

# MEJORAMIENTO DEL ESTATUS ANTIOXIDANTE Y DE LA CALIDAD DEL SEMEN POR SUPLEMENTACIÓN ORAL CON VITAMINAS C Y E EN CARNEROS

## *Improvement of antioxidant status and semen quality by oral supplementation with vitamins c and e in rams*

*Eileen Jennifer Cofré-Narbone<sup>1</sup>, Oscar Alejandro Peralta-Troncoso<sup>1</sup>, Bessie Elvira Urquieta-Mangiola<sup>1</sup>, Luis Alberto Raggi-Saini<sup>1</sup>, Nataly Benavides-Aguila<sup>1</sup> y Víctor Hugo Parraguez-Gamboa<sup>1,2\*</sup>*

*<sup>1</sup>Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile.<sup>2</sup>Facultad de Ciencias Agronómicas, Universidad de Chile. \* Autor responsable: Víctor H. Parraguez. Tel: 56-2-29785548. E-mail: vparragu@uchile.cl*

### RESUMEN

La mayor parte de las condiciones que conducen a la disminución de la fertilidad en los machos están asociadas con estrés oxidativo, donde los niveles supra-fisiológicos de especies reactivas del oxígeno ejercen efectos perjudiciales sobre la espermatogénesis y la función de los espermatozoides. El objetivo del presente trabajo fue evaluar el efecto de la suplementación oral con vitaminas C y E sobre el estado antioxidante y parámetros seminales en carneros. Dieciséis carneros se suplementaron diariamente con 600 mg de vitamina C y 450 UI de vitamina E, durante 30 días (d). Otros dieciséis carneros no se suplementaron, actuando como controles. Se obtuvieron muestras de sangre y se analizaron para medir las concentraciones de vitaminas C y E. Se extrajeron eyaculados que fueron analizados para volumen, concentración de espermatozoides, motilidad total y progresiva, y viabilidad espermática. En el plasma seminal se evaluó la actividad de la superóxido dismutasa (SOD) y de la glutatión peroxidasa (GPx), y la capacidad antioxidante total (TAC). Las concentraciones plasmáticas de vitamina C y E fueron significativamente mayores en los carneros suplementados. SOD, GPx y la TAC también fueron mayores en plasma seminal de los carneros suplementados con vitaminas. El volumen del eyaculado no mostró diferencias entre suplementados y no suplementados, mientras que la concentración espermática, la motilidad total y progresiva, y la viabilidad espermática fueron significativamente mayores en los carneros suplementados. Se concluye que la suplementación oral diaria de vitaminas C y E durante un periodo de 30 d, permite mejorar, tanto los parámetros antioxidantes en el fluido seminal, como la calidad del semen de carneros. Esto constituye una técnica simple y de bajo costo para aumentar la eficiencia del carnero durante la temporada reproductiva, especialmente cuando se utiliza inseminación artificial, pudiendo además ser beneficioso para mejorar la calidad del semen refrigerado o congelado/descongelado.

**Palabras clave:** Semen de carnero; fertilidad; antioxidantes; vitaminas

### ABSTRACT

In males, most of the conditions leading to decreased fertility are associated with oxidative stress, where supra-physiological levels of reactive oxygen species exert detrimental effects on spermatogenesis and sperm function. The goal of the present work was to evaluate the effect of oral supplementation with vitamin C and E on antioxidant status and semen parameters in rams. Sixteen rams were daily supplemented with 600 mg of vitamin C and 450 IU of vitamin E, during 30 days (d). Other sixteen rams were not supplemented, acting as controls. Blood samples were obtained and analyzed for vitamin C and E concentrations. Ejaculates were recovered and analyzed for volume, sperm concentration, overall and progressive motility, and sperm viability. In addition, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and total antioxidant capacity (TAC) were evaluated in the seminal plasma. Plasma concentrations of vitamin C and E were significantly higher in the supplemented rams. Seminal plasma SOD, GPx and TAC were also higher in vitamin supplemented rams. Ejaculate volume showed no difference between supplemented and non-supplemented rams, while sperm concentration, overall and progressive motility, and sperm viability were significantly higher in the supplemented rams. It is concluded that daily oral supplementation of vitamins C and E during a period of 30 d, allows to improve both antioxidant parameters in seminal fluid and semen quality. This constitutes a simple and cheaper technique for increase ram efficiency during breeding season, especially when artificial insemination is used, and may be beneficial to improving the quality of refrigerated or frozen/thawed semen.

**Key words:** Ram semen; fertility; antioxidants; vitamins

Recibido: 12/02/2016 Aceptado: 21/04/2016

## INTRODUCTION

In most of the male infertility/sub-fertility cases, oxidative stress, an imbalanced redox equilibrium where production of reactive oxygen species (ROS) exceeds the antioxidant defenses, has been identified as one of the main mediators causing sperm dysfunction [2, 5]. The ROS, under physiological conditions, play important roles in different processes necessary for sperm maturation and function, such as capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion, allowing proper fertilization process [19, 21, 40]. However, excessive ROS amounts may lead to pathological conditions, exerting detrimental effects on spermatogenesis and/or sperm function [19]. Specifically, negative effects on sperm concentration [3], motility [7] and morphology [3, 9] as well as increases in sperm DNA damage [18] and apoptosis [1] has been reported.

Seminal fluid has endogenous antioxidants in order to protect sperm cells from ROS excesses. This antioxidant system is composed by enzymatic antioxidants as superoxide dismutase (SOD), glutathione peroxidase/glutathione reductase (GPx) and catalase (CAT), and non-enzymatic antioxidants as ascorbate, tocopherol, urate, pyruvate, glutathione, taurine and hypotaurine. Total Antioxidant Capacity (TAC) is the sum of enzymatic and non-enzymatic antioxidants and it is noteworthy that low TAC in seminal plasma has a key role in infertility/sub-fertility [12]. Moreover, SOD is recognized as responsible to maintain a balance between ROS generation and degradation [17].

Vitamins C (ascorbic acid or ascorbate) and E (alpha-tocopherol) are largely known to be required for male reproductive function and spermatogenesis. Vitamin C, in addition to be required as enzymatic cofactor, also acts as an important antioxidant agent by scavenging free radicals (e.g.  $O_2^-$ ,  $OH^-$ ) [23]. Vitamin E is the primary lipid-soluble molecule antioxidant in biological systems [37, 54], acting by inhibiting lipid peroxidation reaction in the membranes [53]. In addition, it has been described that deficiencies of vitamin E causes degeneration of the germinal epithelium [33]. Besides their individual antioxidant properties, ascorbate recycles alpha-tocopherol by repairing its tocopheryl radical, thereby permitting it to function again as a ROS scavenger [13].

The addition of antioxidants to diluents or cryoprotectants media is a well-known method to improve viability and motility of both fresh-stored and cryopreserved ram sperm cells [8, 12, 35]. *In vitro* treatment of sperms with ascorbic acid and alpha-tocopherol has showed to reduce detrimental effects of ROS in human sperm [27].

*In vivo* supplementation with these vitamins is also useful for increasing reproductive traits. Yue et al. [60] showed that rams (*Ovis aries*) fed with a diet supplemented with vitamin E in a final dose of 200 IU per ram for 12 months, improve semen traits and endogenous antioxidant capacity of testicular tissue. On the other hand, early reports indicated that adult ruminants were incapable

of using orally administered ascorbic acid to increase serum concentrations due to ruminal degradation [31]. However, more recent studies showed that multiple oral administration of finely powdered vitamin C increased plasma ascorbic acid concentration in sheep (*Ovis aries*) [24, 42] and cattle (*Bos taurus*) [25]. Thus, vitamin C supplementation in rams, through a diet containing 300 mg  $kg^{-1}$  of this vitamin, showed beneficial effects on semen quality after 4-6 weeks of treatment [28].

Although there are few studies analyzing the effect of individual vitamin C or vitamin E on ram semen traits, there are no reports on the effect of oral supplementation of both vitamin C and E on ram semen parameters. In previous studies in sheep, combined treatments showed to prevent oxidative stress [42] and to improve reproductive parameters [41]. Data in men indicate that increased basal levels of endogenous antioxidant enzymes, mainly SOD, in seminal plasma [57] and other tissues [30] were positively associated with sperm concentration and overall motility. Hence, the aim of the present study was to evaluate the effect of oral vitamin C and E supplementation on antioxidant status and semen and sperm parameters in rams.

## MATERIALS AND METHODS

### Study design and animals

This is an experimental study designed to test the effect of oral supplementation with antioxidant vitamins on ram semen antioxidant status and quality. For this, thirty two one year-old crossbred rams were randomized into two groups, a group with supplementation of vitamins C and E and other non-supplemented that constituted the control group.

### Ethics statement

This study was performed in agreement with the International Guiding Principles for Biomedical Research Involving Animals [15], and the study was 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

Experiments were done during the middle of reproductive season, at the Faculty of Veterinary Sciences, University of Chile, Santiago, Chile (33° 34' 31" S 70° 37' 52" W). Rams (48.4 ± 3.8 kg body weight; 2.8 ± 0.3 body condition, 1-5 scale) were fed with alfalfa (*Medicago sativa*) hay and balanced feed to satisfy requirements [38]; tap water was supplied *ad libitum*. The treated group was composed of 16 rams that were daily supplemented with 600 mg of vitamin C (ascorbic acid) and 450 IU of vitamin E (alpha-tocopherol), during 30 days, supplied directly into the mouth. The remaining 16 rams were the not supplemented control group.

### Vitamins C and E assessment in blood

For evaluating the effectiveness of the treatment for increasing plasma vitamins concentrations, blood samples (10 mL) were taken at d 30 from the left jugular vein, using heparinized syringes. Blood was centrifuged (Hettich, Mikro 200R, Andreas Hettich GmbH & Co.KG, Germany) at 1200 g x 5 min; the obtained plasma was aliquoted and stored at -80°C (Ilshin, DF8514, Ilshin Lab Co. Ltd, Korea) until assayed for vitamins C and E measurement by high-performance liquid chromatography as previously described [42]. Briefly, vitamin C was measured in plasma samples diluted ten-fold in ultra-pure water, by means 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 spectrofluorimetric detection at 290- and 330-nm wavelengths for excitation and emission, respectively.

### Evaluation of semen characteristics

Ejaculates were obtained 30 d after vitamins supplementation starts, using an electro-ejaculator standardized for small ruminants (Minitube e320, Tiefenbach, Germany). This technique was preferred because of the large number of experimental animals, and the temporal and spatial limitations to train rams for the use of the artificial vagina. Furthermore, although some authors indicate that electroejaculation could decrease the volume of ejaculate and sperm concentration [14, 47, 48], others indicate that these changes are of lesser relevance when considering the ease and speed of obtaining semen without the need for prior training of rams, and can be used for breeding soundness examination [32, 34] and for semen conservation techniques [29]. Before starting the electroejaculation procedure, rams were sedated using xylazine (0.2 mg kg<sup>-1</sup> body weight, Xylazine 2%®, Centrovét, 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 ventrally oriented longitudinal electrodes 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 seconds followed by a rest period of 3 seconds. All animals ejaculate between 4 and 8 V. The ejaculates were received in clean graduated glass cups, isolated and protected from sunlight.

Immediately after obtaining the ejaculates, these were evaluated for volume, sperm concentration and overall and progressive motility 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 x 10<sup>6</sup> sperms/mL [20]. Sperm viability was also assessed by CASA, using the Duovital® florescent kit (Proiser, Valencia, Spain). Measurement were done in 4 µL sperm suspension loaded in a 20 µM depth cell counting chamber (Spermtrack®, Proiser, Valencia, Spain) maintained at 37.5°C in a thermal plate, as recommended by the manufacturer.

Afterwards, semen samples were centrifuged at 1500 g x 10 min at 4°C and seminal plasma was stored at -80°C until assayed for superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and total antioxidant capacity (TAC). SOD was measured in 10 µL seminal plasma diluted 1:5 in sample buffer (50 mM Tris-HCl, pH 8.0), using the Superoxide Dismutase Assay Kit® (Cayman Chemical Company, Ann Arbor, USA), following the supplier instructions. GPx was measured in 20 µL seminal plasma, using the Glutathione Peroxidase Assay Kit® (Cayman Chemical Company, Ann Arbor, USA), following the directions of the supplier. Meanwhile, TAC was measured in 10 µL seminal plasma diluted 1:26 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, Ann Arbor, USA), following the supplier instructions. In each assay, the absorbance was measured in a microplate reader (DNM-9602, Perlong Medical Equipment Co. Ltd., Nanjing, China).

### Statistical analysis

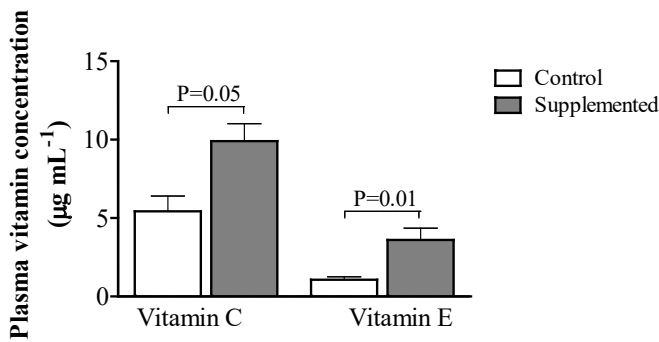
Data obtained from vitamin supplemented and control groups were compared by means of a Mann-Whitney *U*-test [43], using the StatGraphics Centurion XV software, Version 2.15.06 (StatPoint Technologies, Inc., Warrenton, VA, USA). Data are presented as mean ± SEM and differences were considered significant when  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

Overall results in the current study show that oral supplementation with vitamins C and E for 30 d allow to increase the seminal plasma antioxidant status, sperm concentration in the ejaculates and the most important sperm characteristics related to fertility.

### Effects of the treatment on plasma vitamin concentrations

After 30 d, the supplementation results in significant increases of plasma concentration of vitamin C ( $9.9 \pm 1.1 \mu\text{g mL}^{-1}$  in the treated group and  $5.4 \pm 0.9 \mu\text{g mL}^{-1}$  in control rams,  $P=0.05$ ; FIG. 1) and vitamin E ( $3.6 \pm 0.8 \mu\text{g mL}^{-1}$  vs  $1.1 \pm 0.2 \mu\text{g mL}^{-1}$  in treated and control groups, respectively,  $P=0.01$ ; FIG. 1). Previous studies have addressed the need for high doses of vitamin for increasing plasma concentrations of vitamin C in sheep [24]; however, the present study indicates that rams supplemented with 600 mg/d increased to almost twice vitamin C plasma concentration. This result is in agreement with a more recent study in sheep daily supplemented with equivalent dose/kg body weight [42]. Treated rams also increased vitamin E plasma levels, indicating that gastrointestinal uptake after oral administration was followed by plasma accumulation. This is also consistent with our previous studies [42] and those of others who have reported similar plasma levels of vitamin E after comparable periods of oral supplementation in sheep and rams [36, 39, 46]. Overall this data indicate that increased plasma levels of vitamin C and E may be obtained after several d of oral supplementation.

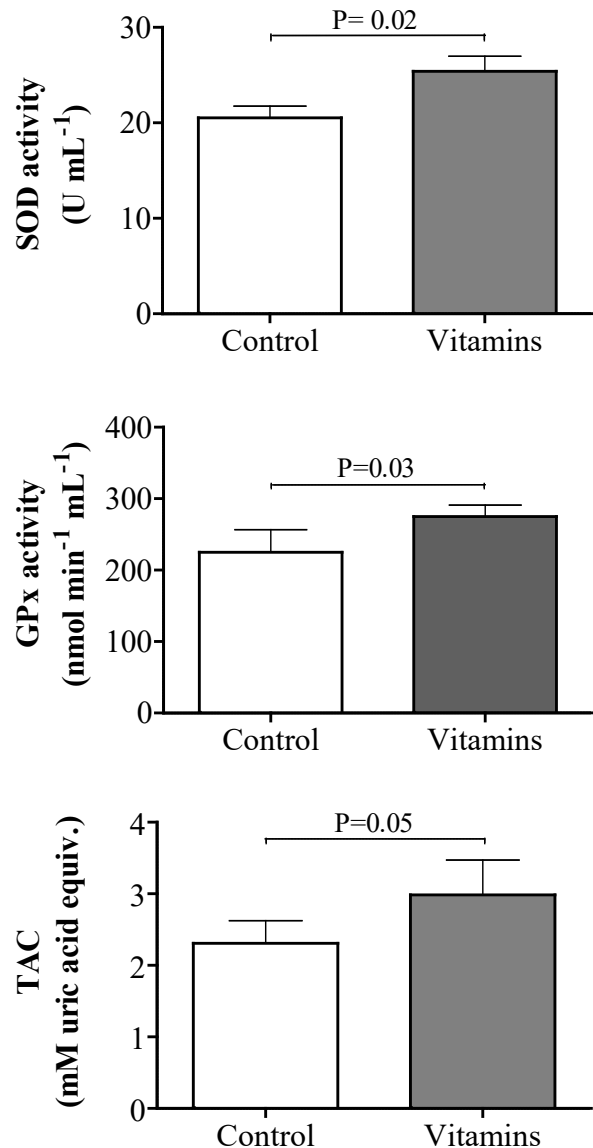


**FIGURE 1. PLASMA CONCENTRATIONS OF VITAMIN C AND E IN RAMS DAILY SUPPLEMENTED WITH VITAMINS C 600 MG AND E 450 IU DURING 30 DAYS. STATISTICAL COMPARISON WAS DONE BETWEEN VITAMINS TREATED AND CONTROL RAMS (MANN-WHITNEY TEST).**

**Effects of the treatment on antioxidant status of seminal plasma**

Seminal plasma antioxidant biomarkers are shown in FIG. 2. Supplemented rams showed a significant increase in SOD ( $25.4 \pm 1.6$  vs  $20.5 \pm 1.2$  U mL<sup>-1</sup> in the control group,  $P=0.02$ ; top panel) and in GPx activities ( $275.3 \pm 15.8$  vs  $225.2 \pm 31.6$  nmol min<sup>-1</sup> mL<sup>-1</sup>, for supplemented and control groups, respectively,  $P=0.03$ ; middle panel). TAC was also increased in seminal plasma by effect of vitamins supplementation ( $3.0 \pm 0.5$  vs  $2.3 \pm 0.3$  mM uric acid equivalents in treated and control rams, respectively,  $P=0.05$ ; bottom panel). These results show a concomitant significant increase in SOD, GPx and TAC levels in seminal plasma, indicating that antioxidant status was improved as a consequence of higher vitamin C and E concentrations in blood plasma. As indicated above, there is no previous information on simultaneous supplementation of vitamins C and E in rams. However, reports on the effect of oral supplementation with vitamin E show an improvement of antioxidant capacity in testis, involving at least an increase of SOD and GPx [26, 60]. Data obtained in men supplemented with vitamin C and E at least three times a week, show high SOD activity in seminal plasma, in addition of higher sperm concentration and motility [57]. The finding in ram semen reported here is extremely important because SOD is considered to be the most important antioxidant in seminal fluid [57] and represents the main part of the enzymatic ROS scavengers in seminal plasma [1, 51, 52]. Previous studies have reported that seminal plasma represents the main antioxidant source for protection against ROS due to reduced endogenous antioxidant activity in the sperm [10, 16]. Spermatozoa are susceptible to oxidation of their plasma membranes due to the presence of polyunsaturated fatty acids [4]. The toxic lipid peroxides are known to cause membrane damage and reduce motility [45]. The reported beneficial effect of antioxidant *in vitro* supplementation to sperm motility [44] may be the consequence of the negative effect of ROS with sperm motility [50]. In addition to membrane impairment, ROS damage to sperm may be associated to effect on DNA including modifications of bases, generation of a basic site,

DNA cross linkage and DNA-protein cross-links [6], which may allow to sperm death. Thus, findings in the present work of higher sperm concentration, motility and viability in ejaculates collected from rams supplemented with vitamins, might be supported by the inhibition of pathological ROS-depending mechanisms described before, due to increased seminal plasma antioxidant status.

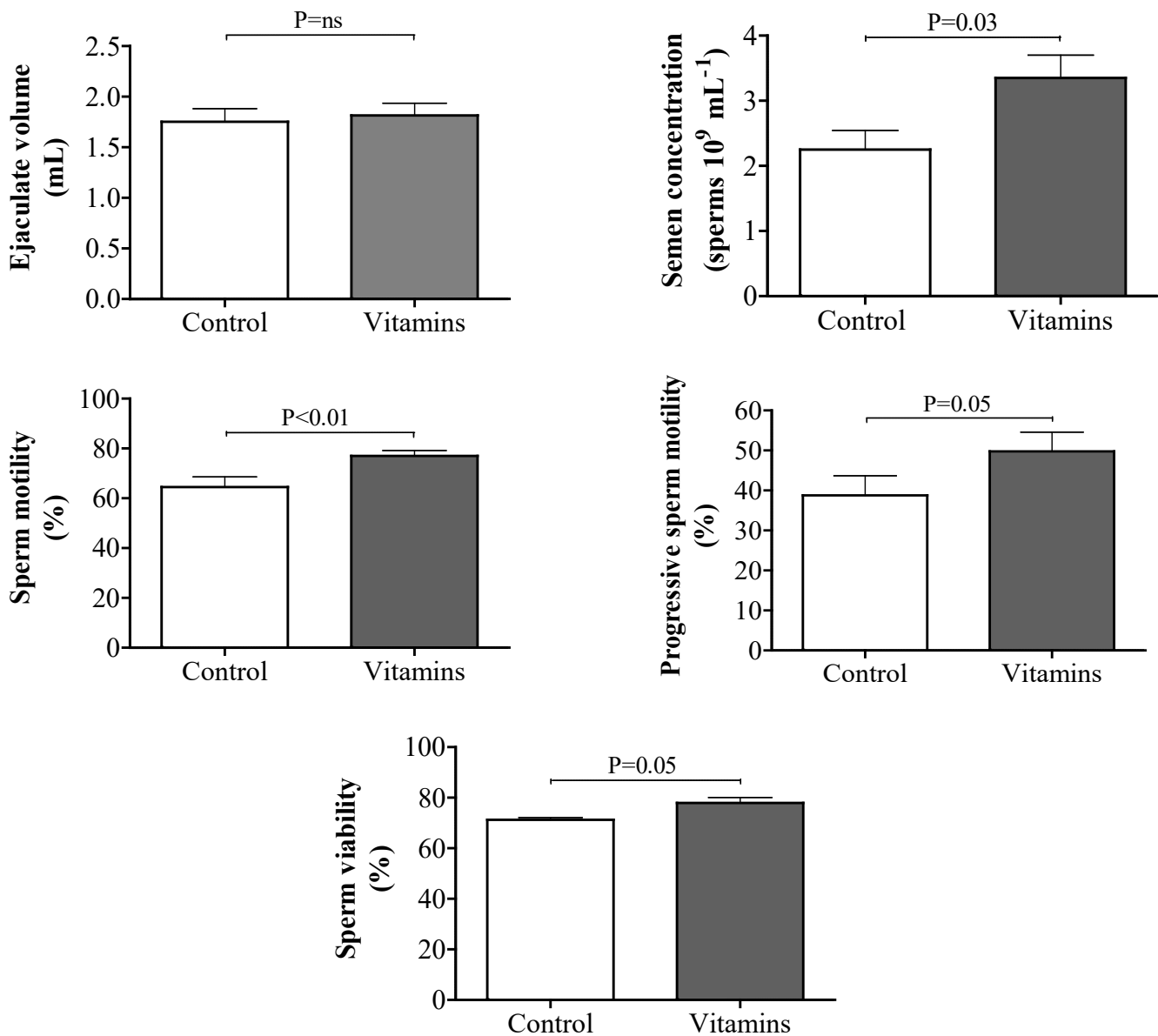


**FIGURE 2. EFFECT OF ORAL SUPPLEMENTATION WITH VITAMINS C AND E ON ANTIOXIDANT STATUS OF RAM SEMINAL PLASMA. TOP PANEL: SUPEROXYDE DISMUTHASE (SOD) ACTIVITY. MIDDLE PANEL: GLUTATHIONE PEROXIDASE ACTIVITY (GPX). BOTTOM PANEL: TOTAL ANTIOXIDANT CAPACITY (TAC). STATISTICAL COMPARISON WAS DONE BETWEEN VITAMINS TREATED AND CONTROL RAMS (MANN-WHITNEY TEST).**

**Effects of the treatment on ejaculates and sperms characteristics**

No effects of the supplementation were observed on ejaculate volume (FIG. 3, top left panel); however, vitamin-treated rams increased sperm concentration compared to untreated controls ( $3.35 \pm 0.35$  vs  $2.25 \pm 0.29$  sperms  $10^9$  mL<sup>-1</sup>, respectively;  $P=0.027$ ; FIG. 3, top right panel). Analyses of sperm motility detected higher percentages of overall ( $77.0 \pm 2.2$  vs  $64.6 \pm 4.1$  %, treated and control groups, respectively,  $P<0.01$ ; FIG. 3, middle left panel) and progressive motility ( $49.8 \pm 4.8$  vs  $38.8 \pm 4.9$  %, treated and control groups, respectively,  $P=0.05$ ; FIG. 3, middle right panel) as a result of the vitamin supplementation. Comparison of

sperm viability in both treated and control groups also showed a beneficial effect of vitamins supplementation ( $77.9 \pm 2.2$  vs  $71.3 \pm 0.9$  %, treated and control groups, respectively,  $P=0.05$ ; FIG. 3, bottom panel). There are no previous data in the literature on a similar antioxidant vitamins supplementation; moreover, few studies have evaluated the effect of oral supplementation of each individual vitamin on sperm parameters in the ram. Among them, previous experiments on addition of vitamin C to ram diets in a dose equivalent to the present study resulted in significant increases in semen volume and sperm concentration, motility and viability after six weeks of treatment, although a tendency to these increases was present from week four [28]. Meanwhile,



**FIGURE 3. EFFECT OF ORAL SUPPLEMENTATION WITH VITAMINS C AND E ON RAM EJACULATE AND SPERMS TRAITS. STATISTICAL COMPARISON WAS DONE BETWEEN VITAMINS TREATED AND CONTROL RAMS (MANN-WHITNEY TEST).**

ram diets supplemented with different concentrations of vitamin E have shown to increase ejaculate volume, sperm concentration and sperm motility with daily supplementation of at least 200 IU of vitamin E per animal per day during 12 months [60]. Moreover, supplementation of vitamin E to rams has also showed to increase sperm acrosomal proteases, which may improve fertilization capacity [46]. In other species like bulls (*Bos taurus*) [55], rabbits (*Oryctolagus cuniculus*) [59] and boars (*Sus scrofa domestica*) [11], vitamin E supplementation has also improved seminal traits, like ejaculate volume, sperm concentration and total motile sperms. In humans, it has been recently reported that administration of multivitamin formula including vitamin C and E to infertile men with varicocele, improved sperm parameters including reduction in sperm DNA fragmentation and increase in total number of sperm cells, although exerted no effect on sperm motility and viability [22]. Importantly, vitamin supplementation to men undergoing *in vitro* fertilization (IVF) resulted in improvement in sperm parameters including sperm count and motility [56].

An interesting point in the present study is that significant changes were observed in semen quality, despite the relatively short-treatment period. It might be perhaps difficult to explain, since the overall process of spermatogenesis in the ram lasts about 47 d [49]. However, it is known that small increases in the amount of ROS in the testis can lead to exacerbation of apoptosis process not only during spermatocytogenesis, but also during spermiogenesis [6], which constitutes the later stages of spermatogenesis. In addition, Yeni et al. [58] reported a significant increase in sheep biomarkers of oxidative stress and decreased endogenous antioxidant enzymes during the breeding season compared to other seasons, in ram seminal plasma. Thus, the improvement of antioxidant status may control these adverse effects of ROS, avoiding sperm losses and increasing the passage of these cells to the epididymis for maturation. Likewise, sperm maturation during passage through the epididymis also requires an adequate level of antioxidants to ensure adequate motility and fertilizing capacity of sperm [47], which helps to explain the increase in semen quality observed in the present study.

## CONCLUSION AND IMPLICATIONS

Daily oral supplementation of vitamins C and E during a period of 30 d, allows the improvement of antioxidant parameters in seminal fluid and semen quality in rams. This constitutes a simple and cheaper technique for increasing ram efficiency during breeding season, especially when artificial insemination is used. Furthermore, the obtained increase in seminal antioxidant capacity may be of benefit for improving the quality of refrigerated or frozen/thawed semen.

## ACKNOWLEDGEMENT

The authors thank the veterinary students' residents at the laboratory of Animal Physiology for helping during animal management and sampling. This work was supported by grant FONDECYT 1130181, CONICYT, Chile.

## BIBLIOGRAPHICS REFERENCES

- [1] AGARWAL, A.; SAID, T.M. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. **BJU Int.** 95:503-507. 2005.
- [2] AGARWAL, A.; VIRK, G.; ONG, C.; DU PLESSIS, S. Effect of oxidative stress on male reproduction. **World J. Mens Health** 32:1-17. 2014a.
- [3] AGARWAL, A.; SHARMA, R.K.; SHARMA, R.; ASSIDI, M.; ABUZENADAH, A.M.; ALSHAHRANI, S.; DURAIRAJANAYAGAM, D.; SABANEH, E. Characterizing semen parameters and their association with reactive oxygen species in infertile men. **Reprod. Biol. Endocrinol.** 12: 33. 2014b.
- [4] AITKEN, R.J.; FISHER, H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. **Bioassays** 16:259-66. 1994.
- [5] AITKEN, R.J.; ROMAN, S.D. Antioxidant systems and oxidative stress in the testes. **Oxid. Med. Cell. Long.** 1:15-24. 2008.
- [6] AITKEN, R.J.; KOPPERS, A.J. Apoptosis and DNA damage in human spermatozoa. **Asian J. Androl.** 13:36-42. 2011.
- [7] ATHAYDE, K.S.; COCUZZA, M.; AGARWAL, A.; KRAJCIR, N.; LUCON, A.M.; SROUGI, M.; HALLAK, J. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. **J. Androl.** 28:613-620. 2007.
- [8] AZAWI, O.I.; HUSSEIN, E.K. Effect of vitamins C or E supplementation to Tris diluent on the semen quality of Awassi rams preserved at 5 °C. **Vet. Res. Forum** 4:157-160. 2013.
- [9] AZIZ, N.; SALEH, R.A.; SHARMA, R.K.; LEWIS-JONES, I.; ESFANDIARI, N.; THOMAS, A.J. JR.; AGARWAL, A. Novel association between sperm reactive oxygen species production, sperm morphological defects, and sperm deformity index. **Fertil. Steril.** 81:349-354. 2004.
- [10] BILODEAU, J.F.; CHATTERJEE, S.; SIRARD, M.A.; GAGNON, C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. **Mol. Reprod. Dev.** 55:282-288. 2000.
- [11] BRZEZIŃSKA-ŚLEBODZIŃSKA, E.; ŚLEBODZIŃSKI, A.B.; PIETRAS, B.; WIECZOREK, G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. **Biol. Trace Element Res.** 47:69-74. 1995.
- [12] BUDAI, C.; EGRSZEGI, I.; OLAH, J.; JAVOR, A.; KOVACS, A. The protective effect of antioxidants on liquid and frozen stored ram semen – **Review. Anim. Sci. Biotech.** 47:46-52. 2014.

- [13] BUETTNER, G.R. The pecking order of free radicals and antioxidants, lipid peroxidation, alpha-tocopherol and ascorbate. **Arch. Biochem. Biophys.** 300:535-43. 1993.
- [14] CAMERON, R.D.A. Semen collection and evaluation in ram. The effect of method of stimulation on response to electroejaculation. **Aus. Vet. J.** 53:380-383. 1977.
- [15] COUNCIL FOR INTERNATIONAL ORGANIZATIONS OF MEDICAL SCIENCES AND THE INTERNATIONAL COUNCIL FOR LABORATORY ANIMAL SCIENCE. International guiding principles for biomedical research involving animals. 2012. Online: <http://www.cioms.ch/>. 05.04.16.
- [16] DAWRA, R.K.; SHARMA, O.P. Effect of seminal plasma antioxidant on lipid peroxidation in spermatozoa, mitochondria and microsomes. **Biochem. Int.** 11:333-339. 1985.
- [17] DE LAMIRANDE, E.; JIANG, H.; ZINI, A.; KODAMA, H.; GAGNON, C. Reactive oxygen species and sperm physiology. **Rev. Reprod.** 2:48-54. 1997.
- [18] DESAI, N.; SHARMA, R.; MAKKER, K.; SABANEKH, E.; AGARWAL, A. Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. **Fertil. Steril.** 92:1626-1631. 2009.
- [19] DU PLESSIS, S.S.; AGARWAL, A.; HALABI, J.; TVURDA, E. Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. **J. Assist. Reprod. Genet.** 32:509-520. 2015.
- [20] EMMANVERDI, M.; ZHANDI, M.; ZARESHAHNEH, A.; SHARAFI, M.; AKABAR-SHARIF, A. Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. **Reprod. Dom. Anim.** 48:899-904. 2013.
- [21] FORD, W.C.L. Regulation of sperm function by reactive oxygen species. **Hum. Reprod. Update** 10:387-399. 2004.
- [22] GUAL-FRAU, J.; ABAD, C.; AMENGUAL, M.J.; HANNAOUI, N.; CHECA, M.A.; RIBAS-MAYNOU, J.; LOZANO, I.; NIKOLAOU, A.; BENET, J.; GARCÍA-PEIRÓ, A.; PRATS, J. Oral antioxidant treatment partially improves integrity of human sperm DNA in infertile grade I varicocele patients. **Hum. Fertil.** 19:1-5. 2015.
- [23] HALLIWELL, B.; GUTTERIDGE, J.M.C. Antioxidant protection by low-molecular-mass agents: compounds derived from diet. In: **Free radicals in biology and medicine**. 3rd Ed. New York, USA: Oxford University Press, Pp 200-219. 1999.
- [24] HIDIROGLOU, M.; BATRA, T.R.; ZHAO, X. Comparison of vitamin C bioavailability after multiple or single oral dosing of different formulations in sheep. **Reprod. Nutr. Dev.** 37:443-448. 1977.
- [25] HIDIROGLOU, M. Technical note: forms and route of vitamin C supplementation for cows. **J. Dairy Sci.** 82:1831-1833. 1999.
- [26] HONG, Z.; HAILING, L.; HUI, M.; GUIJIE, Z.; LEYAN, Y.; DUBING, Y. Effect of Vitamin E supplement in diet on antioxidant ability of testis in Boer goat. **Anim. Reprod. Sci.** 117:90-94. 2010.
- [27] HUGHES, C.M.; LEWIS, S.E.; MCKELVEY-MARTIN, V.J.; THOMPSON, W. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. **Hum. Reprod.** 13:1240-1247. 1998.
- [28] JAFAROGHLI, M.; ABDI-BENEMAR, H.; ZAMIRI, M.J.; KHALILI, B.; FARSHAD, A.; SHADPARVAR, A.A. Effects of dietary n-3 fatty acids and vitamin C on semen characteristics, lipid composition of sperm and blood metabolites in fat-tailed Moghani rams. **Anim. Reprod. Sci.** 147:17-24. 2014.
- [29] JIMÉNEZ-RABADÁN, P.; GARCÍA-ÁLVAREZ, O.; VIDAL, A.; MAROTO-MORALES, A.; INIESTA-CUERDA, M.; RAMÓN, M.; DEL OLMO, E.; FERNÁNDEZ-SANTOS, R.; GARDE, J.J.; SOLER, A.J. Effects of vitrification on ram spermatozoa using free-egg yolk extenders. **Cryobiol.** 71:85-90. 2015.
- [30] KHASSAF, M.; MCARDLE, A.; ESANU, C.; VASILAKI, A.; MCARDLE, F.; GRIFFITHS, R.D.; BRODIE, D.A.; JACKSON, M.J. Effect of vitamin C supplements on antioxidant defense and stress proteins in human lymphocytes and skeletal muscle. **J. Physiol.** 549:2:645-652. 2003.
- [31] KNIGHT, C.A.; DUTCHER, R.A.; GUERRANT, N.B.; BECHTEL, S. Destruction of ascorbic acid in the rumen of dairy cows. **J. Dairy Sci.** 24:567-577. 1941.
- [32] MARCO-JIMENEZ, F.; PUCHADES, S.; GADEA, J.; VICENTE, J.S.; VIUDES-DE-CASTRO, M.P. Effect of semen collection method on pre- and post-thaw Guirra ram spermatozoa. **Theriogenol.** 64:1756-1765. 2005.
- [33] MASON, K.E.; MAUER, S.I. Reversible testis injury in the vitamin E-deficient hamster. **J. Nutr.** 105:484-493. 1975.
- [34] MATTHEWS, N.; BESTER, N.; SCHWALBACH, L.M.J. A Comparison of ram semen collected by artificial vagina and electroejaculation. **S. A. J. Anim. Sci.** 4:28-30. 2003.
- [35] MAXWELL, W.M.C.; STOJANOV, T. Liquid storage of ram semen in the absence or presence of some antioxidants. **Reprod. Fert. Develop.** 32:353-360. 1996.
- [36] NJERU, C.A.; MCDOWELL, L.R.; WILKINSON, N.S.; LINDA, S.B.; WILLIAMS, S.N. Pre- and postpartum supplemental dl-alpha-tocopheryl acetate effects on placental and mammary vitamin E transfer in sheep. **J. Anim. Sci.** 72:1636-1640. 1994.

- [37] NOURI, M.; GHASEMZADEH, A.; FARZADI, L.; SHAHNAZI, V.; GHAFARI NOVIN, M. Vitamins C, E and lipid peroxidation levels in sperm and seminal plasma of asthenoteratozoospermic and normozoospermic men. **Iranian J. Reprod. Med.** 6:1-5. 2008.
- [38] NATIONAL RESEARCH COUNCIL (NRC). Nutrient requirements of small ruminants. National Academic Press, Washington D.C., USA. Pp. 244-270. 2006.
- [39] OCHOA, L.; MCDOWELL, L.R.; WILLIAMS, S.N.; WILKINSON, N.; BOUCHER, J.; LENTZ, E.L. Tocopherol concentrations in serum and tissues of sheep fed different sources of vitamin E. **J. Anim. Sci.** 70:2568-2573. 1992.
- [40] O'FLAHERTY, C. Redox regulation of mammalian sperm capacitation. **Asian J. Androl.** 17:583-590. 2015.
- [41] PARRAGUEZ, V.H.; ATLAGICH, M.A.; BEHN, C.; BRUZZONE, M.E.; RAGGI, L.A. Fertility in ewes at high altitude: Comparison between animals with log- and short-time residence at high altitude and the effect of antioxidants vitamins. **Reprod. Dom. Anim.** 41:372. 2006.
- [42] PARRAGUEZ, V.H.; ATLAGICH, M.; ARANEDA, O.; GARCÍA, C.; MUÑOZ, A.; DE LOS REYES, M.; URQUIETA, B. Effects of antioxidant vitamins on newborn and placental traits in gestations at high altitude: comparative study in high and low altitude native sheep. **Reprod. Fert. Dev.** 23:285-296. 2011.
- [43] PAULSON, D.S. Nonparametric statistics. In: **Applied statistical designs for the researcher**. Marcel Dekker, Inc. New York, USA, Pp 519-606. 2003.
- [44] PEÑA, F.J.; JOHANNISSON, A.; WALLGREN, M.; RODRIGUEZ-MARTINEZ, H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. **Anim. Reprod. Sci.** 78:85-98. 2003.
- [45] RAO, B.; SOUFIR, J.C.; MARTIN, M.; DAVID, G. Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. **Gamete Res.** 24:127-34. 1989.
- [46] REKKAS, C.; KOKOLIS, N.; BELIBASAKI, S.; TSANTARLIOTOU, M.; SMOKOVITIS, A. Effect of a-tocopherol on plasma testosterone and plasminogen activator activity or inhibition in ram spermatozoa. **Theriogenol.** 53:751-760. 2000.
- [47] ROBAIRE, B.; HINTON, B.T.; ORGEBIN-CRIST, M.C. The epididymis. In: **Physiology of reproduction**. 3<sup>rd</sup> Ed. Knobil, E.; Neill, J.D. (Eds). Elsevier. Pp 1071-1148. 2006.
- [48] ROBAYO, I.; MONTENEGRO, V.; VALDÉS, C.; COX, J.F. CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration in ruminant cervical mucus. **Reprod. Dom. Anim.** 43:393-399. 2008.
- [49] SHARPE, R.M. Regulation of the spermatogenesis. In: **The physiology of reproduction**. 2<sup>nd</sup> Ed. Knobil, E.; Neill, J.D. (Eds). Raven Press Ltd. NY. EEUU. Pp 1363-1343. 1994.
- [50] SHI, T.Y.; CHEN, G.; HUANG, X.; YUAN, Y.; WU, X.; WU, B.; LI, Z.; SHUN, F.; CHEN, H.; SHI, H. Effects of reactive oxygen species from activated leucocytes on human sperm motility, viability and morphology. **Androl.** 44:696-703. 2012.
- [51] SHIVA, M.; GAUTAM, A.K.; VERMA, Y.; SHIVGOTRA, V.; DOSHI, H.; KUMAR, S. Association between sperm quality, oxidative stress, and seminal antioxidant activity. **Clin. Biochem.** 44:319-324. 2011.
- [52] SIKKA, S.C.; RAJASEKARAN, M.; HELLSTROM, W.J.G. Role of oxidative stress and antioxidants in male infertility. **J. Androl.** 16:464-468. 1995.
- [53] SILVA, P.F.N. Physiology of peroxidation process in mammalian sperm. Faculty of Veterinary Medicine, Utrecht University. PhD Thesis. 178 pp. 2006.
- [54] VATASSERY, G.T.; KRESOWKY, A.M.; ECKFELDT, J.H. Vitamin E concentration in human blood plasma and platelets. **Am. J. Clin. Nutr.** 37:1020-1024. 1983.
- [55] VELASQUEZ-PEREIRA, J.; CHENOWETH, P.J.; MCDOWELL, L.R.; RISCO, C.A.; STAPLES, C.A.; PRICHARD, D.; MARTIN, F.G.; CALHOUN, M.C.; WILLIAMS, S.N.; WILKINSON, N.S. Reproductive effects of feeding gossypol and vitamin E to bulls. **J. Anim. Sci.** 76:2894-2904. 1998.
- [56] WIRLEITNER, B.; VANDERZWALMEN, P.; STECHER, A.; SPITZER, D.; SCHUFF, M.; SCHWERDA, D.; BACH, M.; SCHECHINGER, B.; HERBERT ZECH, N. Dietary supplementation of antioxidants improves semen quality of IVF patients in terms of motility, sperm count, and nuclear vacuolization. **Int. J. Vitam. Nutr. Res.** 82:391-8. 2012.
- [57] YAN, L.; LIU, J.; WU, S.; ZHANG, S.; JI, G.; GU, A. Seminal superoxide dismutase activity and its relationship with semen quality and SOD gene polymorphism. **J. Assist. Reprod. Genet.** 3:549-554. 2014.
- [58] YENI, D.; GUNDOGAN, M.; CIGERCI, I.H.; AVDATEK, F.; FIDAN, F. Seasonal variation of oxidative stress parameters in ram seminal plasma. **J. Anim. Vet. Adv.** 9:49-54. 2010.
- [59] YOUSEF, M.I.; ABDALLAH, G.A.; KAMEL, K.I. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. **Anim. Reprod. Sci.** 76:99-111. 2003.
- [60] YUE, D.; YAN, L.; LUO, H.; XU, X.; JIN, X. Effect of Vitamin E supplementation on semen quality and the testicular cell membranal and mitochondrial antioxidant abilities in Aohan fine-wool sheep. **Anim. Reprod. Sci.** 118:217-222. 2010.