

Alternatives to enable the transcervical artificial insemination in sheep

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Abstract: The present study aimed at assessing alternatives to enable the transcervical artificial insemination (AI) in sheep. 114 Santa Inês ewes were distributed into five groups: G1 – Transcervical AI with no use of cervix enlarger (n = 23); G2 – Transcervical AI with 500IU oxytocin intramuscular (IM) injection, 11h prior to AI (n = 23); G3 – Transcervical AI with 1 mg estrogen IM injection, 11h prior to AI, (n = 22); G4 – Transcervical AI with 0.0375 mg prostaglandin F2 α (PGF2 α) IM injection, 11h prior to AI (n = 23) and G5 – Laparoscopic AI (n = 23). Ewes had their estrus synchronized using intravaginal sponges containing medroxyprogesterone acetate for six consecutive days and were inseminated with fresh diluted semen 50h after sponge withdrawal. The data were submitted to normality analysis, followed by appropriate statistical tests for each variable. There was a significant difference (P<0.05) concerning the timing of AI, being shorter for group 5, with average of 4`13`` if compared to groups 1, 2, 3 and 4 (5`54``; 5`37``; 5`37`` and 5`27``, respectively). Pregnancy rate for group 5 was 68.18%, which was higher than in groups 1, 2, 3 and 4 (30.4%, 8.6%, 0.0% and 13.0%, respectively). There was no difference (P>0.05) for plasma cortisol concentration after insemination among groups (29.95 ng/mL average). Laparoscopic insemination was more efficient since it showed higher pregnancy rate and was quicker to perform. It also had the same stress level as the transcervical inseminations.

Key words: Pregnancy, Dilators, Santa Inês.

Alternativas para viabilizar a inseminação artificial transcervical em ovelhas

Resumo: O presente estudo teve como objetivo avaliar alternativas para permitir a inseminação artificial (IA) transcervical em ovinos. Foram utilizadas 114 ovelhas Santa Inês distribuídas em cinco grupos: G1 - IA transcervical sem uso de relaxante cervical (n = 23); G2 - IA transcervical com injeção intramuscular de oxitocina de 500UI (IM), 11h antes da IA (n = 23); G3 - IA transcervical com aplicação de 1 mg de estrógeno IM, 11h antes da IA, (n = 22); G4 - IA transcervical com aplicação de 0,0375 mg de prostaglandina F2 α (PGF2 α) IM, 11h antes da IA (n = 23) e G5 - IA laparoscópica (n = 23). Realizou-se a sincronização de estro das ovelhas usando esponjas intravaginais contendo acetato de medroxiprogesterona durante seis dias consecutivos e a IA foi realizada 50 horas após a retirada da esponja com sêmen fresco diluído. Os dados foram submetidos à análise de normalidade, seguida de testes estatísticos adequados para cada variável. Houve diferença significativa (P<0,05) em relação ao momento da IA, sendo menor tempo para as fêmeas do grupo 5, com média de 4`13`` se comparado aos grupos 1, 2, 3 e 4 (5`54``; 5`37``; 5`37`` e 5`27``, respectivamente). A taxa de gestação para o grupo 5 foi de 68,18%, maior que nos grupos 1, 2, 3 e 4 (30,4%, 8,6%, 0,0% e 13,0%, respectivamente). Não houve diferença (P>0,05) para concentração plasmática de cortisol após inseminação entre os grupos (média de 29,95 ng / mL). A inseminação laparoscópica foi mais eficiente, uma vez que apresentou maior taxa de gestação e foi mais rápido para realizar. Também teve o mesmo nível de estresse que as inseminações transcervicais.

Palavras chave: Gestação, Dilatador, Santa Inês.

Introduction

The anatomical characteristics of the cervix of the sheep species make AI expansion difficult, as the cervical canal is a rigid, tubular structure formed by several tortuous prominences (cervical rings) that are arranged in different planes and positions obliterating the lumen (Casali et al., 2017). Numerous attempts have already been made to overcome the cervical barrier and reach the uterus with the aim of increasing delivery rates (Moura et al., 2011).

The most practiced access for intrauterine AI in sheep is laparoscopic (Silva-Meirelles et al., 2017), however, this technique requires expensive equipment and specialized labor, whose implementation in a herd is conditional on the adopted production system and cost-benefit ratio. Fertility rates between 40% and 70% can be achieved by using frozen semen with laparoscopic intrauterine deposition (Cardoso et al., 2009).

As an alternative for performing AI in sheep, we use the transcervical approach, which is a non-surgical method and has lower costs (Rabassa et al., 2007), whose fertility rates are around 14% to 71% (Rabassa et al., 2007, Cardoso et al., 2009 & Aral et al., 2010) compared to the laparoscopic technique. But the smaller opening of the cervical canal at the time of estrus prevents or hinders its transposition (Kaabi et al., 2006), justifying the use of some substances to promote cervical relaxation and opening, such as hormones, relaxin, oxytocin, and prostaglandins, among others (Santos et al., 2009).

Hence, the objective of this study was to evaluate some alternatives to enable the transcervical AI in the sheep by checking their pregnancy rates, execution timings, physiological parameters and plasma cortisol concentrations.

Material and methods

This experiment took place from April to July 2010, in São João farm, Entre Rios –Bahia, located at a latitude of 11°56'31" south and a longitude of 38°05'04" west, at an altitude of 162 meters, with temperature average annual of 26.5 Celsius, rainfall of 1.200mm annual and hot and semi-humid climate (Koppen-Geiger climate classification: As). Since it is a tropical region, the animals presented reproductive activity

throughout the whole year. The procedures used in this work are in accordance with the standards of animal welfare.

A total of 114 non-seasonal Santa Inês ewes were randomly distributed into five groups of 23 animals each. Their age (1-4 years old), body weight (42.2 ± 7.8 kg), body condition score (2.0 to 3.0) according to Morand-Fehr and Hervieur (1999), and parturition (1 to 3) were used to homogenize the groups.

Ewes were kept in semi-intensive management condition grazing *Brachiaria sp.* and *Pennisetum purpureum* during the day and taken to stalls of wood slat floor where they were fed 200g of maize bran and soya bean based concentrate. Animals were offered mineral salt and water ad libitum during the whole experimental period.

Ewes were randomly placed into five groups (G): G1 – Transcervical AI with no use of cervix enlarger (n = 23); G2 – Transcervical AI with 500IU oxytocin intramuscular (IM) injection (Placentina™, UCB, Brazil), 11h prior to AI (n = 23); G3 – Transcervical AI with 1mg estradiol benzoate IM injection (Estrogin™, Farmavet, Brazil) 11h prior to AI, (n = 22); G4 – Transcervical AI with 0.0375mg prostaglandin F2 α (PGF2 α) IM injection (Prolise™, Tecnopec, Brazil), 11h prior to AI (n = 23) and G5 – Laparoscopic AI (n = 23), which is the technique that is more commonly used in sheep. The inseminations were carried out by the same inseminator.

In order to synchronize the estrus of all ewes, it was used intravaginal sponges containing 60mg medroxyprogesterone acetate (MPA; Progespon™, Syntex, Argentina) for six consecutive days. At sponge withdrawal, 0.5mL d-cloprostenol (PGF2 α analogous, Prolise™, Tecnopec, Brazil) and 300IU equine chorionic gonadotropin (eCG) (Novormon™, Syntex, Argentina) were injected intramuscularly.

Transcervical AIs were performed by the same technician 50h after sponge withdrawal; animals were kept at standing position in an elevated drafting crate. A 20cm vaginal speculum with light source (similar to a three-blade anoscope) was used to localize the cervix caudal ostium. Cervix was clamped with a 25cm Allis clamp and pulled towards the vulvar vestibulum. An AI applicator for ewes (Aplicador Expansor

Ovino™, Alta Genetics, Brazil) was used to pass through the cervix rings.

Laparoscopic AIs were performed by the same technician with ewes under sedation (0.3mL IM 2% Xylazine chlorhydrate; Rompun™, Bayer, Brazil) and restrained in a stretcher in dorsal decubitus. They were kept at 1m from the ground in Trendelenburg position. Ventral abdomen received local infiltration anesthesia (2% lidocaine, Anestésico L™, Eurofarma, Brazil) in two sites caudally to the navel, and on both sides of the linea alba.

The laparoscope (Endoview™, Germany, Alemanha) was inserted in the abdominal cavity through two 10mm trocars at the anesthetized sites. In one trocar the optical part was inserted and in the other one it was inserted a 30cm-stainless steel blunt edged rod to help the manipulation and localization of the uterus. Then, using an intrauterine insemination pipette (Aspic™, IMV, France), half of the insemination dose was deposited in the mid part of the uterine horn.

Fresh diluted semen from three Dorper males were collected with the use of an artificial vagina and used in all the artificial inseminations. Only semen of at least score 3 of swirling, 70% progressive motility and score 3 of vigor, according to Brazilian College of Animal Reproduction [CBRA] (1998) were used for the calculation of inseminating doses and dilution. Holding Plus™ medium (Nutricell, Brazil) was used to dilute semen to a concentration of 150×10^6 spermatozoa per inseminating dose and then aliquots were loaded into 0.25mL straws. During inseminations semen was kept in hot water bath at 37 °C.

The following parameters were assessed: type of cervix caudal ostium, site of semen deposition in the female genital tract, type of cervix mucus, cervix opening, timing of AIs, rectal temperature (RT), respiratory frequency (RF), heart rate (HR), plasma cortisol concentration and pregnancy rate.

Type of cervix caudal ostium, site of semen deposition, cervix mucus and cervix opening were only assessed in the transcervical inseminations. The other parameters were evaluated in all AIs.

Cervix caudal ostium was classified as duckbill, rosette, crater, slit and flap according to Aisen (2008). Semen deposition sites were divided into superficial (at the cervix ostium),

middle (passing through 2 or 3 cervix rings), deep (between the one before last and the last cervix rings) and intrauterine. Mucus was classified as clear, milky, streaked and cheesy according to Aisen (2008). Cervix opening was classified as a little opened (smaller than the width of the insemination pipette), intermediate opening (same width of an insemination pipette) and wide opened (bigger than the width of an insemination pipette).

Transcervical AI timings were measured from the moment that the animal was placed in the drafting crate, until she was taken away from it. As for the laparoscopic inseminations timing was measured from the moment that the stretcher was placed onto the trestle until the moment that the stretcher was taken away from it. It was used a digital chronometer to register their timings.

In order to assess the ewe's stress, it was measured RT, RF and HR. RT was assessed using a digital clinical thermometer that was introduced at a 5cm depth inside the rectum of the ewes. As for the RF (mov/min), it was assessed by direct stethoscope auscultation at the laryngotracheal region. HR (beats/min) was assessed by direct auscultation on the left thorax region by the location of the aortic arch.

Blood sample (5mL) was collected from jugular vein in EDTA vacutainer tube in order to assess plasma cortisol concentration. Two blood samplings were done, being the first one six days before the AIs (at the moment of sponge withdrawal) and the second one soon after the AIs. Blood samples were centrifuged at 3000 rpm for 15 min to obtain blood plasma, then blood plasma was aspirated and stored in plastic tubes that were kept at -20 °C in a deep freezer for later analysis. The chemiluminescence method and a commercial kit (Access™, Beckman Coulter, EUA) were used according to the manufacturer's instructions in order to determine the plasma cortisol concentrations.

Pregnancy rate (PR) was determined in transrectal ultrasound examination (TUE) (Pie Medical™, Àquila Vet model) 35 days after AI. It was used a 6.0 MHz probe and pregnancy was confirmed by the presence of a heart-beating embryo.

The Shapiro Wilk test was used to assess the normality of the data collected. The variables with normal distribution (TR and HR) were evaluated with the Analysis of Variance and the means compared in the Statistical Analysis

Sistem [SAS] (2004) of the Tukey test, with a significance level of 5%. For the other variables, the Generalized Linear Models (SAS, 2004) were used.

Results and discussion

As for the type of cervix caudal ostium, there was no significant difference ($P>0.05$) among Transcervical AI groups (Table 1). If all

groups were evaluated at once the predominant cervix caudal ostium type would be the rosette (48.9%) (Figure 1).

There was no difference among transcervical-inseminated groups regarding cervix opening ($P>0.05$) (Table 1). However, when it comes to the type of cervix of all groups, 90% of all ewes presented either little-opened cervix or intermediate opening. This emphasizes the fact that in this species the cervix presents high occlusion (Figure 2).

Table 1 - Type of cervix caudal ostium, cervix opening and semen deposition site of Santa Inês ewes subjected to Transcervical AIs.

Parameter	Transcervical Insemination			
	Without cervix enlarger	Oxytocin	Estrogen	PGF2 α
Type of cervix caudal ostium (%)				
Duckbill	19.0	27.3	15.0	10.0
Crater	14.3	18.2	10.0	25.0
Flap	14.3	4.5	15.0	35.0
Rosette	52.4	50.0	60.0	30.0
Slit	0.0	0.0	0.0	0.0
Cervix opening (%)				
Little opened	52.2	47.8	31.8	39.1
Intermediate opening	39.1	43.5	59.1	47.8
Wide opened	8.7	8.7	9.1	13.1
Semen deposition site (%)				
Superficial	26.1	26.08	22.7	26.1
Middle	30.4	34.78	13.7	26.1
Deep	17.4	26.08	22.7	0.0
Intrauterine	26.1	13.04	40.9	47.8

General Linear Models Methodology.

Figure 1 - Types of cervix caudal ostium in Santa Inês ewes subjected to Transcervical AI.

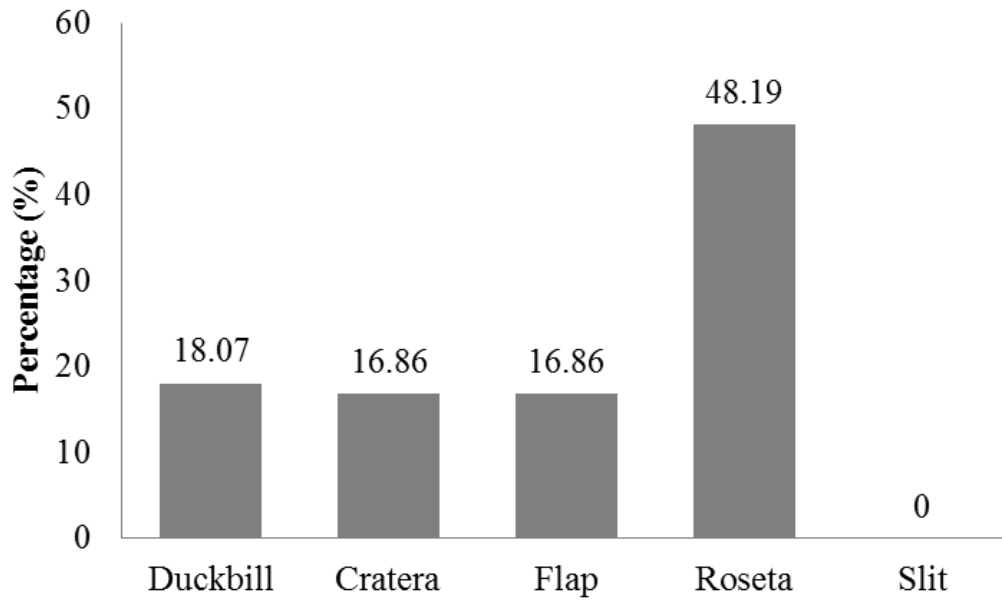
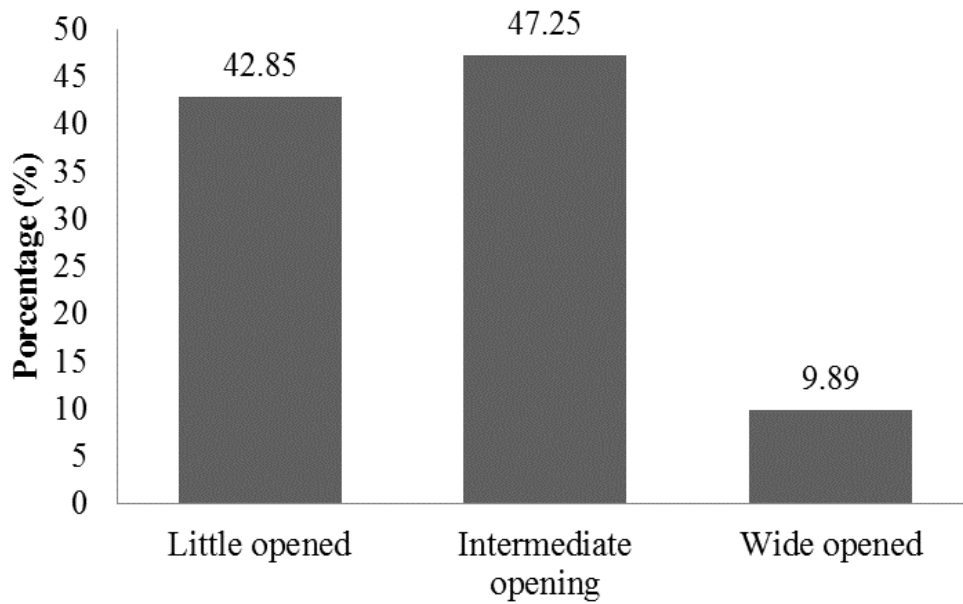
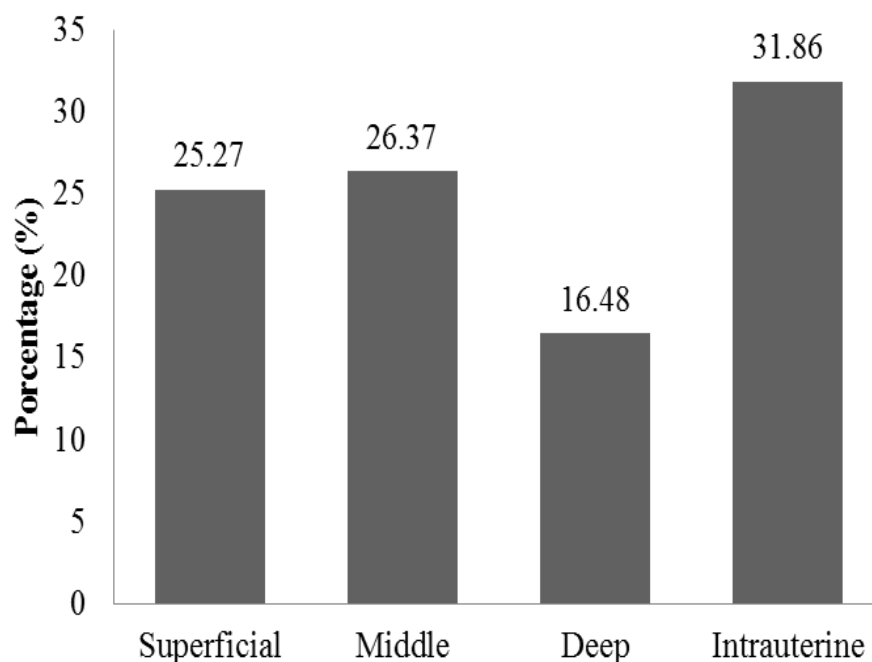


Figure 2 - Cervix opening of Santa Inês ewes subjected to Transcervical AI.



There was no difference ($P>0.05$) among transcervical-inseminated groups when it comes to the semen deposition site (Table 1) (Figure 3).

It is worth noting that animals from G3 presented more flaccid and swollen cervix with erythema if compared to the other groups.

Figure 3 - Semen deposition site in the female genital tract of Santa Inês ewes subjected to transcervical AI.

As for the AI timing, G5 was quicker than the other groups ($P<0.05$) (Table 2). The highest pregnancy rate was observed in G5 ($P<0.05$) (Table 2) whereas G3 presented the lowest pregnancy rate. The highest rectal temperature

was found in G1 ($P<0.05$) whereas G4 and G5 presented the lowest rectal temperature. The animals of group G5 presented the the smaller heart rate and highest respiratory frequency ($P<0.05$) (Table 3).

Table 2 - AI timing and pregnancy rates in Santa Inês ewes subjected to transcervical and laparoscopic AIs

Parameters	Transcervical Insemination				Laparoscopic Insemination
	Without Cervix enlarger	Oxytocin	Estrogen	PGF2 α	
Timing (min)	5'54'' \pm 1' ^a	5'37'' \pm 1' ^a	5'37'' \pm 2' ^a	5'27'' \pm 2' ^a	4'13'' \pm 1' ^b
PR (%)	30.4 ^b	8.6 ^b	0.0 ^c	13.0 ^b	68.1 ^a

PR= Pregnancy rate.

Averages followed by the same letters have no difference in GENMOD ($P<0.05$).

Table 3 - Physiological parameters and plasma cortisol measurement before and after AIs (transcervical and laparoscopic) in Santa Inês ewes

Parameters	Transcervical Insemination				Laparoscopic Insemination
	Without enlarger	Oxytocin	Estrogen	PGF2 α	
RT ¹ (°C)	38.5±0.4 ^a	38.0±0.5 ^b	38.0±0.4 ^b	37.2±0.5 ^c	37.6±0.7 ^{bc}
HR ¹ (beats/min)	78.6±11.6 ^a	79.0±9.5 ^a	73.8±9.8 ^a	80.7±9.3 ^a	55.0±10.4 ^b
RF ² (mov/min)	38.4±7.7 ^a	38.6±6.2 ^a	37.6±6.7 ^a	38.5±10 ^a	58.8±23.8 ^b
Cortisol before ¹ (ng/mL)	14.1±16.6 ^a	4.1±4.5 ^b	4.8±5.5 ^b	3.2±4.3 ^b	10.7±8.3 ^{ab}
Cortisol after ¹ (ng/mL)	30.7±14.6	27.0±26.0	31.1±17.3	30.2±13.9	30.5±17.0

RT= Rectal Temperature; HR= Heart Rate and RF= Respiratory Frequency.

¹Parameters with normal distribution were assessed by GLM. Averages followed by the same letters do not differ by the Tukey's test with 5% of significance.

²Parameters with no normal distribution were assessed by GENMOD. Averages followed by the same letters do not differ with 5% of significance.

As far as plasma cortisol concentration (before the AIs) is concerned, there was significant difference among groups ($P<0.05$). G1 and G5 had similar cortisol concentrations and they were higher than the ones from the other groups (Table 3). As for the plasma cortisol concentrations after AIs, there was no difference among groups (Table 3). There was difference between plasma cortisol concentrations sampled before and after AIs ($P<0.05$).

The first barrier to be broken in artificial insemination of sheep is the external cervical ostium. In this study, the most common types of external cervical ostia were rosette, followed by duckbill, crater and flap, similar findings to those described by Guimarães et al. (2019) and by Franco et al. (2014), who when working with Santa Inês ewes, reported that the type of external cervical ostium most found was rosette.

When it comes to the type of cervix caudal ostium of the ewes, several differences were found in terms of their frequency, and that may occur due to the methodology that was used to observe them. Most authors make such observation in anatomical specimens of slaughterhouses, and that may differ from in vivo studies (Taqueda et al., 2011). However, Kershaw et al. (2005) stated that the type of cervix caudal ostium do not have significant influence upon the fertility rate in ewes and they also concluded that

it is not possible to correlate the type of cervix caudal ostium with characteristics such as size, number of rings and thickness.

Based on the assessment of the cervix opening in which over 90% of the ewes presented little opened to intermediate opening, it can be said that this species present high degree of cervix occlusion; since there was no influence of cervix enlarger in the semen deposition site inside the female genital tract it can be inferred that the chemical cervix enlarger used in this experiment did not facilitate either the opening of the cervix or the passing through the cervix. The semen deposition in deeper sites was consequently not eased.

Taqueda et al. (2011), in one study using Santa Inês ewes of synchronized estrus, performed transcervical AI with frozen semen and deposited semen inside the uterus in 39.3% of all inseminations, which is similar to the result of the present study.

As far as the average AI timing is concerned, the laparoscopic inseminations took about 4min e 13seg whereas the transcervical ones took 5min e 38seg in average. Hence, laparoscopic inseminations were 1min e 25seg faster than the others and that would enable the insemination of a bigger number of ewes and reduce the cost per insemination. Taqueda et al. (2011), in the same study mentioned above,

observed that 53% of the inseminations took between 4 and 5 minutes to finish, and such timing did not have any influence on fertility rate.

Guimarães et al. (2019) reported shorter time (2min and 34s) for cervical penetrability of treated sheep misoprostol, a synthetic prostaglandin E1 analog (PGE1), being highly efficient in inducing cervical opening and stimulating contractile activity of the myometrium. Also Magalhães et al. (2012) obtained shorter passage time (1 min and 53s) when performing transcervical artificial insemination, using misoprostol and terbutaline sulfate as dilators. Moura et al. (2011) reported superior results to this study, which performed the seminal applicator passage in the time of 6min and 15s.

The highest PR was found in the laparoscopic inseminated group, which proves the efficiency of such method in the ovine because sperm is deposited inside the uterus. G1, G2 and G4 presented similar results, which demonstrates that the use of chemical enlargers did not contribute to the improvement of PR. G3 presented 0.0% PR and it might have occurred due to their estrus delay if compared to the other groups. The clear aspect of their vaginal mucus at the moment of the AI evidences their estrus phase.

The 68% pregnancy rate corroborates the fertility rates described in the literature for laparoscopic AI, which range from 40% to 75% (Rabassa et al., 2007 & Cardoso et al., 2009). Still according to some studies, artificial laparoscopic insemination enables the use of cryopreserved semen, which generally has lower fertility than fresh semen for vaginal or cervical insemination (Casali et al., 2017). And, according to Casali et al., (2017) and Consalter et al. (2017), with intrauterine AI, higher pregnancy rates are obtained than with cervical insemination also with fresh semen. According to the researchers, intrauterine deposition facilitates the passage of sperm cells towards the oocyte encounter.

All RT values were below 39.1 °C, which is the physiological RT for the ovine, according to Swenson and Reece (1996). Sheep are homeothermic animals, that is, they have the ability to control body temperature within a narrow range (Hafez, 1995), and rectal temperature is a good physiological parameter of body temperature (Oliveira et al., 2005). The impact of heat on physiological variables results in a 3% increase in

rectal temperature when a 1 °C increase in RT would be sufficient to reduce performance in most domestic animals (McDowell et al., 1974).

The HRs of G1, G2, G3 and G4 were within the normal range of 60 to 80 heartbeats per minute for the ovine (Reece, 1996). On the other hand, G5 presented values lower than the normal range possibly owing to the sedation with xylazine. According to Prado et al. (2000), xylazine causes quick induction and analgesia having hypotension and bradycardia as undesirable effects.

All RF values were above the ewe's physiological value of 16 to 34 mov/min (Swenson & Reece, 1996). However, Neiva et al. (2004), Oliveira et al. (2005) and Santos et al. (2006), showed that over 90% of the Santa Inês sheep in the Brazilian northeast region presented results that were above 34 mov/min. According to Cruz (2012), respiratory rate is a variable that is better than rectal temperature to explain physiological changes caused by thermal stress.

Differences found in plasma cortisol concentrations of serum collected before and after AIs suggest that the increase in the second moment sampling would be connected to the stress induction regardless of the technique. Houdeau et al. (2002) also found no difference in plasma cortisol concentrations in ewes despite the selected technique. Nevertheless, sampling blood after the AIs revealed similar stress level for all techniques.

Conclusion

Laparoscopic artificial insemination was the most efficient method due to its higher pregnancy rate. It was also faster to perform and presented the same stress induction level of transcervical inseminations.

The cervix enlargers employed in this experiment are not recommendable in Transcervical inseminations of Santa Inês ewes due to the fact that they do not improve either the crossing of the cervix rings or their pregnancy rates.

Statement of Animal Rights

The project used in this study is in accordance with the Brazilian guideline for the care and use of animals for scientific and didactic

purposes of the National Animal Experimental Control Council.

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