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Relationship between superovulation and embryo production with ovarian follicular population before superovulatory treatment in Brazilian Bergamasca sheep

[*Relação da resposta superovulatória e produção embrionária com a população folicular ovariana antes do tratamento superovulatório de ovelhas da raça Bergamácia Brasileira*]

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ABSTRACT

The objective of this study was to quantify the superovulatory response and embryo production of Brazilian Bergamasca sheep and to evaluate the link to the follicular condition before superovulatory treatment, as a reference for selection of donors with potential for superovulation. Follicular population of twenty-three sheep was evaluated by ultrasound during metestrus phase of the estrous cycle and divided into groups of low, medium and high follicular population. Subsequently, they were synchronized, superovulated with 133mg of pFSH, mated and subjected to embryo collection. The superovulatory response (9.0±3.3 vs 10.7±6.2 vs 13.8±7.1) and embryo production (4.0±3.8 vs 2.6±2.0 vs 1,8±4.0) were similar between groups (P>0.05). There was a positive correlation between the number of follicles during the metestrus phase and the number of corpus luteum with premature regression (PLR) (0.52) and a negative correlation between the recovery rate and PLR (-0.44) (P<0.05). The sheep that presented PLR had more follicles during metestrus (16.9±7.8 vs 12.7±3.2) and lower embryo recovery rate (38.8±29.3 vs 72.2±29.9) than those with functional CLs (P<0.05). Follicular quantification during metestrus phases was unable to identify donors with high embryo production. Animals with PLR had a larger follicular population during metestrus and lower embryo recovery rate.

Keywords: conservation, embryo, superovulation, genetic resources, reproduction

RESUMO

O objetivo deste trabalho foi quantificar a resposta superovulatória e a produção embrionária de ovelhas Bergamácia Brasileira e relacioná-las com a condição folicular antes do tratamento superovulatório, como referência para seleção de doadoras com potencial para superovulação. Vinte e três ovelhas foram avaliadas quanto à população folicular por ultrassonografia na fase de metaestro do ciclo estral e divididas em grupos com baixa, média e alta população folicular. Posteriormente foram sincronizadas, superovuladas com 133mg de pFSH, acasaladas e submetidas à coleta de embriões. A resposta superovulatória (9,0±3,3 vs. 10,7±6,2 vs. 13,8±7,1) e a produção embrionária (4,0±3,8 vs. 2,6±2,0 vs. 1,8±4,0) foram semelhantes entre os grupos (P>0,05). Houve correlação positiva entre o número de folículos no metaestro e o número de corpos lúteos com regressão prematura (RPCL) (0,52) e correlação negativa entre a taxa de recuperação e RPCL (-0,44) (P <0,05). As ovelhas que apresentaram RPCL tiveram mais folículos no metaestro (16,9±7,8 vs. 12,7±3,2) e menor taxa de recuperação embrionária (38,8±29,3 vs. 72,2±29,9) do que as que apresentaram CLs funcionais (P<0,05). A quantificação folicular nas fases de metaestro não foi capaz de identificar doadoras com alto potencial de produção embrionária. Animais com RPCL tiveram maior população folicular no metaestro e menor recuperação de embriões.

Palavras-chave: conservação, embrião, superovulação, recursos genéticos, reprodução

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INTRODUCTION

Brazilian Bergamasca sheep were introduced in Brazil in the early 20th century, more precisely in the 1930s during the wave of Italian immigration. They are large, white, flocked, polled, hardy sheep with dangling ears. After several generations of natural selection and due to the geographical variety of their country of origin, they developed genetic and phenotypic characteristics that differentiated them from their ancestors, making them a locally adapted breed.

Considering the reduction of its population and its contribution to the Santa Inês breed, the main breed created in Brazil, conservation of the breed is necessary. Among the most used conservation methods is the preservation of gametes and cryopreserved embryos in germplasm banks (Mariante *et al*., 2011). In order to make the conservation of Brazilian Bergamasca possible, studies are needed to quantify the superovulatory response of these animals and to assist in the selection of donors with greater potential for embryo production, increasing the efficiency of conservation actions and reducing their cost.

Ovine superovulation (SOV) consists of ovarian stimulation by means of a hormone protocol for the synchronization of estrus and ovulation for the purpose of developing and maturing a large number of follicles simultaneously, enabling the production and collection of a greater number of embryos. Consequently, SOV as a breeding biotechnology is important for the conservation of sheep breeds that are threatened with extinction (Lopes Júnior *et al*., 2014). Hormone treatments in ovine SOV programs are efficient due to the understanding of the functions and interactions among folliculogenesis, follicle growth, oocyte maturation, ovulation and fertilization (Gibbons *et al*., 2011). However, obstacles are still found in achieving the uniformity of the superovulatory response for the recovery of viable embryos (Bartlewski *et al*., 2016), with factors intrinsic to the animals considered to be responsible (Brasil, 2016).

Studies have demonstrated that the ovarian response is associated with the characteristics of the ovary at the beginning of superovulation where the presence of a large follicle negatively affects the ovulation rate and the number of viable embryos (Gonzalez-Bulnes *et al*., 2000; Gonzalez-Bulnes *et al*., 2002; Veiga-Lopez *et al*., 2005; Mossa *et al*., 2007). In addition, most of these studies recorded that the number of small follicles is significantly related to the rate of ovulation and the number of viable embryos recovered (Gonzalez-Bulnes *et al*., 2000, 2002; Mossa *et al*., 2007).

However, in sheep, the evaluation of antral follicles associated with the production of corpus luteum after superovulation is not well established (Bartlewski *et al*., 2008). Studies carried out with Santa Inês sheep have attempted to link the follicular population of sheep in different phases of the estrous cycle (proestrus, estrus, metestrus and diestrus) with the superovulatory response and obtained quite satisfactory results during metestrus phase, demonstrating the viability of this approach for the selection of embryo donors (Brasil, 2016).

The evolution of methodologies for the pre-selection of embryo donors, especially those locally adapted, threatened with extinction, with a high capacity to respond to superovulation protocols is extremely important so as to reduce the costs of hormone protocols and avoid unnecessary collection procedures in sheep with low potential to respond to superovulation. The objective of this work was to quantify the superovulatory response and embryo production of Brazilian Bergamasca sheep and to relate them to the follicular condition before superovulatory treatment, as a reference for selection of donors with potential for superovulation.

MATERIAL AND METHODS

This Experiment was approved by the Ethics Committee on the Use of Animals within the Embrapa Genetic Resources and Biotechnology unit (*Embrapa Recursos Genéticos e Biotecnologia*) under protocol CEUA / Cenargen 007/2019. The study was carried out in the Experimental Field Sector Fazenda Sucupira belonging to Embrapa Genetic Resources and Biotechnology unit, located southwest of the city of Brasília-DF, at altitudes ranging from 1050 to 1250 meters. The predominant climate is rainy tropical, with dry winter and rainy summer. This region has a rainy tropical climate, with dry winters and rainy summers, type AW in Köppen classification (Alvares *et al*., 2013).

Twenty-three Brazilian Bergamasca sheep, sexually mature and clinically healthy, aged between 2 and 3 years and ECC between 2.5 and 3 (range 1-5; Thompson and Meyer, 1994) were used. The sheep were kept in stalls and fed Tifton hay and commercial feed (300g / sheep / day), with unlimited access to water and mineral salt. For the experiment, all sheep had their estrous cycle synchronized by intramuscular (IM) administration of two doses of 250 µg of sodium D-cloprostenol (Clocio®, sodium D-cloprostenol, Bimeda, Brazil) at an interval of 10 days between applications. Four and a half days after the last application of D-cloprostenol, corresponding to the metestrus phase of the estrous cycle, the animals' ovaries were examined by ultrasound to quantify the follicles ≥2 mm (Brasil, 2016). According to the number of follicles seen in both ovaries, the animals were divided into groups with high (≥18 follicles; G1; n = 5), medium (> 10 <18 follicles; G2; n = 9) and low follicular population (≤10 follicles; G3; n = 5) and subsequently subjected to superovulation and embryo collection.

The division of the groups was defined by the average and standard deviation of the follicular population of all animals, where G1 included animals with follicular population greater than the average standard deviation and G3 included animals with follicular population below the average standard deviation. The ovary evaluation was performed by an experienced operator using real-time, high-resolution, B-mode ultrasound equipment (DP-2200Vet; Shenzhen Mindray Bio-Medical Electronics Co. Ltd., Nanshan, Shenzhen, PR China), equipped with a multifrequency linear transducer at a frequency of 7.5 MHz and adapted for transrectal examination, according to a validated technique for monitoring ovarian follicular dynamics and detection of CL in sheep (Vinoles *et al*., 2004).

The sheep were synchronized with a short protocol where, on Day 0, 250µg of sodium D-cloprostenol (Clocio®, sodium D-cloprostenol, Bimeda, Brazil) was administered intramuscularly (IM) and an intravaginal progesterone device (CIDR, progesterone, Zoetis, Brazil) was inserted and maintained for 7 days. On Day 4 of the protocol, administration of 133mg of pFSH (Folltropin®-V, hormone follicle stimulation, Tecnopec, AHC Inc., Bioniche, Canada) was initiated in eight decreasing doses (20% x 2; 15% x 2; 10% x 2; 5% x 2) with a 12-hour interval. The progesterone device was removed with the application of the seventh dose of FSH. A 250µg dose of sodium D-cloprostenol and 25µg GnRH (Gestran Plus, gonadorelin acetate; Tecnopec, Argentina) was administered IM along with the fifth and eighth dose of pFSH, respectively. Before the start of superovulatory treatment (Day 4) all sheep were submitted to ultrasound examination to count the follicles larger than 2 mm present in the ovaries.

Thirty-six hours after the removal of the CIDR, the ewes were distributed in 5 pens for mating by natural breeding (NM) and kept with the breeders for 24 hours. At the end of the NM, the progesterone device was reintroduced. The rams underwent andrological examinations before the experiment and all were capable of reproduction. Five days after mating, the number of ovulations was verified by laparoscopic procedure. Sheep with <3 CL were considered as females not responsive to superovulation and were not subjected to embryo collection.

The superovulated donors, previously deprived of food and water for 24h, were anesthetized with xylazine (0.10mg/kg IM; Rompun®, xylazine, Bayer, Brazil) and ketamine hydrochloride (3.5mg/kg intravenously; Ketamine, Agener, Brazil). In addition, local anesthesia was administered through a surgical incision (10mL of lidocaine; Lidovet®, Bravet, Brazil). The oocytes / embryos were collected surgically after ventral laparotomy, through a paramedian incision (5cm long) cranial to the udder, to access the reproductive tract.

Each uterine horn was washed with 60mL of embryo recovery medium (DPBS, Cultilab, Brazil), preheated to 37ºC, and supplemented with 1% fetal bovine serum (Cultilab, Brazil). The embryos were recovered in a Petri dish, kept in a holding medium (Holding plus, 0.4% BSA, Embriocare, Cultilab, Brazil), and examined under a stereomicroscope (Olympus SZ; Olympus Optical Co., Ltd., Tokyo, Japan), following the criteria of the International Embryo Transfer Society (Stringfellow, 1998). Embryos that developed up to the morula or blastocyst stages were classified as follows: grade 1 (excellent or good), 2 (good / fair), 3 (poor), and 4 (dead or degenerate). Embryos classified as grade 1 to 3

were considered viable and grade 1 to 2 were considered freezeable.

Statistical analysis was performed using the System for Statistical Analysis (SAEG; Version 9.1: Fundação Arthur Bernardes - UFV - Viçosa, 2007) program, with the purpose of applying parametric and non-parametric tests to analyze the results, with differences considered significant when $P \leq 0.05$. The Lilliefors test was used to assess normality and the Cochran test was used to assess homoscedasticity. ANOVA was used for the results of follicular evaluation during metestrus, the number of corpus luteum, total structures and recovery rate and their means compared by the Tukey test. Embryo parameters, viable, freezing, degenerate embryos and PLR (premature corpus luteum regression) did not show normal distribution and were evaluated by the Kruskal-Wallis test. In addition, Spearman correlations were performed between the parameters of ovarian evaluation, superovulatory response and embryo production.

RESULTS

The results showed a superovulatory response (ε 3 corpus luteum in both ovaries) in 82.61% (19/23) of Brazilian Bergamasca sheep submitted to the SOV protocol, presenting an average recovery rate of 52.10% (113 structures collected / 217 corpus luteum), where 46.20% of these structures were viable embryos, reaching an average of 2.26 ± 2.95 embryos per sheep collected. Follicular evaluations during metestrus (Figure 1) showed that the majority of sheep, 53% (12/23), fell within the average range of follicles (between 10 and 18 follicles). The number of follicles during metestrus differed ($P < 0.05$) between groups with low, medium and high follicular population. However, the variables that quantified the superovulatory response and embryo production were similar ($P > 0.05$) between groups (Table 1).

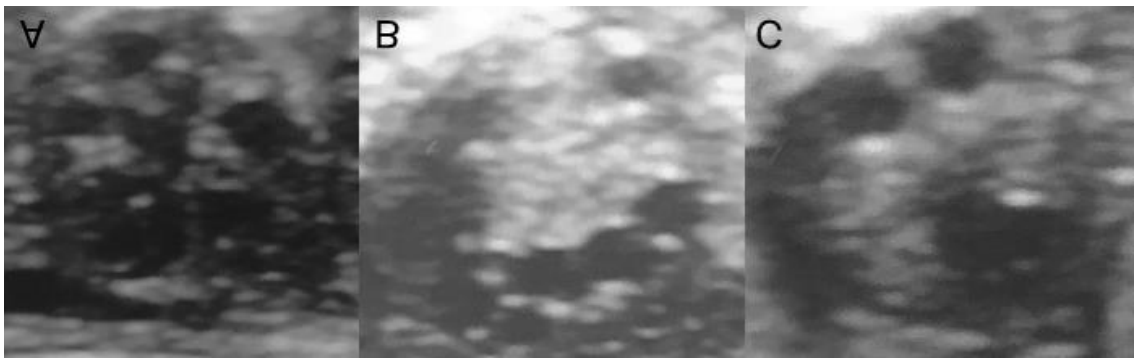

Figure 1. Ovaries of Brazilian Bergamasca sheep with high (A), medium (B) and low (C) follicular population during metestrus phase.

Table 1. Ovarian response and embryo production (mean ± standard deviation) of Brazilian Bergamasca sheep with high (G1), medium (G2) and low (G3) follicular population during metestrus phase, submitted to the superovulation protocol

	G3 (n=5)	G2 (n=9)	G1 (n=5)
Follicles in metestrus	20.4±2.3^{A}	14.8±1.5^{B}	9.6±0.5^{C}
Follicles in superovulation	11.0±7.1	12.7±3.5	11.6±5.8
Corpus luteum	13.8±7.1	10.7±6.2	9.0±3.3
PLR	12.8±8.8	4.9±6.2	2.8±6.3
Total structures	6.2±6.7	5.4±4.9	6.6±1.3
Viable embryos	1.8±4.0	2.6±2.0	4.0±3.8
Freezable embryos	1.6±3.6	2.0±1.8	3.0±2.8
Recovery rate (%)	38.4±35.7	50.9±35.3	77.4±16.4

$^{A, B, C}$ Different letters on the same line indicate significant differences (Anova, Tukey; $P < 0.05$). Premature corpus luteum regression (PLR).

There were positive correlations (P<0.05) between the number of follicles during metestrus and the number of corpus luteum with premature regression; between the number of corpus luteum and total structures recovered; between the number of corpus luteum and PLR; between the rate of recovery and the number of total structures and viable embryos; between degenerate embryos and number of corpus luteum and total structures recovered. There was a negative correlation (P<0.05) between the rate of recovery and premature regression of the corpus luteum (Table 2).

When the animals were divided into those who did and did not undergo premature regression of the corpus luteum, the animals that presented PLR had a greater number of follicles during metestrus and a lower recovery rate (P <0.05). The number of follicles at the beginning of superovulation, corpus luteum, total structures, viable embryos and freezing embryos were similar in sheep with and without PLR (P> 0.05) (Table 3).

Table 2. Pearson's correlation (r-value) between the follicular population and embryo production of Brazilian Bergamasca sheep submitted to superovulation protocol

	METFOL	SOVFOL	CL	TOTEST	REC	FEMB	CEMB	DEG	PLR
METFOL	1	0.13	0.26	-0.14	-0.29	-0.19	-0.18	0.34	0.52*
SOVFOL		1	0.11	-0.01	-0.04	-0.03	0.03	0.31	0.00
CL			1	0.53*	-0.13	0.26	0.25	0.41*	0.66**
TOTEST				1	0.66**	0.49*	0.46*	0.42*	0.03
REC					1	0.43*	0.42*	0.09	-0.44*
VEMB						1	0.98	-0.08	0.15
FEMB							1	-0.08	0.16
DEG								1	0.37
PLR									1

Asterisks indicate significant correlations (Pearson's correlation; * P <0.05; ** P <0.01). Evaluation of follicles during metestrus (METFOL) and on the first day of superovulation (SOVFOL). Corpus luteum (CL). Total structures (TEST). Recovery rate (REC). Viable (VEMB), freezable (FEMB) and degenerate (DEG) embryos. Premature corpus luteum regression (PLR).

Table 3. Ovarian response and embryo production (mean ± standard deviation) of Brazilian Bergamasca sheep with and without early corpus luteum regression (PLR) submitted to superovulation protocol

	With PLR (n=9)	Without PLR (n=10)
Follicles during metestrus	12.7±3.2B	16.9±7.8A
Follicles in SOV	11.6±4.1	12.3±3.5
Corpus luteum	8.7±6.0	13.2±5.0
Total structures	6.2±4.6	5.7±4.9
Viable embryos	2.6±3.1	2.9±3.2
Freezable embryos	2.0±2.4	2.3±2.7
Recovery rate (%)	72.2±29.9A	38.8±29.3B’

[A, B] Different letters on the same line indicate significant differences (Teste t; P <0.05).

DISCUSSION

In the present experiment, a superovulatory response and embryo production of the locally adapted Brazilian Bergamasca sheep was evaluated and correlated with the donor follicular condition before the synchronization and superovulation protocol was initiated. The results achieved can be used as a reference for the breed since no studies were found in the literature that describe the embryo production in Brazilian Bergamasca sheep. The superovulatory response can be considered satisfactory since 82.61% of the sheep responded to treatment with three or more corpus luteum, which is similar to that found by Menchaca *et al*. (2009) in Merinos sheep, Neves *et al*. (2010) and Brasil *et al*. (2016a) in Santa Inês and Brasil *et al*. (2016b) in Morada Nova sheep. There are studies where 20 to 40% of the sheep did not respond to the treatment (D’Alessandro *et al*., 1996; Cordeiro *et al*., 2003). These results show the potential of Brazilian Bergamasca sheep in the use of assisted reproduction tools such as superovulation and embryo collection to support animal conservation and breeding programs.

The overall recovery rate for this work was 52.10%, below that achieved in other studies that

used surgical methods for embryo collection, such as the 63.8% achieved by Lima *et al*., (2012) and the 66% achieved by Brasil (2016) in Santa Inês sheep. Our results suggest that the average of 2.3 embryos collected per sheep can be improved by at least 20% by increasing the recovery rate to levels reached in other studies, enabling its use in conservation and animal breeding programs.

According to Bartlewski *et al*. (2016) the variability in the superovulatory response is still considered critical and is associated with intrinsic (race, follicular population) and extrinsic factors (hormone, dose and protocol, insemination method and interval between treatments) (Amiridis and Cseh, 2012). Although lately the control of extrinsic factors has been greater, the variation in the superovulatory response is still one of the main challenges to overcome, suggesting that intrinsic factors are primarily responsible for the variability (Brasil, 2016), including the follicular development of donors.

Some studies have suggested that the superovulatory response and embryo production is positively correlated with ovarian follicular population (number of follicles ≥2 mm) during metestrus phase (Brasil., 2016) and the beginning of the superovulatory treatment (first application of FSH) (Gonzalez-Bulnes *et al*., 2002; Mossa *et al*., 2007). However, there are studies that have not verified this correlation (Bruno-Galarraga *et al*., 2014).

The results of this study showed that a simple ultrasound analysis for quantifying the population of follicles larger than 2 mm at two different times (metestrus and onset of superovulation) was not efficient in predicting the ability to respond to superovulation and select Brazilian Bergamasca sheep as donors with high potential for embryo production in vivo. A positive correlation was observed only between the follicular population during metestrus and the number of corpus luteum with premature regression and there was no correlation between the number of follicles at the beginning of the superovulatory treatment with variables related to the superovulatory response and embryo production. Furthermore, when the animals were divided into groups with high, medium and low follicular population during metestrus, the results of the superovulatory response and embryo production confirmed the low correlations observed, showing no influence of the follicular population on them.

However, other methodologies are being studied to select sheep with greater potential for embryo production in vivo, suggesting an alternative for future studies with Brazilian Bergamasca in order to achieve more satisfactory results. Pinto *et al*. (2018) demonstrated in Santa Inês sheep that the plasma dosage of anti-Miller hormone can be an efficient alternative in selecting donors with high embryo recovery rates after a superovulation protocol. The premature regression of the corpus luteum is considered a problem inherent to the in vivo production of embryos of the sheep species, occurring between 20 and 40%, reaching up to 75%, of sheep subjected to superovulation, as demonstrated by Lopes Júnior *et al*. (2006) and Oliveira *et al*. (2013), resulting in the recovery of low-quality embryos or even no recovery (Schiewe *et al*., 1991; Cognie, 1999).

In our study 57.9% of the sheep presented premature regression of the corpus luteum, with 31.5% of them producing viable embryos. There was a negative correlation between premature regression of the corpus luteum and the rate of embryo recovery, which interfered with the average number of embryos recovered (2.9 ± 3.2). In addition, the number of regressed corpus luteum had a positive correlation with the number of follicles during metestrus, reducing the average number of viable embryos in ewes with a larger follicular population as compared to those with a lower number of follicles during metestrus (1.8 ± 4.0 vs 4.0 ± 3.8, respectively, $P>0.05$). The high proportion of sheep with premature regression of the corpus luteum must have contributed significantly to the fact that the animals with the largest follicular population during metestrus did not demonstrate better embryo production.

In an attempt to circumvent the negative effects of premature regression of the corpus luteum on embryo production, all embryo donor sheep had the progesterone device (CIDR) reintroduced after NM, as proposed by Fonseca (2005). This strategy allowed 1/3 of the sheep that presented premature regression of the corpus luteum to produce viable embryos. However, the low embryo recovery rate achieved in these animals suggest that cost-benefit assessment should be carried out before adopting this practice.

The set of results achieved in this study shows the potential of using superovulation and embryo collection programs to promote the conservation and improvement of the Brazilian Bergamasca breed. Furthermore, the results of superovulatory response and embryo production can be used as a reference for the breed. Further studies are needed to increase the efficiency of embryo collection programs, which consequently would reduce the cost of embryo production and make it viable to use in conservation and breeding programs.

CONCLUSION

The quantification of the population of antral follicles during metestrus of Brazilian Bergamasca sheep should not be used as a tool for selecting donors with high potential for embryo production in vivo.

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