the conservation of semen quality (Agarwal and Allamaneni, 2011) and have shown the efficacy of antioxidant treatment for improvement semen quality (Agarwal and Sekhon, 2010).

Thus, having in mind the social and economic importance of sheep production for rural populations living at ha (Huddleston et al., 2003), the first aim of the present work was to characterize the effects of short- and long-term exposure to ha hypoxic environment on ram testosterone levels and semen quality. The second objective was to establish whether oral antioxidant vitamin supplementation prevents or attenuates effects associated with ha exposure.

Material and methods

Ethics statement
This study was performed in agreement with the Guide for Care and Use of Laboratory Animals (Eighth Edition, National Research Council, National Institute of Health, Washington, DC, USA). The protocols were approved by the Bioethics Review Committee of the Faculty of Veterinary and Animal Sciences, University of Chile, as well as by the Bioethics Advisory Committee of the Chilean National Commission for Scientific and Technological Research (CONICYT, Chile).

Animal management and sampling
The experiment involved 48 crossbred rams, from the experimental flocks of the University of Chile, with 1 year of age, 45.5 ± 0.9 kg of mean BW and 2.8 ± 0.3 of body condition score in a 1 to 5 scale. In al, 16 native ha rams (group HH; descendants of sheep introduced to the Andean high plateau by Spanish settlers almost 500 years ago) at the beginning of the experiment, were allocated to a single pen at the animal facilities of the International Centre for Andean Studies (INCAS, University of Chile; ~3600 m altitude, barometric pressure ~667 hPa). A total of 32 native la rams (~500 m; barometric pressure ~ 990 hPa), with similar phenotypes, BWs and age to the males in the HH group were also selected. Four days before starting the experimental protocol, half of the la rams (group LH, n = 16) were moved to the INCAS facilities to join the HH rams. The remaining 16 rams were maintained at low altitude (group LL). Animals were fed with alfalfa hay and balanced feed to satisfy requirements (NRC, 2006); water was supplied ad libitum. The experiment began in mid-December, along with the start of the reproductive season in Chile.

Rams in each group were randomly divided into two equal subgroups (n = 8 each) and housed in different pens. Half of the animals remained as controls (maintaining the identification as groups HH, LH and LL), whereas the other half of the rams were supplemented daily with 600 mg of vitamin C (ascorbic acid) and 450 IU of vitamin E (alpha-tocopherol) orally, over 60 days (groups HHV, LHV and LLV). Vitamin doses were calculated on the basis of previous experiments on sheep maintained at similar environmental conditions, where the treatment prevented oxidative stress (Parraguez et al., 2011).

Assessment of vitamin C and E, oxidative stress biomarkers and testosterone in blood
Blood samples (10 ml) were taken from the left jugular vein at 0, 30 and 60 days after starting vitamin supplementation using heparinized syringes. Blood was centrifuged at 1200 × g ×5 min at 4°C; the obtained plasma was stored at −80°C until assayed.

Vitamins C and E were measured by high-performance liquid chromatography as previously described (Parraguez et al., 2011). Briefly, vitamin C was measured in plasma samples diluted 10-fold in ultra-pure water, by means of amperometric detection, using a glassy carbon electrode operated at 800 mV and an Ag/AgCl reference electrode. Vitamin E was measured in ethanol/dichloromethane extracted plasma samples by means of spectrofluorimetric detection at 290 and 330 nm wave-length for excitation and emission.

Malondialdehyde (MDA) and protein carbonyls (PC) were measured in blood plasma as oxidative damage biomarkers for lipids and proteins, respectively. Quantification of MDA was made using a colorimetric kit (TBARS, TCA method, Assay Kit; Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer instructions. Absorbance was read at 540 nm with a microplate reader (Perlong DNM-9602; Nanjing Perlove Medical Equipment Co. Ltd., Nanjing, China). Quantification of PC was made using the Oxiselect™ Protein Carbonyl ELISA kit (Cell Biolabs Inc., San Diego, CA, USA) according to the protocol provided by the manufacturer. Absorbance was read at 450 nm in the microplate reader. Before the assay, plasma samples were diluted in phosphate buffered saline to achieve a protein concentration of 10 µg/ml. In addition, total antioxidant capacity (TAC) in plasma was assessed using the colorimetric Antioxidant Assay Kit.
Assessment of semen quality and semen oxidative status
Ejaculates were obtained at 0, 30 and 60 days after starting vitamins supplementation, using an electro-ejaculator standardized for small ruminants (Minitube e320, Tiefenbach, Germany). This technique was preferred to prevent the eventual absence of sexual libido observed by Monge et al. (1945) in rams exposed to HA. Furthermore, as indicated by Matthews et al. (2003), 'electroejaculation is more practical as it does not require previous training of the rams and can be used for breeding soundness examination'. Before starting the electroejaculation procedure, rams were sedated using xylazine (0.2 mg/kg BW, Xylazine 2%; Centrotev, Santiago, Chile), then the rectum was emptied and the prepuce was washed with saline solution and carefully dried with paper towel. The probe (diameter: 2.5 cm, length: 16 cm) was lubricated with contact gel, introduced into the rectum with the three longitudinal electrodes ventrally oriented and electric ramps were applied. The electric ramps consisted of consecutive increases of 0.5 V, starting from 0 up to a maximum of 8 V. Each voltage was applied for 3 s followed by a rest period of 3 s. All animals ejaculated between 4 and 8 V. The ejaculates were recovered in clean graduated glass cups, which enabled direct volume measurement. Then, a 100 µl semen sample was taken for pH determination using test strips (MColorpHast™; Merck Millipore, Billerica, MA, USA). Subsequently, the collection cups were isolated and protected from direct sunlight. Sperm concentration, overall and progressive motility, and viability were evaluated in a CASA system (ISAS-V®, Proiser, Valencia, Spain). For this, semen samples were diluted in Tris/citric acid/fructose medium to obtain a final concentration of about 50 × 10⁶ sperm/mL (Emmanverdi et al., 2013). Sperm viability was assessed using the Duovital® fluorescent kit (Proiser, Valencia, Spain). Measurements were done in 4 µl sperm suspension loaded in a 20 µm depth cell counting chamber (Spermtrack®, Proiser) maintained at 37.5°C in a thermal plate, as recommended by the manufacturer.

Afterwards, ejaculates were centrifuged at 1500 × g × 10 min at 4°C and seminal fluid was stored at −80°C until assayed for SOD and GSH-Px activities and TAC. Concentrations of SOD were measured in 10 µl seminal plasma diluted 1:10 in sample buffer (50 mM Tris-HCl, pH 8.0), using the Superoxide Dismutase Assay Kit® (Cayman Chemical Company), following the supplier instructions. Concentrations of GSH-Px were measured in 20 µl seminal plasma, using the Glutathione Peroxidase Assay Kit® (Cayman Chemical Company), following supplier guidelines. Meanwhile, TAC was measured in 10 µl seminal fluid diluted 1:10 in assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose), using the Antioxidant Assay Kit® (ELISA commercial kit, Cayman Chemical Company), following the supplier instructions. In the SOD and TAC assays, absorbance was measured in a microplate reader (DMN-9602; Perlong Medical Equipment Co. Ltd., Nanjing, China), meanwhile the GSH-Px assay absorbance was read at 340 nm every minute for 5 min with a microplate spectrophotometer (Epoch®, Winooski, VT, USA).

Statistical analysis
Comparisons among groups for each experimental time (0, 30 and 60 days of vitamins supplementation), were done by analysis of variance, using the general linear model procedure (GLM; SAS Institute Inc., Cary, NC, USA). Comparisons were made using two statistical models. The first model was used to test the effect of altitudinal status, including two cross factors: the place of birth or origin of the animals and the place where the samples were obtained and studied. The second model took into consideration the previous two factors in addition to vitamin supplementation. Interactions among factors were also analysed. When significant differences were found, Duncan’s test was used to determine the groups among which the differences were statistically significant. Significant differences were considered when P < 0.05. The results are expressed as means ± SEM.

Results
Blood plasma concentrations of vitamins C and E, and oxidative stress biomarkers
Blood plasma concentrations of vitamins C and E are shown in Figure 1 (upper left and right panels, respectively). There were no differences at day 0; however, vitamin concentrations in the plasma of all supplemented groups increased at subsequent time points (days 30 and 60). However, vitamin E plasma concentrations did not achieve statistical significance between HH and HHV rams at day 30. The same situation was found for groups LH and LHV at day 60 of supplementation. It is noteworthy that the supplemented group LLV reached the highest values for both vitamins.

Biomarkers of oxidative damage (MDA, PC) and TAC in plasma are shown in Figure 1 (intermediate left and right panels, respectively). In the case of MDA, the values at day 0 were higher in HH animals than in the other groups. At day 30, MDA increased in LH rams, which was prevented in the group with vitamins supplementation (group LHV). At day 60, all the rams exposed to ha showed high levels of MDA, with high dispersion of the values. In the case of PC, there were no differences among groups at day 0. At day 30 and 60, PC had a similar pattern to MDA at day 30, with also high dispersion of the values, especially in ha groups, at day 60. The lowest value for this trait was present in LHV group. Analysis of changes in TAC over time showed, overall, an inverted pattern to PC, being significantly higher.
in LL rams at day 0. At days 30 and 60, TAC significantly decreased in LH group; an effect not observed in group LHV.

**Blood plasma concentrations of testosterone**
Plasma testosterone concentrations are shown in Figure 1 (lower right panel). No significant differences in testosterone levels were found at day 0. At day 30 of treatment, LH rams showed the lowest testosterone value; significantly different from LL rams. At day 60 of treatment, the lowest concentrations of testosterone were observed in LH and LHV animals; significantly different to LL group. No effect of vitamin supplementation was observed on the concentration of testosterone at days 30 and 60.

**Semen quality parameters**
Results on semen characteristics are shown in Figure 2. The mean ejaculate volume was 2.0 ± 0.1 ml, without differences among groups in each experimental time (left upper panel). Assessment of sperm concentration at time 0 showed a lower value in HH rams. At day 30, vitamin supplementation lead to an increased sperm concentration in LLV and LHV rams, but this effect was not present in HHV animals. At day 60, there were no observable effect of vitamin supplementation and only differences according to the altitudinal status were observed; where, animals exposed to ha had lower sperm concentrations (right upper panel).

Overall sperm motility (left intermediate panel) showed no differences among groups at days 0 and 30. At day 60, lower overall sperm motility was present in the four groups exposed to ha. Meanwhile, progressive sperm motility (Figure 2, right intermediate panel) was lower in HH rams at day 0. A marked decrease in progressive motility was observed in LH group at days 30 and 60, which was prevented by vitamin supplementation in the LHV group. Sperm viability is presented in the left lower panel. Lower values for this trait were shown by groups exposed to ha, at three experimental time points. Vitamin supplementation was able to prevent the ha effect at day 30 of treatment, but no such
effect was observed at day 60. Seminal pH values (right lower panel) ranged from 6.5 to 8.3, without differences among groups at day 0; however, significant increases were detected at 30 and 60 days of exposure to ha. No effect of vitamin supplementation was observed for seminal pH.

Semen oxidative status
Parameters of oxidative seminal status are shown in Figure 3. Activities of GSH-Px (upper panel) and SOD (intermediate panel) were significantly reduced in ha rams, situation occurring from day 0 onwards. However, for GSH-Px the effect of ha was more accentuated in LH rams. At day 30, vitamin supplementation reduced ha effect in LH rams for GSH-Px activity. At day 60, vitamin supplementation showed no effect on both antioxidant enzymes. In the case of TAC (bottom panel), only an effect of short-term exposure to ha was observed, thus, decreased TAC was present in LH rams at 60 days of treatment. In the same group of rams, vitamin supplementation increased the TAC, but this effect was observed only at day 30.

Discussion
The results of the present study indicate a state of oxidative stress in rams exposed to ha, with short-term exposed animals being the most affected. Blood concentrations of MDA, CP and TAC are normally used for evaluation of redox status, in the general body or specific tissues (Marrocco et al., 2017). In the present work, blood MDA and CP were increased, while TAC was decreased by exposure to ha, evidencing the state of oxidative stress, mainly in the rams exposed to ha during short time. These results are in agreement with those obtained in pregnant ewes (Parraguez et al., 2011) and humans (Sinha et al., 2010). Moreover, blood and seminal plasma oxidative stress biomarkers show similar patterns in men with reproductive dysfunctions leading to infertility (Benedetti et al., 2012). Oral supplementation with vitamin C and E increased plasma levels of these vitamins, confirming previous data in rams (Cofre et al., 2016), and improved the redox status in those recently exposed to ha.
These results are consistent with those obtained in ewes maintained under different altitudinal conditions, being also associated with prevention against deleterious effects of hypoxia on reproductive parameters (Parraguez et al., 2011), in a similar way to our LHV rams. Previous studies on the effects of the exposure to hypoxic environment in male reproductive function, either for short or long time and either in rams (Monge et al., 1945) or men (Donayre et al., 1968), have been based on the evaluation of semen quality and sexual behavior. The current study also evaluated possible effects of the exposure to hypoxia on plasma testosterone concentrations in rams. Our results indicate that hypoxia has a detrimental effect on testosterone concentration, especially in LH rams. Previous studies in rats have shown that blood testosterone decreased after a few days of exposure to simulated hypoxia (Farías et al., 2008). In men, however, results have shown inconsistent effects on testosterone levels, from increases under acute hypoxia exposure (Barnholt et al., 2006), to no changes (Basu et al., 1997) or decreases (Benso et al., 2007) after long-term exposure. Such differences may be related to different stages of adaptation to the altitude (Gonzales, 2013), a hypothesis consistent with the results obtained in the present study, where the most affected males were those recently exposed to hypoxia.

The present study showed no effects of supplementation with vitamin C or E on testosterone levels in hypoxic rams, which is in agreement with previous data on the use of oral supplementation with only vitamin E (Rekkas et al., 2000). Sperm characteristics were highly sensitive to hypoxia. Sperm viability in LH rams decreased, becoming similar to HH animals, after only 4 days of hypoxia exposure. Progressive and overall sperm motility in LH rams decreased after 30 and 60 days of exposure to hypoxia, respectively. These results are consistent with previous data in men after 7 to 14 days of hypoxia exposure (Donayre et al., 1968). When considering possible causes for these changes in sperm characteristics, it needs is important to know that the overall process of spermatogenesis lasts around 47 days in the ram (Sharpe, 1994) and more than 70 in men (Hess and de Franca, 2008). Hence, these quick effects of hypoxia exposure would be related to deleterious effects of the exposure to hypoxic environment on mature semen. In agreement with this observation, previous studies have found impaired sperm motility and increased apoptosis in mature sperm exposed to small increases in the amount of ROS in testis and epididymis (Aitken and Koppers, 2011; Bansal and Bilaspuri, 2011). The data obtained in the current study would support this hypothesis. Information on seminal oxidative/antioxidant status in individuals exposed to hypoxia is scarcely available in the literature. Studies in humans and rats exposed to natural or artificial hypoxia conditions showed decreased levels/activities of GSH-Px and SOD in different tissues (Dosek et al., 2007), consistent with our findings in seminal fluid. On the other hand, data from studies at altitude showed a positive correlation among TAC and activity of GSH-Px and SOD in seminal fluid and semen quality in both men (Khosrowbeygi et al., 2004) and rams (Cofré et al., 2016), with SOD and GSH-Px having a crucial protective role against ROS in ram-lamb’s semen (Kasimanickam et al., 2006).
Supplementation with vitamin C and E showed short-term beneficial effects on sperm viability, concentration, and progressive motility, especially in LH rams, in agreement with previously reported data in rams maintained at la (Cofré et al., 2016). Previous studies in rams supplemented with vitamin C doses equivalent to the current study, resulted in significantly increased semen volume and sperm concentration, motility and viability (Yue et al., 2014), whilst supplementation with vitamin E increased ejaculate volume, sperm concentration and sperm motility (Yue et al., 2010).

Moreover, results from the current study also suggest short-term beneficial effects of oral vitamin C and E supplementation on the oxidative/antioxidant status of the rams, which is in agreement with previous studies that report a significant increase in GSH-Px and SOD activities in seminal plasma and a significant improvement in semen parameters after treatment of bucks with vitamin E (Majid et al., 2014).

In agreement with the present results obtained in rams, as well as those previously obtained in sheep (Parraguez et al., 2013 and 2014), it is feasible to think that the observed seminal modifications at ha are partially responsible for the low fertility rate of ovine species raised under this condition. This is also supported by similar changes in both the semen and sperm characteristics present in human anomalies leading to subfertility or infertility, which are frequently associated to the presence of oxidative stress (Agarwal and Allamaneni, 2011; Benedetti et al., 2012).

Conclusions

Rams under both short- and long-term exposure to ha conditions have impairments of oxidative/antioxidant status in plasma and seminal fluid, effects which concurrently decrease semen quality. These effects may contribute to the previously reported decreased fertility of ovine species at ha. Oral supplementation with vitamins C and E had beneficial effects on blood and semen oxidative status, as well as some of the male semen characteristics. Thus, oral supplementation with vitamins C and E constitute a simple and cheap alternative to improve semen quality of rams. Future works should assess whether changes in seminal quality affect the rate of fertilization of rams moved to high altitudes.

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