Ovarian follicular growth in Morada Nova sheep supplemented with crude glycerin before mating

Crescimento folicular ovariano em ovelhas Morada Nova suplementadas com glicerina bruta antes do acasalamento

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Abstract

This study was aimed to investigate the follicular growth of Morada Nova sheep supplemented with crude glycerin before mating. Eighteen ewes were subjected to follicular wave and estrus synchronization with three injections of D-cloprostenol administered via IM, on days -14, -7 and -1 before mating. Animals were randomized into two groups, the control (n = 9), which received an oral drench of solution of 200 mL of saline solution, and the glycerin group (n = 9), which was supplied with 200 mL glycerol (n = 9), administered as an oral drench of crude glycerin in saline solution (9:1), 1h after morning feeding. Glycerin was supplied for 6 days, beginning 7 days from the mate. Growth follicular pattern was monitored daily by transrectal ultrasonography during the period of glycerin supplementation. Blood samples were taken during glycerin administration for progesterone and glucose analysis. There was a significant increase (P < 0.001) in blood glucose after supplementation with glycerin (mean of 66.2 ± 1.3 vs 56.5 ± 0.8 mg/dL). The greater availability of energy near to ovulation was beneficial to Morada Nova ewes, leading to a significant increase (P < 0.001) of large follicles (> 3 mm). At diagnosis of pregnancy, it was observed that approximately 87% (13/15) (87.5% control group vs. 85.7% glycerin group) of the ewes were pregnant. The group treated with glycerin presented an increase of more than 40% of prolificacy (1.7 ± 0.3 vs. 1.2 ± 0.2; P=0.21). In conclusion, the monitoring of follicular growth reveals that crude glycerin increases the follicular population of Morada Nova ewes, when applied before mating.

Keywords: Follicle. Crude glycerin. Pregnancy rate. Ovine. Morada Nova.

Resumo

Este estudo objetivou investigar o crescimento folicular ovariano de ovelhas Morada Nova, suplementadas com glicerina bruta antes do acasalamento. Dezoito ovelhas foram submetidas à sincronização da onda folicular e do estro administrando-se três injeções de D-cloprostenol (IM) nos dias -14, -7 e -1 antes da cobertura natural. Os animais foram distribuídos aleatoriamente em dois grupos: grupo controle (n = 9), que recebeu por via oral uma solução de 200 mL de soro fisiológico, e o grupo glicerina (n = 9), que foi suplementado por via oral com 200 mL de glicerol, administrados como uma parte de glicerina bruta diluída em soro fisiológico (9:1) 1h após a refeição matinal. O tratamento com glicerina foi fornecido por 6 dias, iniciando-se 7 dias antes da monta. O crescimento folicular foi monitorado diariamente por ultrassonografia transretal durante todo o período de suplementação. Além disso, amostras de sangue foram coletadas para dosagem de progesterona e glicose durante este período. Após a suplementação com glicerina, foi observado um aumento significativo (P < 0,001) na glicemia neste grupo em comparação ao grupo controle (média de 66,2 ± 1,3 vs 56,5 ± 0,8 mg/dL). A maior disponibilidade de energia próximo à ovulação foi benéfica para ovelhas Morada Nova, levando a um aumento significativo (P < 0,001) no número de folículos grandes (> 3 mm) no grupo suplementado com glicerina. Ao diagnóstico de gestação, foi observado que aproximadamente 87% (13/15) (87,5% grupo controle vs. 85,7% grupo glicerina) das ovelhas estavam prenhes. O grupo tratado com glicerina apresentou um aumento de mais de 40% de prolificidade (1,7 ± 0,3 vs 1,2 ± 0,2; P = 0,21). Em conclusão, o monitoramento do crescimento folicular revelou que a glicerina bruta aumenta a população folicular de ovelhas Morada Nova, quando aplicada antes do acasalamento.

Palavras-chave: *Folículo. Glicerina bruta. Taxa de gestação. Ovino. Morada Nova.*

Introduction

The Brazilian semi-arid region is characterized by low and irregular rainfall, which imposes severe food shortage to livestock production in the dry season, causing malnutrition to the flocks and consequent reduction in the reproductive performance. Much of this region is located close to the equator and thus presents a constant photoperiodic regime. In these conditions, the nutritional offer is the most important environmental condition for the reproductive success. Hair sheep breeds, as Morada Nova, are well adapted to semi-arid climate conditions and exhibit favorable reproductive characteristics, such as high prolificacy and good maternal ability (Facó et al., 2008). However, the reproductive efficiency is dependent of nutritional management strategy that guarantee the expression of this potential (Bomfim et al., 2014).

In order to maintain satisfactory levels of livestock production, food supplementation is traditionally the most used way to minimize the nutritional deficit caused by fluctuation in forage availability. However, the use of cereal-based concentrate exposes farmers to price variations and market offer, often increasing production costs and thus affecting livestock production potential of the region. A solution that has been studied is the use of alternative feedstuffs, such as crude glycerin, a biodiesel production co-product, as energy supplement for ruminants. Brazil is among the largest producers and consumers of biodiesel worldwide, with an effective production exceeding 3 million m^3 in 2014. In that same year, it was generated over 290 thousand m^3 glycerin (ANP, 2015).

The use of crude glycerin in animal nutrition is due to the high energy value of the principal component, glycerol. According to Donkin (2008), about 80-88% of crude glycerin is comprised of glycerol. In ruminants, a part of glycerol can be fermented in the rumen turning into short chain fatty acids, mainly propionate, which in turn is metabolized to oxaloacetate via the citric acid cycle in the liver and can be used to form glucose by the gluconeogenic pathway (Mach et al., 2009). Another portion of the glycerol can be directly absorbed by the rumen epithelium without undergoing fermentation and metabolized in the liver and, by the action of glycerol kinase, it is converted into glucose (Rémond et al., 1993).

In the last years, several studies have focused on the use of this product also aimed at meat production in cattle (Mach et al., 2009), and sheep (Gunn et al., 2010). In relation to its effects on ovarian status, it was reported in Holstein cows a significant difference in the number of large follicles between the glycerol supplemented and control group (Karami-Shabankareh et al., 2013). Nevertheless, there are only few contributions on the application of glycerin in reproduction, especially in small ruminants. In sheep, Gutierrez et al. (2011) used a short flushing system by means of a single dose of glycerol before mating and found an increased ovulation rate.

In recent years, the use of glycerin as energy supplement in sheep, in the short or supershort flushing system (Scaramuzzi et al., 2006; Viñoles et al., 2010), seems interesting, however there is still little information to integrate the effect on follicular development with reproductive response after mating. Given this and considering the potential application of glycerin as an alternative food during critical periods in the Northeastern region, this study aimed to evaluate the follicular growth of Morada Nova sheep supplemented with crude glycerin before mating.

Material and methods

Animals and management

The experiment was conducted at the Agricultural Experimental Farm Dr. Esaú Accioly de Vasconcelos, in the municipality of Guaiúba, Ceará State, located at 4º2 23"S and 38º38'14"W, from May to October. This area, characterized by a constant photoperiod regimen, has a warm, tropical, sub-humid climate with a mean annual rainfall and temperature of 904.5 mm and 26-28º C, respectively, with two distinct seasons: rainy, from February to May, and dry, from June to January. The study was approved by the Ethics Commission for the Use of Animals of the State University of Ceará (CEUA-UECE), under Protocol nº 3450166/2014.

Eighteen pluriparous Morada Nova ewes were chosen from the same flock with age and body weight (mean ± SD) of 50.0 ± 13.2 months and 36.3 ± 6.7 kg. Ewes were grouped according to body condition score (BCS) at lambing by a single trained examiner, assigned a score using a 1-5 scale with steps of 0.25 (Russel et al., 1969). BCS was 3.1 ± 0.4 (mean ± SD), which was equivalent of subcutaneous fat lumbar thickness and lumbar depth mean measured at lumbar region of 4.2 ± 0.2 mm and 16.7 ± 2.9 mm, respectively. Sheep were kept in one sheltered pen measuring 8 × 8 m, where they received mineral salt and water *ad libitum*. Pen was clayed with concrete and faced an east-west direction. During 20 days of nutritional and housing adaptation, ewes were subjected to internal and external parasite treatment and monitoring of the ovarian function assessed by ultrasound examinations (Mindray® DP 2200 VET, Shenzhen, China) as described by Fernandes et al. (2016). Animals received a common feed composed of a mixture of chopped elephant grass (*Pennisetum purpureum*) and a commercial concentrate (10.6% crude protein and 66.8% total digestible nutrients on a dry matter basis). The diet formulation was prepared according to the requirements for maintenance and breeding (NRC, 2007) for adult sheep. The diets were provided twice a day (7 am and 3pm).

Estrus synchronization and mating

Estrus and follicular wave of ewes were synchronized as described by Viñoles et al. (2010). Three injections were administered via IM, with seven day-intervals each (Figure 1), using 100μg D-Cloprostenol (Prolise® - Tecnopec, São Paulo, Brazil). After 24 hours of the 3rd and final application of PGF2α, one Morada Nova ram with fertility previously proven was introduced in each pen. After this first mating, the ewe was removed from the pen and the procedure repeated 24 hours later with a second ram, always of the same breed. After pregnancy diagnosis, non-pregnant females were subjected to a new mating period using the same males. Ewes that were non-pregnant to both mating periods were ruled out from the experiment.

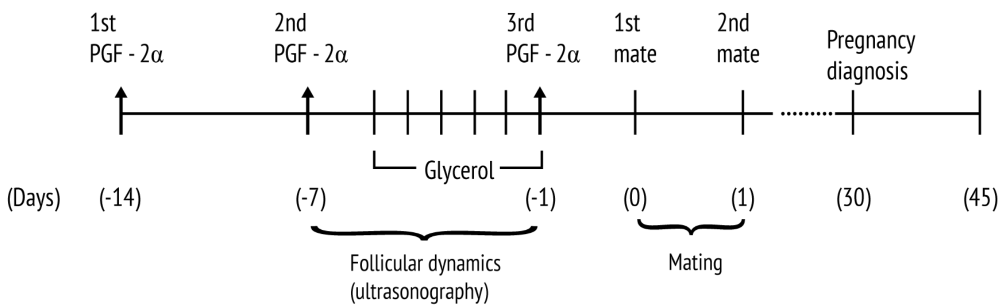


Figure 1 - Drenching of glycerol, hormonal treatment for estrus and follicular wave synchronization and mating.

Glycerin and experimental design

Crude glycerin used in this study was obtained from a biodiesel facility (Petrobrás Biocombustível S.A., Quixadá, Ceará, Brazil), which contained 80% glycerol, 13% water, 7% salt, and 0.1% methanol. Animals received a drench of 200mL saline solution (Control group, n = 9), or 200mL glycerol (Glycerin group, n = 9), administered as a drench of crude glycerin in saline solution (9:1), 1 h after morning feeding. Glycerol in the form of crude glycerin was used as an energy supplementation, supplied for 6 days beginning 2 days after the second PGF2α injection (Figure 1), 7 days from the 1st mate (Viñoles et al., 2010). Each oral dose of crude glycerin was equivalent to 1.03 Mcal of metabolizable energy (Mach et al., 2009).

Ultrasonography

In all animals, the growth pattern of ovarian follicles was monitored daily by ultrasonography during the period of glycerin supplementation. The images were obtained with a real-time ultrasound scanner (Mindray Bio-medical Eletronics Co., LTD, China, model DP-2200Vet), connected with a linear transrectal transducer of 7.5 MHz. The methods for examination of the ovaries by transrectal ultrasonography and for following individual follicles from day to day have been described previously by Viñoles et al. (2004). Briefly, with the female contained in station position, feces were removed and a lubricant gel was introduced into the rectum to avoid damage of the mucosa. With the probe placed into the rectum, it was pushed cranially to find the urinary bladder. After reaching it, the probe was put a few centimeters cranially and then laterally on both directions in order to localize the ovaries. The ovarian follicles were identified by the presence of an anechoic spherical structure with a thin wall (Gonzalez-Bulnes et al., 1994). It was considered as a single follicular wave the pool of follicles that have emerged in a 48 h interval with size ≥ 2 mm (Viñoles et al., 2004). Pregnancy diagnosis was performed on the 30th and 45th days post breeding using the same ultrasound scanner and transducer.

Evaluation of subcutaneous fat loin thickness (SFLT) and loin depth (LD) was carried out before mating and at partum using the same ultrasound device with linear transducer of 5 MHz. To this end, the female was kept in station and the transducer arranged linearly in the region between the 3rd and 4th lumbar vertebrae (Silva et al., 2014). All procedures were performed by the same trained technician, in randomized evaluation. For measurement of structures of interest, including the growth pattern of the ovarian follicles, ultrasonographic examinations were recorded in the form of videos, followed by the capture and measurement of images (with several different projections of ovary) for each structure using the Image J software (Image J, National Institutes of Health, Millersville, USA), which was previously calibrated.

Glucose and progesterone assay

On a daily basis, in all animals, four blood samples were taken respectively 1h before the crude glycerin administration, and 4h, 8h and 12h after supplementation. Glucose levels were immediately measured using a glucose metering (G. Tech Free® SD – Standard Diagnostic), with a measurement range of 10–600 mg/dL. Plasma progesterone levels were analyzed by daily blood sampling during the period of supplementation with glycerol and 24 hours later (seven samples per animal). Blood was obtained from the jugular vein into heparinized tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and centrifuged at 3000 rpm for 15 min.

Progesterone level was measured using a commercial kit (Immulite® 2000 Progesterone, Siemens Healthcare Diagnostics LTDA, Llanberis, United Kingdom) and a specific device (Immulite® 2000, Siemens Healthcare Diagnostics LTDA, Llanberis, United Kingdom). The sensitivity of the assay kit was 0.1 ng mL^{-1}, and the intra and inter-assay coefficients of variation were 9.71% and 12.21% respectively.

Statistical analysis

For body weight, body condition, loin subcutaneous fat thickness, loin depth and lamb birth weight data were analyzed using PROC GLM , and the factors used in the model for analysis of variance (ANOVA) included group (glycerin, control), type of parturition (simple or multiple) and interaction group vs. type of parturition. For the glucose levels, plasma progesterone level, number of follicles and follicle diameter, the model included group, interval of assessment considered (time) and interactions group vs. time. Comparisons between means were determined by t student test. For pregnancy rate, litter size, twinning rate and kidding rate, the effect of group and type of parturition were analyzed by the Kruskal-Wallis ANOVA test. Comparison between numbers was performed using a chi-squared test.

Results and discussion

The present study aimed to evaluate the follicular population of Morada Nova sheep supplemented with crude glycerin before mating. The efficiency of the short flushing on follicular development and ovulation in sheep has been documented (Muñoz-Gutiérrez et al., 2002; Viñoles et al., 2005; Letelier et al., 2008a), however, its regulation is not completely elucidated (Scaramuzzi et al., 2006). In addition, to date, no changes in follicular dynamics have been reported in Morada Nova ewes supplemented with glycerin. Figure 2 shows ultrasound images.

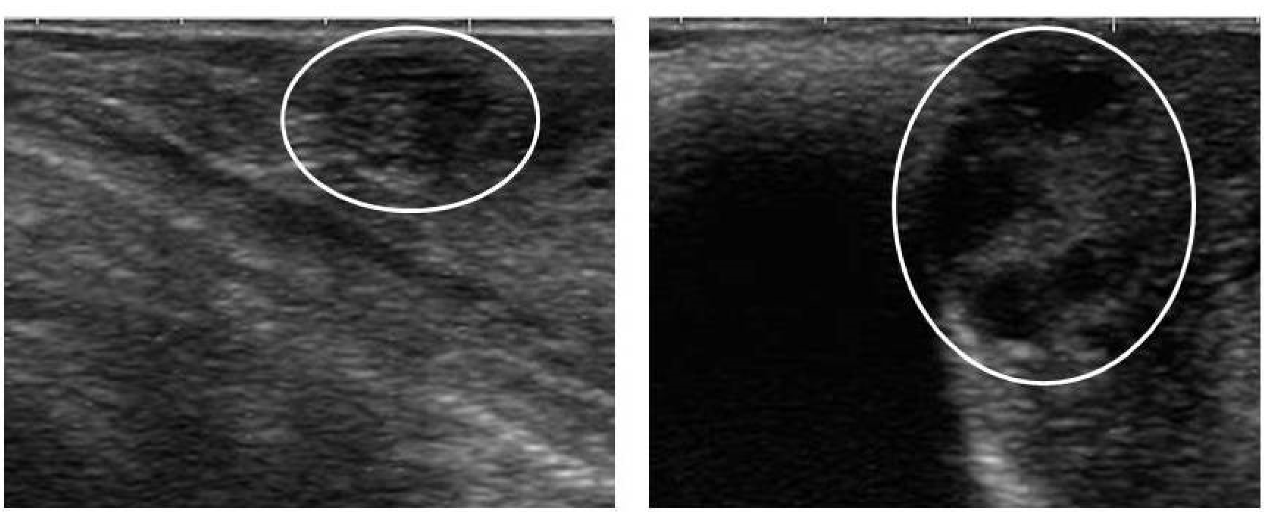


Figure 2 - Representative ultrasonographic images of follicular growth in Morada Nova ewes. The ovary is highlighted with a white circle. Note a wave of emerging follicles (left) and a population of large follicles (right).

The protocol used in this experiment provided a surplus of energy to animals with satisfactory nutritional status (Table 1), and kept in a proper diet to the mating period (NRC, 2007). Briefly, the results confirmed the success of the treatment with glycerol.

The flow of energy released before ovulation stimulated follicular development. The group supplemented with glycerol presented an increase in the number of large follicles (> 3 mm) and, after birth, an increase of more 40% in the number of lambs per ewe lambed (Figure 3).

Table 1 - Reproductive and productive parameters of Morada Nova ewes and lambs treated with glycerol

Parameters		Group*		P – value	
	Control	Glycerin	Group	TP	G x TP
No. of ewes	8	7**			
Breeding					
Body weight (kg)	40.2 ± 3.3	35.6 ± 3.3	0.46	0.73	0.65
BCS (1-5)	3.2 ± 0.2	3.2 ± 0.2	0.87	0.78	0.49
SFLT (mm)	3.8 ± 0.2	4.4 ± 0.2	0.05	0.13	0.78
LD (mm)	17.3 ± 1.7	15.6 ± 0.7	0.44	0.81	0.91
Pregnancy rate (%, n)	87.5 (7/8)	85.7 (6/7)	0.92	-	-
Parturition					
Body weight (kg)	38.4 ± 3.4	38.7 ± 4.1	0.91	0.89	0.74
BCS (1-5)	3.1 ± 0.9	3.0 ± 0.2	0.43	0.21	0.79
SFLT (mm)	4.5 ± 0.4	4.9 ± 0.7	0.17	0.15	0.96
LD (mm)	13.8 ± 1.2	13.9 ± 0.8	0.83	0.91	0.53
Lambing rate (%, n)	85.7 (6/7)	100 (6/6)	0.35	0.50	-
Litter size(n)	1.2 ± 0.2	1.7 ± 0.3	0.21	-	-
Twinning rate (%, n)	14.3 (1/7)	50.0 (3/6)	0.24	-	-
Lamb birth weight, (kg)	2.6 ± 0.3	2.3 ± 0.2	0.62	0.28	0.13

Note: TP = type of parturition; G x TP = group vs. type of parturition; BCS: Body Condition Score; SFLT: Subcutaneous Fat Loin Thickness; LD: Loin Depth. *In each group one ewe was ruled out being negative to a pregnancy diagnosis for two consecutive mating periods ** In this group one animal was ruled out due to a hyperglycemic crisis.

Figure 3C illustrates plasma glucose values measured during administration of crude glycerin. In the period of administration, on average the glucose level was 66.2 ± 1.3 mg/dL in the glycerin group and 56.5 ± 0.8 mg/dL in the control group (P < 0.001). There was a significant increase in blood glucose levels 4 hours after supplementation with glycerin (74.8 ± 2.7 vs. 55.6 ± 1.8; P < 0.001). Studies on the kinetics of ruminal degradation of glycerol in cattle indicate how the metabolization occurs between 4 and 6 hours after administration (Rémond et al., 1993). In this interval, glycerol is fermented into propionate and butyrate (Defrain et al., 2004); the former is used as a gluconeogenic substrate by ruminants (Mach et al., 2009).

The protocol of glycerol dosing was set on the basis of data from previous experiment (Silva et al., 2014). Furthermore, field observations of the animals showed that the repeated administration of 200ml glycerol was relatively well accepted. Thus, blood glucose values recorded in the collection interval (Figure 1) were always lower (< 80 mg/

dL) than those recorded in goats by Silva et al. (2014). Nevertheless, the individual component in the glycemic response should always be taken into account when establishing a protocol for administration of glycerin or another glycogen precursor. In the present experiment, after the second administration, one of the animals presented a pronounced increase in blood glucose (> 100 mg/dL) and, therefore, it was necessary to withdraw it from the test.

Sheep can develop 3 to 4 follicular waves during the interovulatory interval (Bartlewski et al., 2011). Thus, comparing experimental animals, asynchrony between the development stage of the follicular wave and the moment in which the energy supplement is given may represent a serious obstacle for the study of these types of effects (Viñoles et al., 2005). One option is the use of the first wave model (Viñoles et al., 2010). The synchronization protocol and the study of the first wave, as proposed by Viñoles et al. (2010), allows the use of a similar follicular growth model for all animals and groups, thereby increasing accuracy in evaluation of energy supplementation impacts in the short term.

Figures 3A, 3B and 3D grouped the main information recorded during the period of application of crude glycerin. There was no glycerin administration effect on plasma progesterone levels (Figure 3D). As expected, P_4 levels increased after the second administration of prostaglandin, showing higher values on the day of the third dose of PGF-2α, one day before mating, and a decrease after 24 hours, because of the luteolytic action of prostaglandin treatment. This behavior was similar for all the sheep, but two animals, one in each group, did not respond to hormonal treatment. Data from these animals were not considered for analysis (Viñoles et al., 2010).

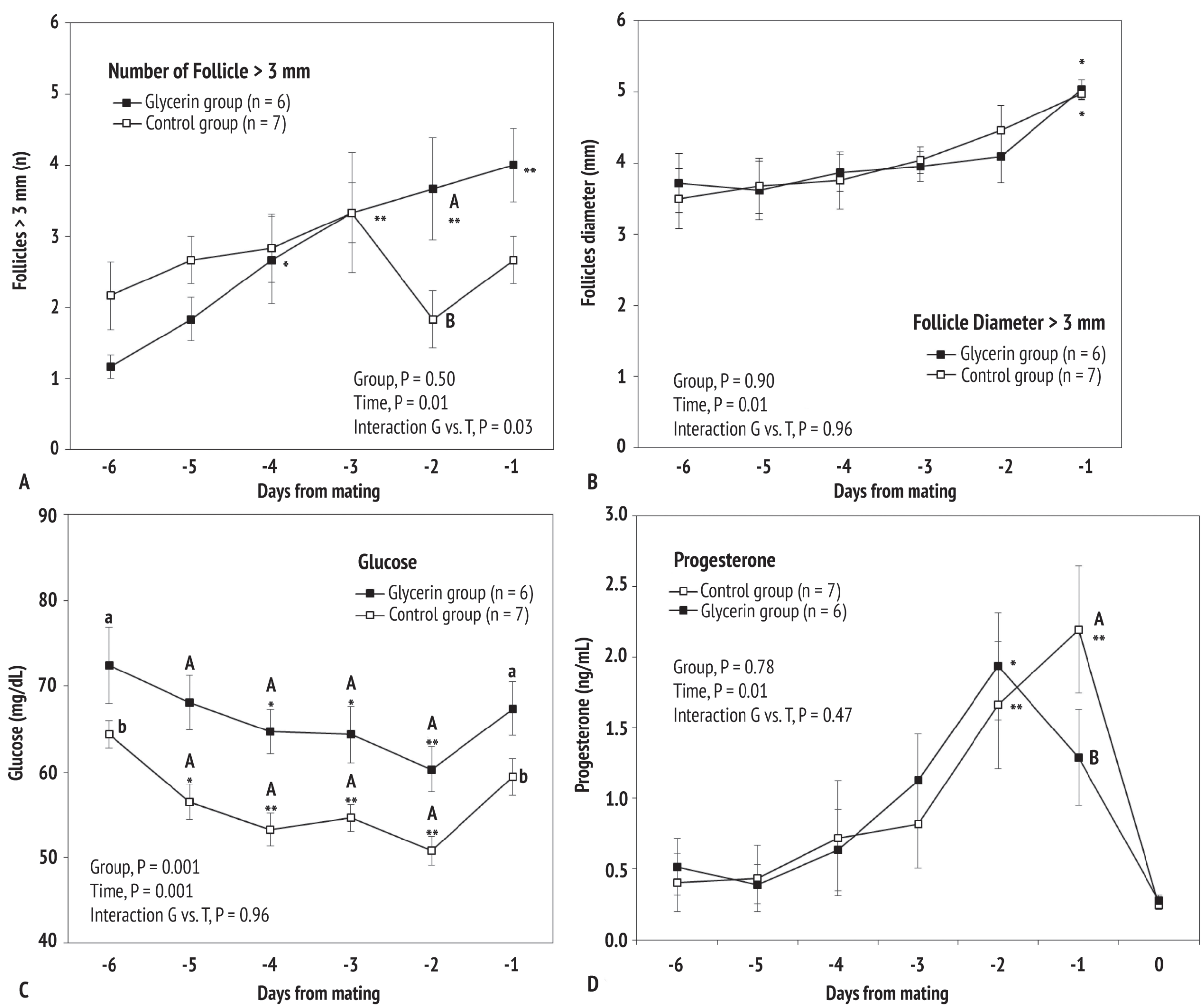
In the group supplemented, throughout the interval of application of crude glycerin, we observed a progressive increase in the number of follicles >3 mm (Figure 3A), unlike that found in the control group. From the third day of glycerin administration (4 days before mating), the number of follicles >3 mm was statistically higher (P = 0.03) at the start of treatment (six days before mating). On the last day of supplementation, one day before mating, the number of follicles was greater than in the first day (4.0 ± 0.5 vs. 1.8 ± 0.2; P = 0.0001), contrary to that in the control group (2.7 ± 0.3 vs. 2.2 ± 0.5; P > 0.05). Two days before mating, the groups were significantly different (3.7 ± 0.7 vs. 1.8 ± 0.4; P < 0.01).

Regarding the characteristics of the first wave (Figure 3B), there was no effect of the treatment on the diameter of the follicles > 3 mm (P = 0.90). The results confirmed that the growth of the first wave during application of glycerin was positive (P < 0.001) and similar in both groups (G vs. T, P = 0.96). Follicular growth rate in the interval considered was respectively 0.3 ± 0.0 mm/day and 0.2 ± 0.0 mm/day for glycerin and control groups, respectively (P = 0.45). The mean diameter on the last day of supplementation, when we recorded the largest follicular diameter (5.0 ± 0.1 mm vs. 5.0 ± 0.1 mm; P = 0.77) resulted as expected, analogously to the follicular wave described by Viñoles et al. (2010) and Bartlewski et al. (2011).

All these results suggest the efficacy of crude glycerin and the dosing protocol used in short-term nutritional regulation on the follicular development in Morada Nova sheep, as also reported for other glucogenic precursors in Manchega breed (Letelier et al., 2008b) and Sarda breed (Berlinguer et al., 2012). Moreover, the data presented lead to attempt that crude glycerin is an effective source of energy supplement also in animals with adequate nutritional status. According to Viñoles et al. (2010), ewes with high body condition are distinguished by their follicular development rates supported by the highest concentrations of metabolic hormones. Even in this condition, a brief energy stimulation allows to alter the hormonal metabolic condition and further stimulate follicular development.

With respect to fertility response, it was observed that approximately 93% (14/15) of the ewes were pregnant. These results indicate that the estrus synchronization protocol combined with the natural mating system at fixed intervals was highly efficient. The application of glycerin during six days before mating demonstrated no influence on the pregnancy rate, which was similar and high (>85%) for both groups (Table 1). Based on the report of Rondina and Galeatti (2010), we consider that a contributing factor for this result is homogeneous and adequate body condition of the experimental animals. The pregnancy rates obtained in this work were comparable to those (91%) reported previously for Morada Nova ewes raised in the Northeastern of Brazil (Villarroel and Fernandes, 2000).

Additionally, in the glycerin group, supplementation before mating showed a positive tendency in terms of fertility, with an increase of more than 40% in prolificacy at lambing (1.7 ± 0.3 vs. 1.2 ± 0.2; P = 0.21). Traditionally, in sheep, flushing has been applied for two to four weeks before mating, depending on body condition of the flock. This type of protocol ensures follicular stimulation (Haresign, 1981), followed by an increased prolificacy (Gunn, 1983). Similar prolificacy rates (1.5) were described for Morada Nova sheep kept in cultivated pastures and receiving concentrate supplementation (Machado et al., 1999).



Note: * P < 0.05, ** P < 0.01 differences from -6th day in each period. A, B P < 0.01 differences between groups. Statistically significant effect of group, time treatments and interaction are given in the figure.

Figure 3 - Number of follicles >3 mm (A), diameter of follicles >3 mm (B) and glucose concentration (C) measured 6 days from the mating, during crude glycerin administration. Progesterone levels (D) were determined 6 days from mating, during the energy supplementation and 24 hours after. Values are given in mean ± SEM.

Conclusion

The monitoring of ovarian follicular growth reveals that crude glycerin as energy supplement for short periods increases the follicular population of Morada Nova ewes, that glycerol doses impacted on the glycemic rate, and that new studies are required using lower doses of glycerol.

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