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Effect of Glutamate Supplementation upon Semen Quality of Young Seasonally Sexual-Inactive Dorper Rams

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ABSTRACT

The aim of this study was to determine if exogenous administration of glutamate to young Dorper rams is able to enhance semen quality under long-day photoperiods in northern Mexico (25° north). Dorper rams (n=10) with homogeneous live weight (LW; 56±0.9 kg), body condition score (BCS; 3.2±0.1 units) and scrotal circumference (SC; 31.9±0.38 cm) were randomly divided into two experimental groups and treated with: i) GLUT (n=5; 7 mg kg^{-1} LW of glutamate, every 3d × 28d, im.) and ii) CONT (n=5; 1 mL of saline, every 3d × 28d, im.). At the end of the experimental period (d28), semen was collected throughout the use of an artificial vagina; different quality and quantity parameters were evaluated. The ANOVA reveled treatment differences ($P<0.05$) regarding sperm concentration with the largest value observed in the GLUT-rams (4,260±95.9 × 10^6 cells) regarding to the CONT-rams (2,828±209.2 × 10^6 cells). Yet, when considering the rest of the response variables which included ejaculation latency (47 ± 15.8 sec), seminal volume (1.1 ± 0.15 ml), total number of ejaculated sperms (4024.5 ± 696.5 × 10^6 cells), mass motility (2.1 ± 0.3 units) and the percentage of sperms alive (57.5 ± 9.4 %), no statistical differences ($P>0.05$) were observed between treatments. Results of this study unveils to glutamate as an interesting molecule positively affecting the spermatogenesis process by increasing the sperm concentration of young Dorper rams during photo-inhibitory reproductive schemes. Results also denote interesting outcomes not only to other animal industries but may also embrace translational applications.

Keywords: Ram, Dorper, reproductive arrest, glutamate, seminal quality

Several environmental factors affect small ruminant reproductive activity, not only in females but also in males (D'Alessandro and Martemucci, 2003; Gonzalez-Bulnes *et al*., 2011; Escareño *et al*., 2013). Certainly, in rams from some breeds and(or) developed in certain areas, not only the libido but also the reproductive activity across the year is modified, being the photoperiod one of the main environmental cues affecting testicular function, reproductive behavior and seminal quality (Roselli *et al.,* 2004; Gonzalez-Bulnes *et al*., 2011: Bravo *et al.,* 2014). To generate such seasonal reproduction pattern, small ruminants are able to perceive the day length through retinal photic receptors which translate this photic input to an endocrine output in the pineal gland throughout the synthesis and secretion of melatonin. In turn, the specific endocrine pattern of melatonin release is a signal perceived at hypothalamic level, activating or deactivating the GnRH pulse generator (Meza-Herrera *et al*., 2011a; Meza-Herrera and Tena-Sempere, 2012; Schlatta and Ehmcke, 2014).

Certainly, once day length increases, a phase of reproductive arrest is observed: the hypothalamic centers become exquisitely sensitive to the negative retroaction from the gonadal steroids, promoting the long day

pattern release of melatonin which inhibits the secretion of the hypothalamic GnRH, compromising, in turn, the hypophyseal release of gonadotropins (Polat *et al.,* 2011; Meza-Herrera and Tena-Sempere, 2012). Upon reduction of gonadal testosterone secretion, the male reproductive function is suppressed, generating a decrease in testicular volume, spermatogenesis output and libido (Bustos and Torres-Díaz, 2012). Because of that, long-day photoperiods during spring and summer are related to a reduction in both quality and quantity of semen production (Gastel *et al.,* 1995; Ibrahim, 1997; Andersen *et al.,* 2011).

On the other hand, the excitatory amino acid glutamate, is considered the main neurotransmitter in the central nervous system (Brann and Mahesh, 1997; Meza-Herrera, 2008). Glutamate influences GnRH secretion because of the action of glutamatergic neurons which trigger a hormonal cascade affecting the hypothalamic-pituitary-gonadal axis (Meza-Herrera, 2012). Such neuroendocrine scenario has been linked to an enhancement in reproductive efficiency in females (Meza-Herrera *et al.,* 2014a,b) as well as in the sexual behavior of males (Andersen *et al.,* 2011), promoting an endocrine milieu prone to an amplified sexual behavior at courtship (Walkden-Brown *et al.,* 1999). Interestingly, the expression not only of ionotropic and metabotropic glutamate receptors (Takarda *et al.,* 2004) but also the presence of multiple glutamate transporters (Lee *et al.,* 2011) at testicular level, suggest that glutamate homeostasis must be critical in the mammalian testis. Nonetheless, the possible action of glutamate supplementation upon the seminal quality and quantity in male sheep under sexual photo-inhibitory schemes is still elusive; this study was designed to respond such research question.

MATERIALS AND METHODS

All the methods and management of the experimental units used in this study were in strict accordance with accepted guidelines for ethical use, care and welfare of animals in research at international (FASS, 2010) and national (NAM, 2002) levels, with institutional approval reference number UAAAN-UL: 1330-8241-2903.

Experimental area and environmental conditions

The study was conducted during spring under natural light conditions of increased photoperiods in an intensive sheep production unit in a semi-desert area of Northern Mexico, at 25° 64' N and 103° 26' W, an altitude of 1,120 m, and annual average temperature from 22° to 24°C.

Management and experimental treatments

Dorper rams (n=10, 11 mo.) were fed alfalfa hay, corn silage and corn grain twice daily (12:00 and 18:00h). Once rams were individually identified and prior to the onset of treatments, LW, BCS and SC were registered; while the BCS was measured by dorsal palpation (Russel, 1984) ranging from the scale of 1 (very thin) to 5 (very fat), the SC considered the measure of widest part of the testes using a flexible tape (Braun *et al.,*1980). In March, rams, which were homogeneous in terms of live weight (LW; 56±0.9 kg), body condition score (BCS; 3.2±0.1 units), and scrotal circumference (SC; 31.9±0.38 cm), were randomly assigned to one of two experimental treatments: i). Glutamate group (**GLUT;** intramuscular injection of 7mg kg^{-1} LW of L-glutamate (pH Eur EMPROVE® exp; MERCK-$C_5H_9NO_4$-art-101791, diluted in distilled water); the solution was prepared as previously described by Meza-Herrera et al. (2011) and applied every 3d × 30d, and ii). Control group (**CONT**, intramuscular injection of 1 mL of physiological saline every 3 d × 30 d). During the experimental period, which lasted from March to April, rams had *ad libitum* access to clean water, shades and mineral salts.

Response variable: measurements of semen quality

On day 28 of the experimental period, semen was collected with the use of an estrus female treated with 2 mg estradiol cipionate (Laboratorios NORVET, Mexico) in order to be mounted by rams. Yet, to proceed with the collection of semen, a standard sheep artificial vagina was used at a 42° C temperature. Previously, the artificial vagina was pre-heated from 30 to 41°C. Once collected, the tubes with the recuperated fresh semen were immediately submerged in a plastic container with water heated at 38°C and transported to the lab for its posterior analyses during the following 10 minutes. The measurements of semen quality considered:

i. Latency to ejaculation (seconds), considered the period of time from the moment at which the ram was exposed to an estrus ewe up to the moment in which the ram ejaculated inside the artificial vagina.

ii. Ejaculated volume (ml), was quantified directly in the gradated collection conic tube with 0.1 ml optically visible intervals.

iii. Sperm concentration was determined through photometric analysis (Spermacue®, 12300/0500 Minitub, Landshut, Germany; Olivera-Muzante *et al.,* 2011) using non-diluted semen and expressed as 10^6 cells per ml.

iv. Total number of ejaculated sperms (units) was calculated considering the sperm concentration per ml and multiplied by the total ejaculated volume, and expressed as 10^6 cells.

v. Mass motility (%), was assessed with the use of an arbitrary 1 to 5 scale; 1=25% to 5=100% motile sperms) as suggested by Mahsud *et al.* (2013). Sperm motility was determined with the use of a pre-heated platform (37°C) using a phase contrast microscopy, X400.

vi. Live sperms (%), sperm viability was assessed by using the eosine-nigrosine staining technique as described by Kafi *et al.* (2004). At least 200 spermatozoa were recorded per slide by light microscopy (1000X), and the percentage of dead (colored pink) and live (unstained) cells were quantified. All the evaluations were made by the same skilled operator.

Statistical analyses

Data were normalized by square root transformation, and the least squares means were analyzed by the General Linear Model (GLM) ANOVA procedure of SAS (SAS Institute Inc, Cary, NC, USA, V9.1). The statistical model to analyze the response variables include the effect of treatment; the results are presented as non-transformed means ± SEM and were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Our working hypothesis stated that the i.m. administration of glutamate would promote a positive effect upon semen quality and quantity of young Dorper rams treated during the natural sexual resting season; our results partially support such hypothesis. Analysis of variance reveled that treatments were not a significant source of variation for most response variables. Nonetheless, the greatest number of sperms per ml (sperm concentration), favored to the GLUT group regarding the CONT group ($P<0.05$; 4,260 ± 96.5 × 10^6 cells *vs.* 2,828 ± 597.2 × 10^6 cells).

Certainly, although most variables were not affected because of glutamate administration, the number of ejaculated sperms per ml favored to the GLUT treated rams, obtaining a 66.4% sperm concentration increase in those young Dorper rams treated with glutamate. To the best of our knowledge, this is the first report that document such findings. A possible explanation of such performance is the previously documented results regarding the positive action of glutamate upon not only the GnRH neurons (Meza-Herrera, 2012), being GnRH the common triggering hormone activating the hypothalamic-pituitary-gonadal function (Andersen *et al*., 2011) but also because of the presence of both ionotropic and metabotropic glutamate receptors and glutamate transporters in the testicular tissue (Takarda *et al.,* 1994; Lee *et al.,* 2011). Here, the interesting situation is that such testicular output triggered by glutamate supplementation, was promoted under long-day photoperiods which inhibit reproductive function at this latitude (25° north).

On this respect, Olney *et al.* (1976) stated that subcutaneous administration of glutamate in mice incremented both frequency and amplitude of GnRH acting in a positive fashion not only on the hypophyseal release of LH and FSH but also regarding the release of gonadal testosterone (Polat *et al.,* 2011). Besides, in the testicular Sertoli cells, both FSH and testosterone are responsible for initiate the spermatogenesis process and testosterone completes the sperm development until it is released to the rete testis (Bustos and Torres-Días, 2012; Dong *et al.,* 2016). Therefore, merging such neuroendocrine and cellular events, our results suggest that in the establishment of both previous scenarios, glutamate treatment may had triggered an increased spermatogenesis output, augmenting the observed sperm concentration in the glutamate-treated rams. Besides, it has been demonstrated the presence of glutamate in the seminal plasma while the seminal amino acid content could serve as oxidizable substrate in the sperm metabolism (Pruneda *et al.,* 2007).

Table 1 shows the results for the classical semen quality parameters. General averages for seminal volume (1.1 ± 0.15 ml), mass motility (2.1 ± 0.3 units) and the percentage

Table 1: Least square means ± standard error for different parameters for sexual behavior and semen quality in young Dorper rams (n=10) supplemented with Glutamate (GLUT) or Control (CONT) under natural photoperiodic conditions during the natural sexual resting season (March to April) in northern Mexico (25˚North)

Item	GLUT (n=5)	CONT (n=5)
Latency to ejaculation (s)	29 ± 8.7[a]	65 ± 23[a]
Volume ejaculated (ml)	1.1 ± 0.1[a]	1.1 ± 0.2[a]
Sperm concentration per ml, ($\times 10^6$ cells)	4,260 ± 96.5[a]	2,828 ± 597.2[b]
Total number of spermatoza ejaculated ($\times 10^6$ cells)	4,397 ± 500.9[a]	3,652 ± 893.9[a]
Mass motility, (1-5 units)	2.1 ± 0.3[a]	2.1 ± 0.3[a]
Live sperms, (%)	62 ± 7.9[a]	53 ± 10.9[a]

[a,b] Values in the same line with different superscript, differ (P<0.05).

of sperms alive (57.5 ± 9.4 %), without observing statistical differences (P>0.05) between treatments. Regarding the total number of sperms per ejaculation, despite the GLUT treated group got the largest value (4397±95.9 vs 3652±609.2), such differences did not reach significance (P>0.05). Yet, it is worth to mention that such lack of significance was probably due to the huge variability of the standard error of the CONT group that, compared to the GLUT group, was greater than 620%. Certainly, while the observed reproductive response was quite homogeneous in the GLUT-treated rams, an extremely large variation for such response variable occurred in the CONT-group. A similar figure can be mentioned with respect to the latency to ejaculation that although no statistical differences observing (P>0.05) between treatments (general averages 47 ± 15.8 s), a 264% increase in the standard error was observed in the CONT-rams regarding those treated with glutamate. Therefore, in some non-defined way, glutamate administration served to reduce the large variation in the sexual and reproductive responses observed in the CON-rams.

At this point, two ideas must be highlighted. The first is that regarding to the length of the experimental period which only considered 28 days. Although we got promising results when considering the positive effect of glutamate administration upon sperm concentration, as previously stated, the other response variables were not affected because of the glutamate inclusion. On this respect, future studies should consider to increase the length of the experimental period (i.e. 40-60 days), especially when considering the duration of the epithelial seminiferous cycle, from the first A1-spermatogonial mitosis up to the release of the spermatozoon to the rete testis (Cardoso and Queiroz, 1988). A second interesting point is that the Dorper rams most of the time display a quite short period of seasonal reproductive arrest (Malejane *et al.*, 2014). Both mentioned situations may had impede to observe statistical differences for the other response variables defining semen quality. Indeed, we would expect to observe a more defined effect of glutamate administration in some response variables, (i.e. sperm motility), since glutamate has shown to positively affect ATP production which plays a relevant role in the sperm metabolism as energy source to promote sperm motility (Susetyarini, 2015).

Different research groups have demonstrated a crucial role of glutamate upon sexual behavior. In adult male rats, increases in cellular glutamate have been related to an enlarged number of ejaculations, a reduced reaction time to ejaculation as well as a diminished period of time to accomplish the next ejaculation (latency to ejaculation) (Dominguez *et al*., 2006). Moreover, it has been stated that both the frequency of ejaculations and the libido are positively correlated with sperm concentration (Mahsud *et al.,* 2013). Such findings can be considered in line with the positive effect of glutamate administration upon sperm concentration observed in this study.

Results of this study unveils to glutamate as an interesting molecule which potentially affected the spermatogenesis process, observing in turn an increased sperm concentration of those young Dorper rams treated with glutamate even under photo-inhibitory reproductive schemes. Although further studies should be designed to

evaluate any possible effect of glutamate supplementation upon sperm morphology, results obtained in this study are promising while also denote interesting outcomes not only to other animal industries but may also embrace interesting translational applications.

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