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

E. Cofré[1], O. A. Peralta[1], A. Raggi[2,3], M. De los Reyes[1], F. Sales[4], A. González-Bulnes[5,6] and V. H. Parraguez[1,2,3†]

[1]*Faculty of Veterinary Sciences, University of Chile, Santa Rosa 11735, 8820808 Santiago, Chile;* [2]*Faculty of Agricultural Sciences, University of Chile, Santa Rosa 11315, 8820808 Santiago, Chile;* [3]*International Centre for Andean Studies, University of Chile, Santa Rosa 11735, 8820808 Santiago, Chile;* [4]*Department of Animal Science, INIA-Kampenaike, Angamos 1056, 6212707 Punta Arenas, Chile;* [5]*Department of Animal Reproduction, INIA, Av. Puerta de Hierro s/n, 28040 Madrid, Spain;* [6]*Department of Veterinary Medicine, University of Sassari, Piazza Università 21 Sassari, Italy*

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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 (Farías *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

† E-mail: vparragu@uchile.cl

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 $1200 \times \mathbf{g} \times 5$ 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

(Cayman Chemical Company), following the instructions of the manufacturer. Absorbance was read at 405 nm in the microplate reader.

Plasma testosterone concentrations were measured by radioimmunoassay (Testo-RIA-CT; DIAsource Immuno Assays S.A., Louvain-la-Neuve, Belgium) in 50 μl of plasma. Measurements were done in duplicate and the minimum concentration detected by the assay was 0.11 ng/ml. The intra- and inter-assay variations coefficients were 4.6% and 6.2%, respectively.

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%®; Centrovet, Santiago, Chile), then the rectum was emptied and the periprepuceal area 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 50×10^6 sperms/ml (Emmanverdi *et al.*, 2013). Sperm viability was assessed using the Duovital® florescent 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 $1500 \times \mathbf{g} \times 10$ 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 : 25 in sample buffer (50 mMTris-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 (DNM-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, and lower left 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 days 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

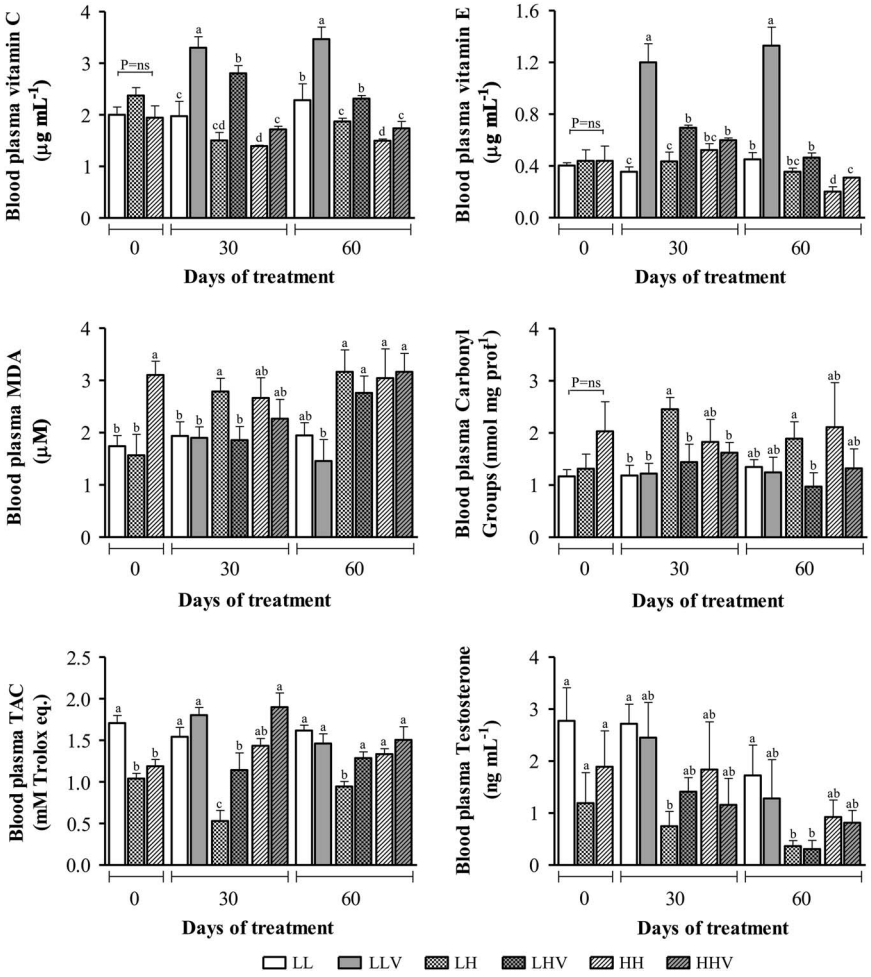

Figure 1 Blood plasma concentrations of vitamins C and E, oxidative stress biomarkers and testosterone in control and vitamin C/E supplemented rams, exposed to high altitude conditions. Ram groups are as follows: HH, rams native to high altitude, kept at high altitude, without vitamins supplementation; HHV, rams native to high altitude, kept at high altitude, with vitamins supplementation; LH, rams native to low altitude, taken to high altitude, without vitamins supplementation; LHV, rams native to low altitude, taken to high altitude, with vitamins supplementation; LL, rams native to low altitude, kept at low altitude, without vitamins supplementation; LLV, rams native to low altitude, kept at low altitude, with vitamins supplementation. Different letters above the bars indicate significant differences among groups in each experimental time (Duncan test, $P < 0.05$).

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

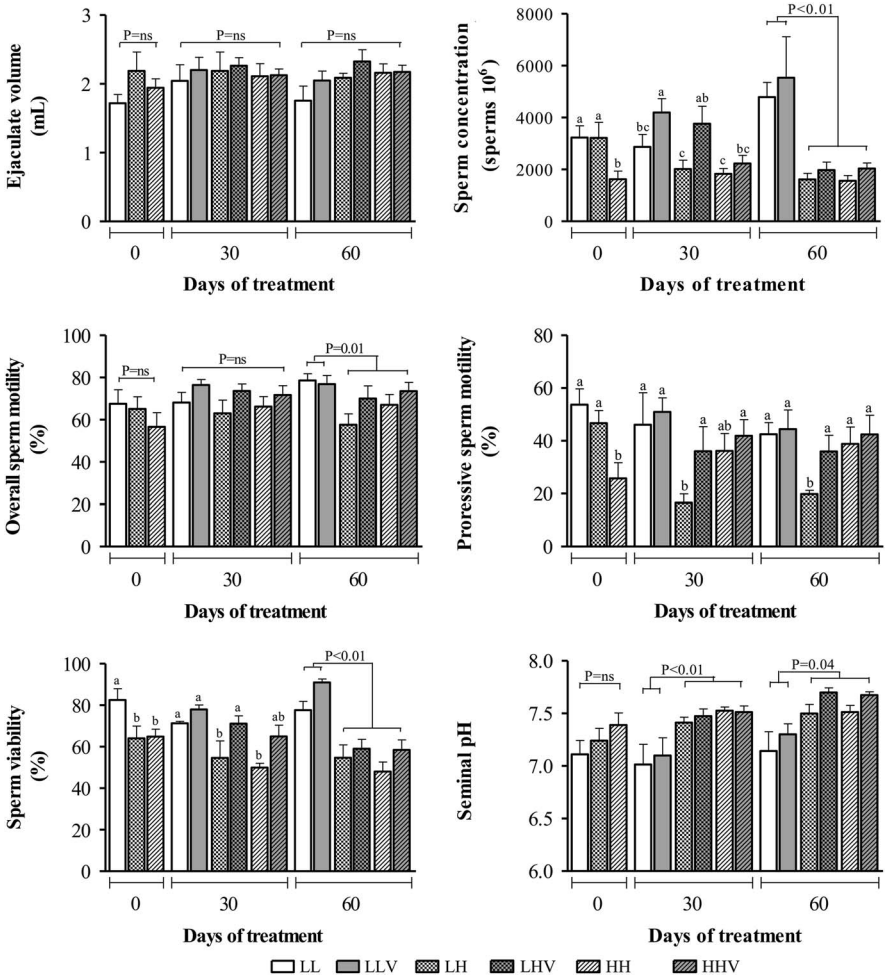

Figure 2 Characteristics of ejaculates obtained from control and vitamin C/E supplemented rams, exposed to high altitude conditions. Rams groups are as follow: HH, rams native to high altitude, kept at high altitude, without vitamins supplementation; HHV, rams native to high altitude, kept at high altitude, with vitamins supplementation; LH, rams native to low altitude, taken to high altitude, without vitamins supplementation; LHV, rams native to low altitude, taken to high altitude, with vitamins supplementation; LL, rams native to low altitude, kept at low altitude, without vitamins supplementation; LLV, rams native to low altitude, kept at low altitude, with vitamins supplementation. Different letters above the bars indicate significant differences among groups in each experimental time (Duncan test, $P < 0.05$).

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* effects in HH 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 (Cofré *et al.*, 2016), and improved the redox status in those recently exposed to *ha*.

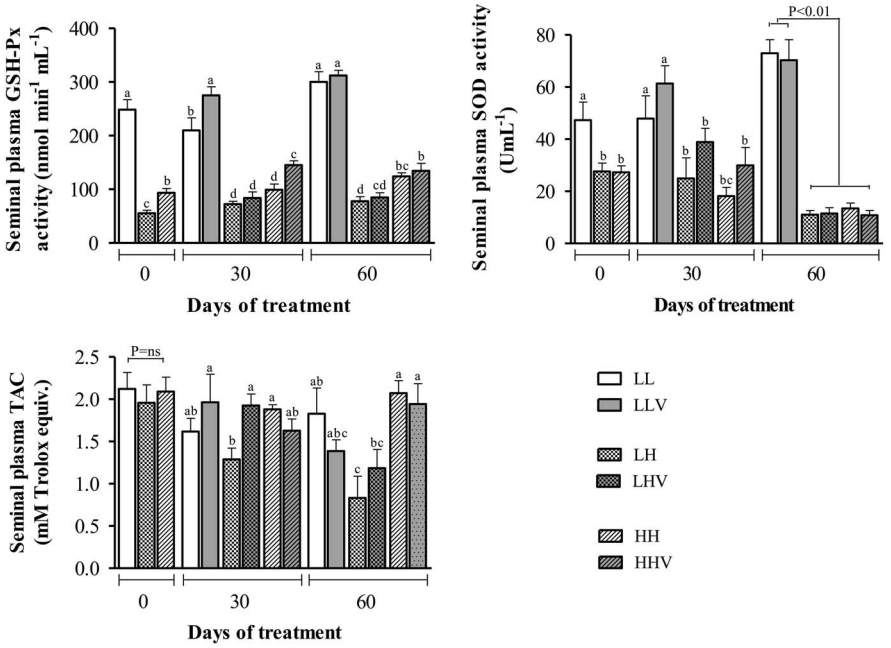

Figure 3 Antioxidant status of seminal plasma from ejaculates obtained from control and vitamin C/E supplemented rams, exposed to high altitude conditions. Rams groups are as follow: HH, rams native to high altitude, kept at high altitude, without vitamins supplementation; HHV, rams native to high altitude, kept at high altitude, with vitamins supplementation; LH, rams native to low altitude, taken to high altitude, without vitamins supplementation; LHV, rams native to low altitude, taken to high altitude, with vitamins supplementation; LL, rams native to low altitude, kept at low altitude, without vitamins supplementation; LLV, rams native to low altitude, kept at low altitude, with vitamins supplementation. Different letters above the bars indicate significant differences among groups in each experimental time (Duncan test, $P < 0.05$).

These results are consistent with those obtained in ewes maintained under different altitudinal conditions, being also associated with prevention against deleterious effects of *ha* on reproductive parameters (Parraguez *et al*., 2011), in a similar way to our LHV rams.

Previous studies on the effects of the exposure to *ha* 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 *ha* on plasma testosterone concentrations in rams. Our results indicate that *ha* 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 *ha* (Farías *et al*., 2008). In men, however, results have shown inconsistent effects on testosterone levels, from increases under acute *ha* 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 *ha.*

The present study showed no effects of supplementation with vitamin C or E on testosterone levels in *ha* 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 *ha*. Sperm viability in LH rams decreased, becoming similar to HH animals, after only 4 days of *ha* exposure. Progressive and overall sperm motility in LH rams decreased after 30 and 60 days of exposure to *ha*, respectively. These results are consistent with previous data in men after 7 to 14 days of *ha* 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 *ha* 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 *ha* is scarcely available in the literature. Studies in humans and rats exposed to natural or artificial *ha* 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 *la* 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 (Jafaroghli *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 quality 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 at *ha*, primarily in short-term exposed animals. Future works should assess whether changes in seminal quality affect the rate of fertilization of rams moved to high altitudes.

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