Reproductive performance and luteal function of Santa Ines ewes inseminated by cervical retraction with fresh or frozen semen

Desempenho reprodutivo e função luteal de ovelhas Santa Inês inseminadas por retração cervical com sêmen fresco ou congelado

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RESUMO

Avaliou-se a inseminação artificial por retração cervical usando sêmen fresco ou congelado disponível comercialmente. Ovelhas Santa Inês foram divididas em cinco grupos. A monta natural (MN) correspondeu ao grupo controle. Quatro grupos foram submetidos a um tratamento hormonal e inseminação artificial por retração cervical (CRI) ou laparoscopia (LAI) usando sêmen fresco ou congelado. Para a CRI, ovelhas foram mantidas em estação. O tempo para penetrar o canal cervical e realizar a CRI foi mensurado, bem como o local de deposição do sêmen e intensidade de reação da ovelha (fraca, moderada, forte). Amostras de sangue foram coletadas para dosagem de progesterona aos dias 0, 3, 5, 12 e 17 (dia 0 = inseminação ou monta). Foram avaliadas as taxas de não retorno ao estro (NRE), gestação (ao dia 35) e fertilidade (nascimento / ovelhas acasaladas). O tempo médio para penetração cervical foi de $52,8 \pm 21,2$s e para a CRI foi de 3:26min $\pm$ 47s. A intensidade de reação à CRI foi fraca ou moderada em 92,3% das ovelhas. Os níveis séricos de progesterona após CRI, LAI e MN foram similares. NRE das ovelhas inseminadas foi similar, exceto em ovelhas com CRI e sêmen congelado, que foi inferior (P<0,05). As taxas de fertilidade foram semelhantes entre CRI e LAI (35,4% e 42,2%, respectivamente). A inseminação artificial por retração cervical com ovelhas em estação se mostrou prática e não alterou o perfil de progesterona, possibilitando índices reprodutivos similares à laparoscopia. No entanto, o uso desta técnica com sêmen disponível comercialmente apresentou baixa taxa de fertilidade.

Palavras-chave: inseminação transcervical, laparoscopia, nível de progesterona, ovino, posição anti-estresse

SUMMARY

The artificial insemination (AI) by cervical retraction using fresh or commercially available frozen semen was evaluated. Santa Inês ewes (n=151) were assigned in five groups. Natural mating (NM) composed the control group. Four groups were submitted to hormonal treatment and timed insemination by cervical retraction (CRI) or laparoscopy (LAI), using fresh or frozen semen. To perform CRI, the ewes were kept in standing position. The time required to penetrate the cervical canal and to perform CRI were recorded; local deposition of semen and the reaction intensity of the ewe (weak, moderate or strong) were measured. Blood samples were collected to perform the progesterone dosage at days 0, 3, 5, 12 and 17 (day 0 = AI/NM). Non-return to estrus rate (NRE), pregnancy (at D_{35}) and fertility (birth / mated ewes) were evaluated. Mean time for cervical penetration and CRI were 52.8 ± 21.2s and 3:26min $\pm$ 47s, respectively. Reaction intensity to CRI was weak or moderate in 92.3% of the ewes. Serum levels of progesterone after CRI, LAI or NM were similar. NRE of the inseminated ewes were similar, except for CRI with frozen semen, which was lower (P<0.05). Fertility rates were similar between CRI and LAI (35.4% and 42.2%, respectively). Artificial insemination by cervical retraction with ewes in a standing position is practical, and does not change the progesterone profile, providing reproductive rates similar to laparoscopy. However, the use of this technique along with commercial frozen semen presents low fertility rate.

Keywords: anti-stress position, laparoscopy, progesterone level, sheep, transcervical insemination

INTRODUCTION

Productive characteristics of sheep farming, such as a short production cycle and exploitation in small areas, are required to meet the increasing demand for food. However, to accelerate production in commercial herds, it is Artificial insemination (AI) enables the dissemination of the best genetic material at the field level. The irregular layout of the cervical rings of ewes hinders the passage of conventional applicators, limiting the use of AI in sheep (Kershaw-Young et al., 2010; El Khalil et al., 2018). Use of vaginal and cervical AI with fresh semen results in acceptable fertilization rates, but the limited number of doses per ejaculate restrict the use of high-quality animals. Use of frozen sheep semen is commercially practical, helps to preserve superior ram and can be used in large-scale, but has been shown to result in low reproductive rates, since the freezing/thawing process compromises

necessary to increase genetic progress by using animals with reproductive characteristics that are suitable for the environment in which they will live. Therefore, studies with Santa Inês ewes are important due to the adaptive value of this sheep breed in different regions (McManus et al., 2010).

the motility and fertilizing ability of the spermatozoa (Masoudi et al., 2017). Laparoscopic AI (LAI) increases fertility rates because it enables intrauterine deposition of semen. However, this technique is difficult to apply commercially because of the high cost of the equipment, need for specialized professionals, and concern for the animals' well-being (Cardoso et al., 2009).

Halbert et al. (1990) developed a transcervical AI method with a curve-tipped probe and by restraining the ewe in a dorsal recumbency; this method is called the Guelph system of transcervical AI. Because this technique may cause lesions

on the cervix (Campbell et al., 1996), various adjustments to the transcervical method were investigated, as adjustments to conventional applicators include the use of cervical dilation drugs (Leethongdee et al., 2010; Álvarez et al., 2012). In addition, the reproductive rates obtained using transcervical AI with frozen semen are quite variable, ranging from 10% to 36% (Halbert et al., 1990; Richarson et al., 2012). Thus, our objective was to evaluate the feasibility of adapted AI by cervical retraction (CRI) in Santa Inês ewes kept in a standing position by using fresh or commercially available frozen semen.

MATERIAL AND METHODS

The experimental procedures were approved by the Ethics Committee on Animal Experimentation, State University of South-west Bahia (Protocol No. 68/2014).

The study was conducted at the "Estação Experimental Fazenda Almada", sheep sector, Municipality of Ilhéus-BA (14°47′20″S, 39°02′56″W), with a mean annual rainfall of 1,988 mm, temperature of 23.4 ± 1.5°C, and relative humidity of 85.4 ± 4.0%. A total of 151 multiparous Santa Inês ewes were used (age, 4–6 years; mean live weight, 52.2 ± 4.6 kg; and mean body condition score, 3.4 ± 0.4, scale 0–5). One Santa Inês ram and four vasectomized males were used. This flock was managed on grazing patches of *Brachiaria humidicola* from 8:00 AM to 4:00 PM; their diets were supplemented with elephant grass (*Pennisetum purpureum*) and corn- and soy-based concentrates (15% Crude Protein) at a mean quantity of 400 g/animal/day. Mineral salt (Guabiphos Ovinos AE®, Guabi, Brazil) and water were provided *ad libitum*. The ewes were randomly divided into five groups and synchronized using intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon®, Zoetis, Brazil) for a period of 12 days and 200 IU of eCG (Folligon®, MSD Saúde Animal, Brazil) at sponge withdrawal. For the four groups, two AI techniques, CRI or LAI, were combined with the use of fresh (Fr) or commercially obtained frozen (Fz) semen, i.e., CRIFr (n=25), CRIFz (n=40), LAIFr (n=24), LAIFz (n=40). Estrus response was identified using vasectomized rams at a male:female ratio of 1:5 twice a day, for 72 h after protocol. AI was performed 53 ± 1 h after eCG administration. The fifth group was the control group, which consisted of synchronized ewes subjected to natural mating in the subsequent natural estrous cycle (NM, n=22). The subsequent natural mating (NM) was performed by a ram of proven fertility, being one service per ewe in estrus.

For CRI, the ewes were kept in standing position by using a cattle crush with ventral supporting boards, and their hind limbs were immobilized with straps. After sanitizing the vulvar region, a 15-cm vaginal speculum and a light source were used to view the cervical os. Retraction of the cervix to the vulva opening and fastening were performed using two 25-cm-long Allis tweezers. Subsequently, a 12-cm metal applicator containing a mandrel (Aplicador Expansor Ovino®, Alta Genetics, Brazil) was used to traverse the cervical rings as much as possible; then, a 0.25-mL straw containing fresh or frozen semen was introduced. Considering possible stress of this contention, the reaction intensity of each ewe was assessed using the following classifications: "weak" (little or no movement, little or no bleating, and minimum reaction to the introduction of the speculum or applicator), "moderate" (some attempt to escape containment, with isolated and sporadic reactions to the introduction of the speculum or applicator), and "strong" (continuous attempts to escape containment, with an

immediate and frequent reaction to the introduction of the speculum or applicator).

For LAI, the ewes were subjected to a 24-h fasting period; after abdominal trichotomy and antisepsis, the ewes were secured onto a special gurney in the dorsal-oblique decubitus position at 60° with their caudal portion suspended. Local anesthesia was administered (2% lidocaine; Anestésico L®, Pearson, Brazil) at two abdominal points close to the uterine horns; after two small incisions, the puncture was made with trocars, and cannulas were inserted for laparoscopic access (Karl Storz®, Germany), for the handler to access the uterus, and for the semen applicator with a sheath needle. Once the uterine horns were located, insemination was performed and half a dose (0.25 mL) was deposited in each uterine horn. Both AI techniques were performed by the same insemination technician and support team.

Fresh semen samples were obtained from the same proved ram as NM; collection was performed using an artificial vagina, and the semen was immediately evaluated. Ejaculates that had at least 3×10^9 spermatozoa/ml, 70% spermatozoa with progressive motility and vigor 5 (1–5), were diluted using a solution of coconut water (50%), distilled water (25%), and 5% sodium citrate (25%) to obtain 200.10^6 spermatozoa/dose.

Frozen semen samples of Santa Inês ram were commercially obtained from one AI center (100.10^6 total spermatozoa/dose). Averages of progressive motility and vigor were 46.7% and 4 at thaw, 23.3% and 3.7 after thermo-resistance test (35ºC/3h), respectively (transcribed information from reports issued by AI center). For AI, the semen was thawed by immersing in water at 37ºC for 30 s. The rate of non-return to estrus was determined using vasectomized males (male:female ratio of 1:5) for a 21-day period, post-AI/NM (NRE), twice a day; pregnancy diagnosis or visualization of the embryonic vesicle with no embryo was performed using ultrasonography with an 8-MHz rectal transducer (Falco Vet 100, Pie Medical, Netherlands) 35 days after AI or NM. The fertility rate was determined at birth (births/inseminated or mated ewes).

To determine the progesterone (P_4) profile, blood samples were collected from 10 ewes in each group by venipuncture of the jugular vein with needles and vacuum blood-collection tubes without anticoagulant (BD Vacutainer®, England) on days 0, 3, 5, 12, and 17 relative to the day of AI (CRI or LAI treatments) or the day of estrus (NM treatment). The samples were centrifuged at $1500 \times g$ for 10 min, and the serum was stored in a freezer at –20°C. The hormone concentration was determined using Radioimmunoassay with duplicate samples at the Animal Reproduction Laboratory, Federal Fluminense University, Rio de Janeiro. For P_4 dosing, a IM1188 RIA kit (Beckman Coulter®, Immunotech, Czech Republic) was used, with 0.05 ng/mL sensitivity and intra-assay and inter-assay coefficients of variation of 6.5% and 7.2%, respectively.

Data of estrus response, semen deposition, reaction intensity of the ewes, non-return to estrus, pregnancy and fertility rates were compared using the chi-square test (GraphPad Prism, version 6, GraphPad Software, California, USA). The Student-Newman-Keuls test (PROC GLM, SAS version 9.1) was used to compare the mean (±SD) values for insemination time, cervix penetration time, progesterone serum level; differences were considered significant at P < 0.05.

RESULTS

The estrus response was similar for AI groups (P > 0.05), mean of 90.7%

(117/129) for estrus detection. Estrus between 36-48h after protocol occurred in 90.6% of the ewes (106/117).

The time required for cervix penetration and confirming the location for semen deposition was around 60 s (Table 1); however, there was wide individual variation, the shortest being 13 s and the longest, 97 s. The total time taken for CRI and release of the ewes was 3:26 min for fresh and frozen semen treatments. The time required to thaw the straw did not influence the total time for CRI when using frozen semen.

The intrauterine semen deposition rate prevailed in both groups (fresh and frozen semen), but did not show significant difference compared with cervical semen deposition rate (P > 0.05). In 92.3% of the ewes, the reaction intensity was weak or moderate (Table 1).

Table 1. Cervical penetration time (CPT), artificial insemination by cervical retraction time (CRIT), deposition semen rate and reaction intensity in Santa Inês ewes

Semen type	CPT (s)*	CRIT (min ± s)*	Deposition semen rate (%)**		Reaction intensity (%)***		
			Cerv	IU	Wea	Mod	Str
Fresh (n=25)	45.1 ± 17.5	3:07 ± 57	32.0	68.0	72.0[a]	20.0[b]	8.0[b]
Frozen (n=40)	60.4 ± 19.9	3:45 ± 35	37.5	62.5	75.0[a]	17.5[b]	7.5[b]
Mean (n=65)	52.8 ± 21.2	3:26 ± 47	35.4	64.6	73.8[a]	18.5[b]	7.7[b]

Cerv.: cervical; IU: intrauterine; Wea: Weak, Mod.: moderate, Str: strong.
* P>0.05, SNK test. ** P>0.05 Chi-square test. *** P<0.05, same row. Chi-square test

The naturally mated ewes showed the best reproductive results compared to the artificially inseminated ewes (P < 0.05). In the inseminated ewes, non-return to estrus rate was lower when cervical retraction was combined with commercial frozen semen (P < 0.05); however, no differences between the techniques were observed (CRI vs. LAI, Table 2).

Table 2. Non-return to estrus (NRE), pregnancy and fertility rates in Santa Inês ewes subjected to different techniques of artificial insemination or natural mating

Group	% NRE	% Pregnancy	% Fertility
CRIFr	64.0[b] (16/25)	64.0[ab] (16/25)	60.0[ab] (15/25)
CRIFz	27.5[c] (11/40)	22.5[c] (9/40)	20.0[c] (8/40)
LAIFr	62.5[b] (15/24)	58.3[b] (14/24)	58.3[b] (14/24)
LAIFz	45.0[bc] (18/40)	35.0[bc] (14/40)	35.0[bc] (14/40)
NM	90.0[a] (20/22)	85.0[a] (19/22)	85.0[a] (19/22)
CRI mean	41.5 (27/65)	38.5 (25/65)	35.4 (23/65)
LAI mean	51.6 (33/64)	43.7 (28/64)	42.2 (27/64)

CRIFr: Artificial insemination by cervical retraction (CRI) with fresh semen; CRIFz: CRI with commercial frozen semen; LAIFr: AI by laparoscopy (LAI) with fresh semen; LAIFz: LAI with commercial frozen semen; NM: natural mating.
The number in parenthesis indicates the observation frequency in each group
Values with different letters in the same column differ by --Chi-square test (P<0.05).

Use of fresh semen resulted in better pregnancy and fertility rates than that of frozen semen (P < 0.05, Table 2). There was no difference between AI technique (CRI or LAI).

The serum progesterone levels (P_4) were similar in ewes fertilized by AI (CRI or LAI) and NM, with mean values of 0.71, 1.14, 2.5, 5.17, and 5.92 ng/mL on days 0, 3, 5, 12, and 17 following AI/NM, respectively. In the non-pregnant ewes, the P_4 profile was also similar (0.71, 1.17, 2.63, 5.24 and 0.78 ng/mL) regardless of the type of fertilization (AI or NM), with a typical P_4 curve being evident in the luteal phase of the estrous cycle. When pregnant and non-pregnant ewes were compared, a significant difference in circulating P4 concentrations was observed only on D17, when the mean progesterone concentration for non-pregnant ewes was <1.0 ng/ml (Fig. 1).
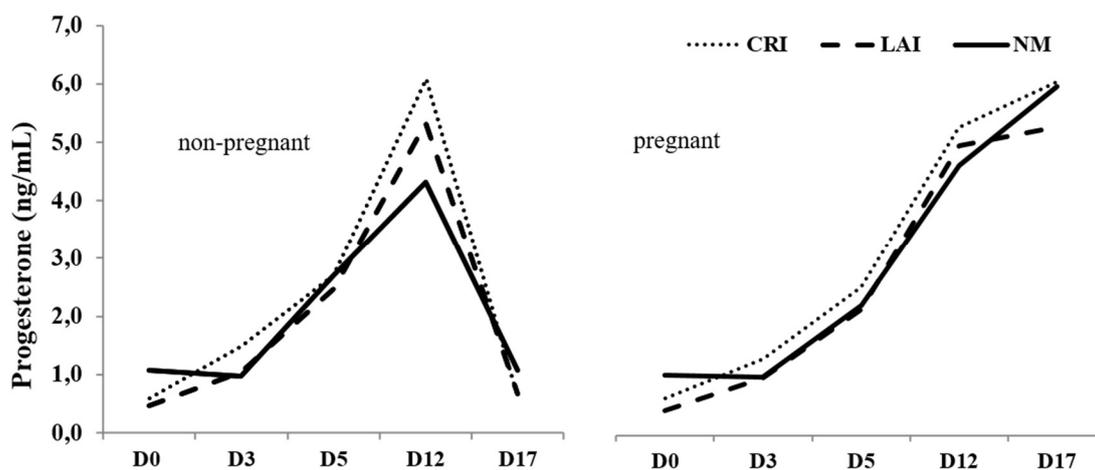

Figure 1. Serum progesterone levels of non-pregnant or pregnant Santa Inês ewes after natural mating (NM), AI by cervical retraction (CRI) or laparoscopy (LAI). D17, P < 0.05, SNK test)

DISCUSSION

Considering CRI performance, some studies have reported times of 3–8 min/ewe (Windsor et al., 1994; Casali et al 2017); this variation is attributed to factors such as breed, age and postpartum period, as well as the skill of the inseminator (Kershaw-Young et al., 2010; Richardson et al., 2012). This study showed that the lesser time spent performing AI can be attributed to the ewe being contained in a more practical position (standing) than the dorsal recumbency. The fact that there was no time difference between AI with fresh or frozen semen was because thawing was conducted simultaneously with the cervical penetration procedures.

Intrauterine semen deposition was performed according to the methods described in the literature, which reports transcervical AI varying between 45% and 90% under the direct influence of the anatomy of the cervix, i.e., ostium type, length, number of rings and organization (Windsor et al., 1994; Kershaw et al., 2005). Penetration rates can be improved with the use of cervical dilators, such as oxytocin (Masoudi et al., 2017) or prostaglandin E2 (Bartlewski & Candappa, 2015), but this do not represent an increase in fertility rates. Casali et al. (2017) achieved intrauterine deposition only in 7.7% of ewes using the same technique of cervical retraction, but with the hindquarters upward maintained on an easel.

In CRI treatments, the intensity of weak/moderate reaction was 92.3%, suggesting that the form of restraining the ewe in standing position was effective, as it did not compromise their welfare during handling. The dorsal recumbency at angles from 60° to 80° reported for several researches (Halbert et al, 1990; Rekha et al., 2016) is an abnormal orientation for sheep; it requires a greater prior fasting period (24 h), and there is a risk of ruminal content aspiration (Ferranti et al., 2013).

The best reproductive rates observed in naturally mated ewes emphasizes the good physiological condition of the ewes and andrological condition of the rams, in addition to effective breeding management, which has a good effect on the flock's performance (McManus et al., 2010). When used as a reference, these results provided greater accuracy in terms of evaluating AI techniques and the influence of semen type. The estrus response was similar for AI groups, therefore, it did not influence the results of reproductive performance.

The AI technique does not influence the non-return to estrus rate in ewes; however, low performance was observed when frozen semen was used. The freezing/thawing process causes ultrastructural, biochemical, and functional damages to spermatozoa, thereby reducing their transport and fertilization capacities (Zarei et al., 2018). This fact was proven when the rates of non-return to estrus were compared using the type of semen, 63.3% vs. 32.3% for fresh and frozen semen, respectively.

The use of fresh semen with 200.10^6 spermatozoa/dose was successful in both AI techniques for pregnancy and fertility (64.0%, 60.0% for CRI and 58.3%, 58.3% for LAI, respectively). Other studies show pregnancy rates with transcervical AI around 54h after P4-

eCG treatment in different breeds, such as Australian Merino, 48% (Olivera-Muzante et al., 2011) or Corriedale, 48,6% (Santos Neto et al., 2015) and 53.1% (Casali et al., 2017). Thus, considering that the ewe inseminated with fresh semen showed a higher performance to those inseminated with frozen semen and similar results between AI techniques, the influence of the CRI based on the parameters evaluated in this study was not verified.

The commercially obtained frozen semen provided low fertility results when CRI was used (20%). Several researchers have reported a high variation in pregnancy rates observed after CRI with frozen semen (values between 19% and 55%) and attributed it to the influence of various factors, such as breed, age, period from prior lambing, hormonal protocol and repeatability of the technique (Windsor et al, 1994; Kershaw et al., 2005).

The AI fertility results were also low when frozen semen and laparoscopy were used, and they were lower than those reported in other studies, which showed values between 60% and 77% (Cardoso et al., 2009; Olivera-Muzante et al., 2011). Thus, commercially obtained frozen semen constituted a limiting factor for obtaining satisfactory reproductive rates, even when the deposition occurred directly in the uterine horns (LAI).

In serum P_4 there was no observed influence of the AI technique in pregnant and non-pregnant ewes. Normality in the secretory pattern of P_4 is essential for the provision of growth factors and binding of glycoproteins required during the embryo implantation stage, which occurs between days 13 and 19 of pregnancy (Barnes, 2000; Brooks et al., 2014). Some researchers have suggested that manipulating and retracting the cervix during AI may increase $PGF_{2\alpha}$ production, thereby causing changes in the uterine environment through migration of neutrophils and Th_1 cytokines and, consequently, luteolysis and embryonic death (Wulster-Radcliffe & Lewis, 2002).

The possible stress of containment and cervical manipulation during CRI could activate hypothalamic-ptuitary-adrenal axis and inhibit GnRH, LH or progesterone secrections (Emsen et al., 2011; Dobson et al., 2012). However, we were not able to verify influence of CRI on the functionality of the corpus luteum, from its formation (ovulation occurred) until 17 days after fertilization (embryo implantation period). The normal function of the corpus luteum suggests that possible inflammatory changes due to CRI are limited to the cervical canal, which could undermine steps before embryonic development, such as spermatic transport and fertilization. This situation potentially aggravated when frozen semen is used (Hawk, 1983; Sayre & Lewis, 1997), resulting in low fertility rates.

AI by cervical retraction in Santa Inês ewes kept in standing position, using a cervical expanding applicator, proved to be technically viable because it did not interfere with the welfare of the ewe or the progesterone secretory pattern post AI. Use of fresh semen was successful, but commercially available frozen semen does not provide a satisfactory fertility rate, regardless of the insemination technique. Use of AI by cervical

retraction results in fertility rates similar to those obtained with intrauterine insemination by laparoscopy.

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