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Does supplemental LH changes rate and time to ovulation and embryo yield in Santa Ines ewes treated for superovulation with FSH plus eCG?

Suplementação com LH modifica a taxa e momento da ovulação e a produção de embriões em ovelhas Santa Inês superovuladas com FSH e eCG?

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ABSTRACT

The objective was to evaluate if supplemental LH given at the end of FSH treatment would synchronize the time of ovulation and increase the ovulation rate and embryo yield in Santa Ines ewes. Twenty superovulatory (SOV) programs were accomplished in cross-over design (60d interval). On D0, a CIDR device was inserted, and the device was replaced with a new one 7 days later, when 37.5µg of d-cloprostenol was administered. On D12, we started the SOV treatment, administering 256mg of pFSH 8 times, 12h apart. On D14, the CIDR was removed, and 200IU of eCG and 37.5µg of d-cloprostenol were administered. On D15, the ewes were allocated into one of two groups, a Control group (n=10) that received no supplemental LH and a LH group (n=10) treated with 7.5mg of LH 24h after CIDR removal. Artificial inseminations (AI) were performed 42 and 48h after CIDR removal. The ovarian structures were evaluated by laparoscopy immediately before each AI and 5 days later (D21) when the embryos were collected. The LH ewes ovulated more frequently (P=0.05) before 42h than between 42 and 48h. Treatment with LH tended to increase the frequency of CL and to decrease the anovulatory follicles (P=0.08). The supplemental LH increased the frequency of ewes with a high SOV response (≥ 11 CL; P=0.05). In conclusion, supplemental LH increased the frequency of ewes with high SOV response and ovulating prior to 42h, however, there was no synchrony between ovulations. The supplemental LH also decreased the frequency of anovulatory follicles, although the ovulation rate and embryo yield were unaffected.

Key words: *superovulation, LH, ovulation, embryo, sheep.*

RESUMO

O objetivo deste trabalho foi avaliar se a suplementação com LH ao final do tratamento gonadotrófico sincroniza o tempo das ovulações e incrementa a taxa de ovulação e produção de embriões em ovelhas Santa Inês. Vinte programas de superovulação (SOV) foram realizados em delineamento cross-over (intervalo de 60 dias). No D0, um CIDR foi inserido, sendo trocado por um novo sete dias após, quando 37,5µg de d-cloprostenol foram administradas. No D12, iniciou-se o tratamento com 256mg de pFSH em 8 administrações (12/12h). No D14, o CIDR foi retirado, 200UI de eCG e 37,5µg de d-cloprostenol foram administradas. No D15, as ovelhas foram alocadas em um dos dois grupos: Controle (n=10), sem suplementação com LH, e LH (n=10), tratado com 7,5mg de LH, 24h após a remoção do CIDR. Inseminações artificiais (IA) foram realizadas 42 e 48h após a remoção do CIDR. As estruturas ovarianas foram avaliadas por laparoscopia imediatamente antes de cada IA e 5 dias após, quando os embriões foram colhidos. As ovelhas que receberam o LH tiveram maior frequência de ovulações antes de 42h (P=0,05). O tratamento com LH tendeu em incrementar a frequência de CL e diminuir a de folículos anovulatórios (P=0,08). A suplementação com LH incrementou (P=0,05) a frequência de ovelhas com alta resposta superovulatória (≥ 11 CL; P=0,05). Em conclusão, a suplementação com LH incrementou a frequência de ovelhas com alta resposta e ovulações antes de 42h depois da remoção do CIDR, entretanto, não houve sincronia entre as ovulações. A suplementação diminuiu a frequência de folículos anovulatórios, embora a taxa de ovulação e a produção de embriões permaneceram inalteradas.

Palavras-chave: *superovulação, LH, ovulação, embriões, ovinos.*

INTRODUCTION

Small ruminants make an important contribution to the Brazilian economy. The Brazilian

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sheep flock includes more than 16 million animals, mainly (~65%) of the Santa Ines breed (IBGE, 2008). Multiple ovulation and embryo transfer (MOET) is a reproductive technology that facilitates genetic improvement, and its commercial application in Brazilian Santa Ines sheep has increased dramatically in recent years.

High individual variability in the superovulatory (SOV) response and the transferable embryo yield is common in ewes and compromises the success of MOET programs (MENCHACA et al., 2010). The high variability of responses is notably the main challenge to increase the efficiency of MOET programs in sheep (OLIVEIRA, 2011). Specifically, the yields are decreased by the lack of or reduction in ovulatory response after stimulation with an exogenous hormone supply. This occurs in approximately 20-30% of the ewes (GONZALEZ-BULNES et al., 2000). A possible cause may a deficient or inexistent preovulatory LH surge (GONZALEZ-BULNES et al., 2003) or the presence of non-responsive follicles due to a down-regulation of the granulosa and theca LH receptors (LOPEZ-DIAZ & BOSU, 1992). COGNIÉ et al. (1986) suggested that the administration of LH at the end of SOV treatment with exogenous FSH might increase the ovulation rate and the number of recovered embryos. Very few studies (PICAZO et al., 1996; D'ALESSANDRO et al., 2005) on MOET have focused on increasing the ovulation rate and the number of viable embryos per donor by modifying the FSH/LH ratio at the end of an FSH treatment. The effects of this procedure are still unclear and may differ between different genotypes (COGNIÉ et al., 1986; PICAZO et al., 1996). The literature shows that breed differences in follicle growth, ovulation rate and fluctuations of FSH and LH lead to differential responses to exogenous gonadotrophins (AMMOUN et al., 2006; SIMONETTI et al., 2008), emphasizing the need for specific treatments for each breed. The breed factor accounted for approximately 30% of the variability in the embryo yields obtained in response to FSH treatments (VIVANCO et al., 1994). For these reasons, it is not possible to predict changes in the ovulation rate and embryo yield related to modifying the FSH/LH ratio in the SOV protocol for Santa Ines ewes.

Additionally, supplemental LH in SOV protocols that employ a combination of FSH plus eCG has not yet been studied. The use of moderate dose of eCG in SOV treatment is very common in sheep and based on the function of final maturation of follicles (MENCHACA et al., 2010). The long half-life of eCG contributes for modification of ratio FSH:LH and complete the final pre-ovulation growth of follicles, however, it is believed that it is not able to directly induce a preovulatory LH peak. Thus, the objective of this study was to evaluate whether supplemental LH given at the end of the FSH treatment changes the time to ovulation (synchronous ovulation) and increases the ovulation rate and embryo yield in Santa Ines ewes.

MATERIAL AND METHODS

The study was conducted at a commercial farm in southwestern Brazil from May through November 2007. Ten non-pregnant pluriparous Santa Ines ewes with a body condition score of 3.95±0.32 (1 to 5 point scale; RUSSEL et al., 1969) and 60.3±10.74kg of body weight were used as embryo donors. The animals were maintained on Coastcross (***Cynodon*** sp.) pasture with free access to mineralized salt and water. To accomplish a crossover design study, all of the ewes were subjected to two SOV protocols over a 60-day interval, totaling twenty MOET programs. The control SOV protocol used follow the traditional pattern of commercial use in Brazil (GUSMÃO, 2006). On a random day of the estrous cycle (D0), the ewes received a progesterone-releasing (P4) intravaginal device (CIDR®; Pfizer-New Zealand) that was replaced with a new one 7 days later, totaling 14 days of treatment. On D7, 37.5µg of d-cloprostenol (Prolise®, Arsa-Argentina) i.m. were administered. The SOV treatment started 48h (D12) before the CIDR removal and consisted of 256mg of pFSH (Folltropin®, Bioniche-Canada; ratio FSH:LH of 5.25:1) i.m., administered in 4 decreasing doses, with each dose repeated once after 12h (48, 48, 36, 36, 24, 24, 20 and 20mg of pFSH) for a total of 8 administrations. The P4 device was removed at the time of the sixth pFSH administration. At this time (D14), the ewes received 200IU of eCG (Novormon®, Syntex-Argentina) and 37.5µg of d-cloprostenol i.m. On D15, the ewes were assigned to one of the groups (control: without LH; LH: with LH). The Control sheep (n=10) received no additional treatment, whereas the LH ewes (n=10) were treated with 7.5mg of LH (Lutropin®, Bioniche-Canada; ratio FSH:LH constantly low) 24h after CIDR removal (D15). Fixed-time artificial inseminations (FTAI) using frozen-thawed semen were performed 42 and 48h after CIDR removal.

The protocols for SOV used avoid the need for estrus detection (FTAI). However, the observation of the onset of estrus (i.e., concentration or dispersion among animals) was used as an auxiliary tool to evaluate the ewes response to the treatment. At the end of the P4 treatment, the estrus detection was accomplished using teasers (vasectomized rams) following the proportion of the 1:4 donors. The pectoral regions of the males were painted with colored grease, and they were kept with the ewes for two days. When the females were mounted, the ewes were color-tagged with the grease. Identification of the tagged females

was done three times daily (6:00, 12:00 and 18:00h), during the two days (D15 and D16).

Intrauterine AI was performed by laparoscopy (Karl Storz, Alemanha). A single batch, 0.25mL straw of frozen-thawed semen (sperm concentration approximately $100x10^6$/palette) from a single ram was used per uterine horn in each AI. Previous evaluation of the sperm for physical and morphological parameters had been conducted to assure the semen quality. The laparoscopies were performed under sedation and local anesthesia at local abdominal puncture points. Concurrently with the AI and embryo collection procedures (42h, 48h, and 7 days after CIDR removal, respectively), the ovarian structures were evaluated by laparoscopy. In the first two evaluations, the number of large follicles (LF), preovulatory follicles (POF), follicles that had undergone ovulation (ovulated, OF; visualization of a hemorrhagic site), and corpus luteum (CL) were determined. The LF and POF differ by size (approximately 4-5.5mm and $\geq$6mm of diameter, respectively). At the last assessment, the presence and the number of anovulatory follicles (AF) and CL were registered and the occurrence of ovulation was observed. The AF were follicles that reached the preovulatory size and were still present on the ovaries at this evaluation (i.e., no ovulation) according to HEIDARI et al. (2010). Ovulation was considered to have occurred when a LF or POF that had been previously observed was no longer present, and a CL had appeared on the same ovary where these follicles had been detected during the previous laparoscopy.

Seven days after the CIDR removal, the ova/embryos were collected by laparotomy under anesthesia. Each uterine horn was flushed with 60mL of flushing media (DPBS®, Cultilab, Brazil) at 37ºC. Briefly, the flushing media was injected via a 20G catheter inserted at the proximal portion of the uterine horn and collected via a n.8 Foley catheter that had been inserted at the external bifurcation of each uterine horn. The recovered structures were classified according to the degree of development, and the embryos were maintained in holding media (Holding plus®, Cultilab, Brazil). Morphological evaluation was performed under a stereomicroscope (40X magnification) following the IETS recommendations. The SOV response was classified using three scores (adapted from CORDEIRO et al., 2003) as follows: (0) ewes with 4 or less CL, characterizing a lack of response to the SOV treatment; (1) ewes with 5 to 10CL, characterizing intermediate SOV response; and (2) ewes with 11 or more CL, characterizing high response to the SOV treatment. According to the time to ovulation, the ewes were classified as: (i) ewes that ovulated before 42h, (ii) those that ovulated between 42 and 48h, and (iii) ewes that ovulated 48h after CIDR removal.

The data analysis was performed using the SAS v 9.2 (SAS/STAT Institute Inc., Cary, NC, USA). Tests for normality of residuals and homogeneity of variances were conducted for each variable. All dependent variables related to estrus (i.e., the onset of estrus), ovarian structures (i.e., the number of LF, POF, OF AF and CL), time to ovulation (i.e., the frequency of ewes that ovulated before 42h, between 42h and 48h, and after 48h after CIDR removal), SOV response (i.e. the ovulation rate, the number of CL, the ovulation failure rate, and the number of AF) and embryo yield (i.e., the number and rate of the structures recovered, viable embryos, unfertilized ova and degenerated embryos) were analyzed by ANOVA using the GLM procedure and compared using a Tukey test. The values are presented as mean±S.E.M. The statistical significance was accepted when $P \leq 0.05$ and was defined as tendency when $P>0.05$ and <0.10. A Pearson correlation analysis was realized between the ovulation time and the onset of estrus.

RESULTS

The data on each ovarian structure evaluated by laparoscopy 42h, 48h and seven days after CIDR removal are shown in table 1. The total number of ovulatory follicles was greater in the LH group than in the Control group (P=0.02). Regarding the follicular status in each evaluation time point, the frequency of LF at 42h after CIDR removal was greater in the Control ewes, whereas in ewes of LH group, POF and OF were more frequent (P<0.001). The mean number of LF per ewe was also greater in the Control ewes, whereas the mean number of POF was greater in LH ewes (P<0.001). Overall, a higher percentage of follicles in an advanced stage of development was observed in LH ewes than in the Control ewes.

As for the evaluation performed 48h after CIDR removal, the follicular stages of development were more homogenously distributed among the groups compared to the first evaluation. However, the frequency of LF was still greater in the Control ewes than in the LH ewes (P<0.001), and the frequency of POF (P=0.0003) and OF (P=0.02) were greater in the LH ewes than in the Control ewes, as described in the previous evaluation time point.

The supplemental LH tended to increase the frequency of CL seven days after CIDR removal and to reduce the frequency of AF in the LH ewes (P=0.08). However, a similar mean number of AF and CL per ewe was found in both groups 7 days after CIDR removal. Considering the ovulatory response, similar rates of

Table 1 - Mean±SEM and percentage of large follicles (LF), preovulatory follicles (PO), follicles that had undergone ovulation (ovulated) and corpus luteum (CL) 42 and 48 hours after the removal of the device from Santa Ines ewes subjected to a superovulation program with (LH group) or without (control group) supplemental LH.

Time	Item	------Control group (n=10 animals; 135 follicles)------		------LH group (n=10 animals; 158 follicles)------	
		% (n)	Mean	% (n)	Mean
42h	Follicles/animal		13.5±3.4^{a}		15.8±5.2^{a}
	LF (4 – 5.9 mm)	63.0%^{A} (85/135)	4.2±3.1^{C}	25.9%^{B} (41/158)	2.1±2.8^{D}
	PO (= 6 mm)	17.8%^{A} (24/135)	1.2±2.3^{C}	40.5%^{B} (64/158)	3.2±2.8^{D}
	Ovulated	17.8%^{A} (24/135)	1.2±2.3	31.6%^{B} (50/158)	2.5±3.2
	Corpus Luteum	1.5% (2/135)	0.1±0.4	1.9% (3/158)	0.2±0.4
48h	LF (4 – 5.9 mm)	54.1%^{A} (73/135)	3.7±2.7^{C}	25.9%^{B} (41/158)	2.1±2.8^{D}
	PO (= 6 mm)	12.6%^{A} (17/135)	0.9 ±1.0	30.4%^{B} (48/158)	2.4±2.5
	Ovulated	14.1%^{A} (19/135)	1.0±1.6	24.7%^{B} (39/158)	2.0±2.2
	Corpus Luteum	19.3% (26/135)	1.3±2.4	19.0% (30/158)	1.5±3.1
7d	Anov. Follicles	22.2%^{a} (30/135)	3.0±1.0	14.6%^{b} (23/158)	2.3±0.5
	Corpus Luteum	77.8%^{a} (105/135)	10.5±1.2	85.4%^{b} (135/158)	13.5±1.5

Different letters on the same row indicate differences at P<0.10 [tendency; a ≠ b (comparing the percentages)] or P<0.05 [A ≠ B (comparing the percentages) and C ≠ D (comparing the means)].

ovulation (P=0.63) and ovulation failures (P=0.48) were detected between the groups (Table 2). When the SOV response was evaluated, none of the ewes were classified as score 0 (4 or less CL). The LH ewes were mainly (70%) classified as score 2 (≥11CL), and the remaining (30%) as score 1 (between 5 and 10CL), whereas half of the Control ewes were classified as score 2 and half as score 1. The supplemental LH produced a greater frequency of ewes with a high SOV response (P=0.05).

As for time to ovulation, 40% of the Control ewes ovulated 48h, whereas 30% ovulated before 42h and 30% between 42h and 48h after the CIDR withdrawal. For LH group, 60% of the ewes ovulated earlier (i.e., before 42h), whereas 10% ovulated between 42h and 48h, and 30% ovulated 48h after CIDR removal. However, because large individual variation observed among animals in the same group, there was no difference between groups (P=0.86). When statistical analysis was performed in each group, the frequency of LH ewes that ovulated before 42h was higher (P=0.05) than those that ovulated between 42h and 48h.

All of the ewes exhibited estrus over the 2 days immediately after CIDR removal. The onset of estrus was 25.2±6.2h after CIDR removal in the Control ewes and 28.8±12.6h in the LH ewes (P=0.7). Additionally, a low correlation (P>0.001, r=0.25) was found between the onset of estrus and the time to ovulation. The indexes of embryo yield showed similar patterns between groups (P>0.05, Table 2). However, the number of unfertilized ova tended (P=0.09) to be greater in LH group than in the Control. The minimum and maximun values shown in table 2 mark the high variability of superovulatory response and embryo yield, in both groups.

DISCUSSION

In the current study, the first laparoscopic evaluation showed that the ovarian follicles of LH group were classified as more advanced stages of development than Control. These data suggest that the supplemental LH accelerated the development and the maturation of the follicles, which is supported by the anticipation of ovulation observed in this group. In a previous study, the interval between sponge removal and the preovulatory LH surge was 37.2±0.7h (ranging from 24 to 48h), whereas the mean time for the onset of ovulation was 65.4±0.7h (ranging from 47 to 79h) in ewes subjected to SOV using only FSH (VEIGA-LOPEZ et al., 2008). According to SIMONETTI et al. (2008), the preovulatory LH peak occurs approximately 47.4±1.9h after sponge removal when FSH is administered during four days without the use of eCG. The same authors reported the occurrence of a delayed preovulatory LH surge when eCG was not administrated. In the present study, the preovulatory LH surge probably occurred earlier than described by these authors, as the OF and CL were observed 42h after CIDR removal, regardless of the group. An anticipated time to ovulation was found in this study compared to previous works (SIMONETTI et al., 2008;

Table 2 - Indexes of the superovulatory response and embryo yield in Santa Ines ewes subjected to a superovulation protocol with (LH group) or without (Control group) LH supplementation.

Variables	Control group			LH group			P
	Mean	Values		Mean	Values		
		Min	Max		Min	Max	
No. of MOET	10			10			
No. of CL/flushed donor	10.5 ± 1.2	5	15	13.5 ± 1.5	7	20	0.80
Ovulation rate (%)	78.7 ± 7.2	41.7	100	84.8 ± 3.1	70	100	0.63
No. of anovulatory follicles	3.0 ± 1.0	0	8	2.3 ± 0.5	0	5	0.51
Ovulation failure rate (%)	3.4 ± 1.2	0	8.8	2.5 ± 0.6	0	5.3	0.48
No. of structures recovered/flushed per donor	6.1 ± 1.5	1	14	8.4 ± 1.7	2	15	0.98
Recovery rate (%)	51.7 ± 10.2	7.1	93.3	59.2 ± 7.8	28.6	88.2	0.92
No. of viable embryos	3.8 ± 1.4	0	13	4.2 ± 1.7	0	15	0.95
Viability rate (%)	64.2 ± 14.5	0	100	39.0 ± 10.9	0	73.3	0.36
No. of unfertilized ova	1.7 ± 1.1	0	9	2.0 ± 1.0	0	9	0.09
Unfertilization rate (%)	18.3 ± 12.6	0	90	32.0 ± 13.5	0	100	0.34
No. of degenerated embryos	0.7 ± 0.2	0	2	2.2 ± 0.6	0	5	0.82
Degeneration rate (%)	17.4 ± 10.1	0	100	26.1 ± 8.4	0	83.3	0.79

VEIGA-LOPEZ et al., 2008). This event may be related to the use of the association of FSH and eCG. Independently there was also anticipation of ovulation in response to supplementation of LH. Shown by the higher frequency of LH ewes that ovulated before 42h than between 42h and 48h. The onset of estrus also occurred earlier than reported by SIMONETTI et al. (2008; 40±2.0h) and CORDEIRO et al. (2003; 36±2.7h), highlighting the probable effect of eCG on anticipating estrus and ovulation. Thus, it is reasonable to assume that the timing of the FTAI should be adjusted depending on whether eCG is administered. Ewes subjected to MOET programs using FTAI that receive eCG may need to be inseminated earlier than those not receiving the eCG. Another factor that may have influenced the anticipation of the onset of estrus is the continued exposure of the ewes to the teasers during the period of estrus observation. When in continued contact with ewes, the male has a complete opportunity to express its full sexual behavior, including courtship and mating (ROMANO et al., 2001). Similar consideration should be taken when using hormones to induce ovulation (i.e., GnRH and LH). In previous studies, the use of GnRH after sponge removal concentrated the time to ovulation and increased the ovulation rate in ewes subjected to SOV (AKINLOSOTU & WILDER, 1993). However, in the current study, the use of LH was insufficient to concentrate the time to ovulation of the follicles from the same ewe and among the ewes, but it also seems to anticipate ovulation. Considering that the best results in fertility are determined by the relationship between the time to ovulation and the AI (LOPÉZ SEBASTIÁN et al., 2006). As the ewes received the FTAI at the same time in both treatments, the anticipation of the time to ovulation may be responsible for the higher number of unfertilized ova (P=0.09) observed in LH group, suggesting the need to adjust the time to AI when LH is given.

Overall, the ewes' response to the SOV treatment was satisfactory (4 or more CL) in all MOET programs. However, the LH treatment improved the frequency of ewes with a high SOV response (11 or more CL). The supplementation of LH also tended to increase the frequency of CL seven days after CIDR removal and to decrease the frequency of AF, but similar rates and numbers of ovulation and anovulatory failure were achieved. When other breeds of sheep (Manchega, Churra and Merino) were treated with LH at the end of the SOV treatment, the ovulation rate was unaltered (PICAZO et al., 1996). Additional, is important to emphasize that the number of ovulations in Santa Ines ewes was higher than in other breeds mentioned (5.2, 7.0 and 5.9 for Manchega, Churra and Merino, respectively). This fact, among others already reported, confirming the variation between breeds. However, in another study, decreasing the FSH dosage, combined with a higher LH dosage (FSH/LH ratio of 1.6 vs. 1.0, 0.6 and 0.3), increased the ovulation rate in dairy ewes in comparison to the constant FSH/LH ratio (D'ALESSANDRO et al., 2005). Thus, the importance of changing the FSH/LH ratio in SOV protocol and its effects on the ovulation rate are still controversial and vary according to the breed. In the present study, there was high individual variability in the SOV response, which may be partially responsible for the lack of improvement in the ovulation rate and the embryo yield in LH group. These results discredit our hypothesis that supplemental LH could improve the ovulation rate and reduce the variability responses among the ewes.

In conclusion, the supplementation with LH during protocols for SOV with FSH plus eCG increased the frequency of Santa Ines ewes showing a high SOV response and ovulating prior to 42h after

CIDR removal, however, there was no synchrony between ovulation. It also decreased the frequency of AF, but the ovulation rate and the embryo yield were unaffected.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The (protocol number 21131).

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