INFLUENCE OF ANATOMICAL AND TECHNICAL ASPECTS ON FERTILITY RATE BASED ON SHEEP TRANSCERVICAL ARTIFICIAL INSEMINATION PERFORMANCE

INFLUÊNCIA DE ASPECTOS TÉCNICOS E ANATÔMICOS NOS ÍNDICES DE FERTILIDADE BASEADO NO DESEMPENHO DA INSEMINAÇÃO ARTIFICIAL TRANSCERVICAL EM OVINOS

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SUMMARY

The aim of this work was to examine both the influence of anatomical and technical aspects on fertility rate of sheep based on the performance of transcervical artificial insemination (TCAI). Transcervical artificial insemination was performed with traction of the cervix in 122 ewes using frozen semen from 11 rams, both Santa Ines breed. The data collected were: type of external cervical opening (CO) (P - papilla; FL - flap; DB - duckbill, S - spiral; RO - rosette), duration of cervical manipulation (2-3, 4-5 and 6-7 minutes), degree of difficulty in cervical transposition (low, moderate, high) and presumed semen deposition site (SC - superficial cervical; DC - deep cervical; IU - intrauterine). The influence of these variables on pregnancy rate was evaluated. Cervical opening type and duration of cervical manipulation had no influence ($p>0.05$) on fertility. The degree of difficulty in cervical manipulation influenced ($p<0.05$) pregnancy rate, since insemination classified as low grade had 52% of pregnancy, while those classified as high recorded only 20%. The presumed site of semen deposition influenced significantly ($p<0.05$) fertility. Pregnancy rates of deposition at each site were: UI – 45.8%, DC – 25.7%; SC – 15.4%. As expected, deeper depositions resulted in higher fertility. In conclusion, the performance of TCAI did not depend on the anatomical classification of external cervical opening of ewe and the duration of cervical manipulation within the range tested (2-7 minutes). The TCAI may have higher fertility rates if difficulties in the application were reduced and the semen deposition was deeper.

KEYWORDS: Cervix. Superficial opening. Cervical traction. Sheep.

RESUMO

Este trabalho teve como objetivo analisar a influência de aspectos técnicos e anatômicos no desempenho da inseminação artificial transcervical (IATC), sobre a fertilidade em ovinos. Foram realizadas inseminações artificiais transcervicais com tração da cérvix em 122 ovelhas sendo utilizado sêmen congelado de 11 carneiros, todos os animais da raça Santa Inês. Durante a inseminação foram coletados dados como o tipo de orifício superficial da cérvix (OS: P - papila; FL – flap; BP - bico de pato; ES – espiral; FD - flor desabrochada), duração da manipulação cervical (2 a 3, 4 a 5 e 6 a 7 minutos), grau de dificuldade na transposição cervical (baixo, moderado e alto) e local presumido de deposição do sêmen (CS – cervical superficial; CP– cervical profundo; IU – intrauterino). Avaliou-se a influência destas variáveis sobre a fertilidade refletida pela taxa de prenhez. Os tipos de OS e a duração da manipulação cervical não apresentaram influência ($p>0,05$) sobre a fertilidade. O grau de dificuldade na manipulação cervical influenciou ($p<0,05$) a taxa de prenhez: fêmeas que apresentaram baixo grau obtiveram 52%, enquanto aquelas com alto grau obtiveram apenas 20% de prenhez. Da mesma forma, o local presumido de deposição do sêmen influenciou significativamente ($p<0,05$) a taxa de fertilidade. As taxas de prenhez em cada local de deposição foram as seguintes: IU - 45,83%; CP – 25,7%; CS - 15,4%. Como era esperado, deposições mais profundas acarretaram em maior fertilidade. Conclui-se que o desempenho da IATC independe da classificação anatômica do orifício superficial cervical da ovelha e da duração da manipulação cervical dentro do intervalo testado (2 a 7 minutos). A IATC pode apresentar maiores taxas de fertilidade desde que se diminua a dificuldade em sua aplicação e se promova a deposição do sêmen mais profundamente.

PALAVRAS-CHAVE: Cérvix. Orifício superficial. Tração cervical. Ovino.

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INTRODUCTION

Transcervical artificial insemination (TCAI) is a powerful tool that allows a rapid genetic progress in sheep because it is cheap and easy to execute (SAYRE & LEWIS, 1997). However, success rate of TCAI is low, especially when associated with frozen semen (WINDSOR, 1994; PERRY et al., 2010), since the process of cryopreservation of sperm damages the structure, especially the mitochondria, the plasma membrane and the acrosome, and leads to premature induction of training processes and acrosome reaction (WINDSOR & WHITE, 1995; AZEVEDO, 2007). On the other hand, the success of insemination programs associated with genetic improvement depends on the use of frozen semen, since fresh semen once harvested lasts approximately 24 hours, which limits the number of females to be used (HAFEZ & HAFEZ, 2004). Another reason for this low success rate is the anatomy of sheep cervix, which consists of a tortuous channel with intense internal folding and misaligned rings that hinder intrauterine semen deposition (BICUDO et al., 2003).

Several authors have proposed methods and techniques of artificial insemination in sheep using frozen semen that involves maneuvers to transpose the cervix; however, few satisfactory results have been reported, especially when compared to those obtained by laparoscopic artificial insemination (SOUZA, 1993; ANEL et al., 2005). Among these techniques, artificial insemination with cervical traction should be highlighted. Souza (1993) compared TCAI in Corriedale sheep with and without cervical traction and found that the use of traction resulted in a larger number of intrauterine deposition.

Nevertheless, few authors have sought to examine more broadly which factors are contributing to low success rate of TCAI. Factors that may potentially influence artificial insemination success rate using frozen semen, such as the anatomy of the cervical cavity surface (HALBERT et al., 1990; KERSHAW et al., 2005); the place where the semen in deposited in the genital tract of sheep during insemination (WINDSOR, 1994; NAQVI et al., 1998), duration of cervix manipulation (SAYRE & LEWIS, 1997; MATOS et al., 2008), have been little explored, especially for Santa Ines sheep. This study aimed to further evaluate the influence of anatomical and technical factors related to the application of transcervical artificial insemination in Santa Ines sheep.

MATERIAL AND METHODS

Animal management

The experiment used 11 rams and 122 sheep of Santa Ines breed in good clinical and nutritional condition. The sheep aged between 10 months and 6 years old, were both pluriparous (n=86) and nulliparous (n=36), weighed between 40 and 70 kg and body condition score varied between 2.5 and 4.0, on a scale from 1.0 to 5.0. The rams used were between 15 months and 7 years old, weighed between 60 and 120 kg BW and scored between 3.0 and 4.5 for body condition.

Estrus synchronization

The females were divided into two same size groups and subjected to two estrus synchronization protocols (P1 and P2) in order to assess whether progestogen insertion time influences the variables tested. P1 protocol consisted of inserting a sponge impregnated with 60 mg of medroxyprogesterone acetate (MAP) and during withdrawal, 350 UI of equine chorionic gonadotropin (eCG) was applied, during 12 days. While P2 protocol consisted of application of chloprostenol (PGF2α) on day zero (D0), placing the MAP on day two (D2), removal of the sponges, and intramuscular injection of 350 UI of eCG and 45 µg de chloprostenol (PGF2α) on day six (D6) and intramuscular injection of 25 µg of Lecirelin (GnRH) on day eight (D8) (BISCARDE et. al., 2009; BISCARDE, 2010). In both protocols, females were subjected to estrus detection by two vasectomized rams twice a day.

Artificial insemination

Females underwent transcervical artificial insemination (TCAI) with cervical traction according to technique described by Halbert et al. (1990), for semen application, a mini applicator expander for sheep was used (Alta Genetics – patent N.ºMU8502569-0). The sheep were contained using a trunk type stretcher developed by Matos et al. (2008), in which the animal was initially in quadrupeds and later tilted at a 45º angle to the ground, so that posterior limbs were higher than the head. After that, the technician inserted a previously lubricated speculum, then located and clamped the cervix using a 25-cm Allis forceps that was fixed on the tissues adjacent to the cervix. With the help of a second Hallis clamp, fingers and thumb, the cervix was manipulated for the introduction of the expander applicator followed by the insemination itself.

Anatomical classification of the sheep cervical opening (CO)

Sheep cervical opening (CO) was classified in all inseminated animals according to methodology proposed by Halbert et al. (1990) and later modified by Kershaw et al. (2005), as follows: P - papilla; FL - flap; DB - duckbill, S - spiral; RO – rosette. This classification was done visually by the technician using a vaginoscope and confirmed at the time of insemination.

Duration of cervical manipulation

The period during which the cervix was manipulated was recorded using a chronometer and it was defined as the time elapsed between the first clamping for cervix traction and the end of semen deposition in the sheep genitals. This period was completely random for each insemination; however, a maximum of seven minutes was the limit to attempt to

achieve full cervical transposition and intrauterine deposition of the semen. To analyze the data, the insemination procedures were grouped into three categories according to how long they lasted: 2 to 3, 4 to 5 and 6 to 7 minutes.

Degree of difficulty of cervical transposition

The technicians performing the insemination classified TCAI degree of difficulty according to how difficult it was to transpose the cervix in order to reach the uterus body, thus allowing intrauterine semen deposition. When insemination was finished the technicians subjectively rated the degree of difficulty according to the following three levels: low, moderate and high.

Presumed semen deposition site

Soon after artificial insemination, the technician presumed where the semen was deposited in the sheep uterine tract. Three deposition sites were preset before insemination: superficial cervical (SC), semen was presumably deposited by the third cervical ring; deep cervical (DC), semen was deposited after the third cervical ring and intrauterine (IU), semen was deposited in the lumen of the uterus.

Pregnancy diagnosis

Females were examined by ultrasonography to confirm pregnancy from 39 to 48 days after insemination, using a two-dimensional General Electrics (GE) unit, with 5 MHz linear transducer and the transrectal ultrasound technique. With the results, pregnancy rate of ewes was calculated and this parameter was used to evaluate the performance of TCAI and the influence of the variables studied.

Statistical analysis

Statistical analysis was performed using MINITAB, version 1.5. Comparisons between groups were made by chi-square test and mean ratios were compared by Z test, both at 5% significance.

RESULTS AND DISCUSSION

From 122 ewes that were inseminated, 37 resulted in pregnancy, while 85 (69.7%) were negative. There was no significant difference ($p>0.05$) in the success rate among different technicians performing the insemination.

Anatomical classification of the cervical opening (CO)

The cervical openings already described by Halbert et al. (1990), Souza (1993) and Kershaw et al. (2005) for other breeds, were also identified in Santa Ines sheep. The frequencies of spiral (S), duckbill (DB) and flap (FL) were low and insufficient to participate in the statistical analysis. Spiral was seen in only three animals (2.4%), followed by (DB) seen in 14 females (11.5%) and FL in 20 females (16.4%). Only the rosette (RO) type and papilla (P) resulted in a number of pregnancies that allowed statistical analysis using Z test to compare the means and determine their influence on pregnancy rates. The rosette (RO) type was seen in 36 females (29.5%) and 12 (9.8%) of them were pregnant after insemination. While the P type was observed in 49 sheep (40.2%) and 21 were successfully impregnated (17.2%). However, artificial insemination success rates were not significantly different ($Z = -0.90$; $p = 0.368$) between the two cervical opening types, RO and P.

Halbert et al. (1990) evaluated 100 sheep of different wool breeds and reported RO and flap (FL) cervical opening types in 35% each, followed by S and RO in 15% each. The authors stated that CO types did not influence fertility rate and concluded that it is not possible to correlate CO type with other characteristics such as size, number and thickness of rings. Although the results of Halbert et al. (1990) with respect of RO and FL cervical opening types are similar to the ones reported in this paper, the frequency of other types differed, which can possibly be explained by anatomical differences inherent to each particular breed. These authors used the breed Suffolk and their cross-breeds Cheviot, Dorset, Leicester, Clun Forest and Hampshire cross-breeds, while this study used only Santa Ines breed, which makes the experimental group more homogeneous.

The cervical opening distribution types differed from the one reported by Souza (1993), who identified only four types (FL, RO, DB and S). The author evaluated 272 sheep cervix, where 138 were Corriedale breed and 134 Ideal, and reported high FL frequency, 69.12%; followed by RO, 25.73%; DB, 3.68% and S, 1.47%. The different frequency of cervical opening types can be explained by the differences in the breeds used, between wool breed sheep, Corriedale and Ideal, and no wool sheep Santa Ines. According to this author, CO did not influence fertility rate, demonstrating that this variable might not be an indicator of the difficulty to transpose the cervix and therefore, does not influence transcervical insemination success rate in sheep.

Kershaw et al. (2005) evaluated 297 cervix from no defined breed (NDB) sheep and reported high frequency of FL, 36% of the total, followed by DB, 25.9% and RO, 18.9%. The remaining openings were divided among the other types: P, 12.5% and S, 6.7%. Cervical opening results reported in this study also differed from the ones by Kershaw et al. (2005). These authors reported that CO was influenced by age rather than by the phase of estrus cycle, and that the type RO was more frequent in young sheep, while type P was more frequent in older sheep.

In this study, CO type was also influenced by parturition order and consequently by age. Among nulliparous, the most frequen type was P, with 69.7% and types FL, RO and DB with 9.1%; 12.1% and 9.1%; which shows the difference between type P and others. Type ES was not found among nulliparous sheep.

Among the pluriparous, distribution was more homogeneous, and RO was more prevalent and found in 35.9% of the animals, followed by P, 29.2%. Types FL, DB and S were respectively found in 19.1%; 12.4% and 3.4% of the animals, respectively.

The differences found in the frequency of cervical opening types can also be attributed to the methodology used. While most authors have made this classification directly in the anatomical parts of slaughtered animals, in this work this was done in a living animal using a vaginal speculum.

Duration of cervical manipulation

Table 1 summarizes the percentage of animals successfully impregnated or not with respect to duration period of cervix manipulation during TCAI of Santa Ines sheep.

Duration of insemination procedure had no effect on fertility ($p>0.05$). Although not significant, there was a trend towards declining fertility as handling time increased, which can be seen in the higher number of pregnant females in the group with shorter cervix handling period. Between 4 and 5 minutes, it is seen and intermediate number of positives and negatives, while the shorter period 2 to 3 minutes had the highest number of pregnancies. Therefore, additional experiments should be conducted to test a wider range and greater number of time intervals to confirm this trend.

Sayre and Lewis (1997) performed TCAI in 75 sheep of Suffolk, Dorset and Hampshire breeds, which resulted in zero pregnancy. Among possible causes, the authors cited the possibility of cervix handling to impact negatively fertility rate and semen transport in the female reproductive tract. The authors also hypothesize the existence of a spermicidal substance resulting from cervical handling. The use of catheters and applicators can cause sudden damage to sensitive areas of the cervix and increased levels of endogenous oxytocin in response to adrenergic stimulation and this is maybe related to reduced fertility (ANEL et al. 2006). Matos et al. (2008) reported that while studying Santa Ines sheep, females that had longer cervix handling period had also low fertility success rate, thus corroborating the hypothesis.

Based on this evidence, a limitation in cervix handling time during TCAI should increase fertility success rate. A 5-minute handling time showed a better balance between the two types of possible diagnosis (positive and negative) and it is maybe appropriate to set this as the time limit to deposit the semen into the uterus of sheep during TCAI.

Presumed semen deposition site

Presumed semen deposition site influenced negatively ($P<0.05$) pregnancy rate. Intrauterine deposition resulted in higher fertility rate compared to other sites, which is in agreement with results reported by Windsor (1994), Naqvi et al. (1998) and Paulenz et al. (2005). On the other hand, no significant difference was observed in fertility rate ($P>0.05$) between superficial and deep cervical deposition. The results obtained for this parameter are shown in Table 2.

Table 1 - Success rate of TCAI with respect to cervix handling period in Santa Ines sheep.

Pregnancy diagnosis	**Cervix handling period (minutes)**			
	2-3	4-5	6-7	Total
Positive	45,8% (11)	30,8% (20)	18,2% (6)	(37)
Negative	54,2% (13)	69,2% (45)	81,8% (27)	(85)
Total	100% (24)	100% (65)	100% (33)	(122)

Chi-square = 5. 041; degrees of freedom = 2.

Table 2 - Fertility rate with respect to semen presumed deposition site during TCAI in Santa Ines sheep.

Pregnancy diagnosis	**Semen presumed deposition site**		
	Superficial cervical	Deep cervical	Intrauterine
Positive	15,4% (6) [b]	25,7% (9) [b]	45,8% (22) [a]
Negative	84,6% (33) [a]	74,3% (26) [a]	54,2% (26) [b]
Total	32,0% (39)	28,7% (35)	39,3% (48)

Chi-square = 9.936; degrees of freedom = 2; p = 0.007.
Values followed by different letters in the same row differ significantly ($p<0.05$).

Table 3 - Pregnancy rate with respect to degree of difficulty to transpose the cervix during TCAI in Santa Ines sheep.

Pregnancy diagnosis	Degree of difficulty to transpose the cervix		
	Low	Moderate	High
Positive	52.0% (13)[a]	34.0% (11)[b]	20.0% (13)[b]
Negative	48.0% (12)[b]	66.0% (21)[a]	80.0% (52)[a]
Total	100% (25)	100% (32)	100% (65)

Chi-square = 9,086; degrees of freedom = 2.
Values followed by different letters in the same row differ significantly ($p<0.05$).

The number of resulting pregnancies was significantly different among the groups deep and superficial cervical deposition and intrauterine deposition, which shows the importance of semen deposition site on fertility rate. Naqvi et al. (1998) reported higher pregnancy rates for females that had semen deposited after the fourth cervical ring (28.5%) and inside the uterus (27.2%) as well, compared to deposition in the cervical opening (0%). These results are in agreement with the present study; however, the same pregnancy rates for both deep cervical and intrauterine deposition were not observed in this study, which can be explained by sheep age difference, since Naqvi et al. (1998) worked with pluriparous females only, breed and insemination pipette differences.

Breed is often cited as a crucial factor in cervical morphology and, therefore, also important in determining how easy it will be to insert the applicator during TCAI. Donovan et al. (2004) suggested that breed may be a relevant factor on the success of TCAI technique, since there may be physical differences that are determinant in the anatomical structure of the cervix. This possibility was confirmed by Kaabi et al. (2006) who analyzed 365 reproductive tract of 4 breeds (Churra: n = 95; Assaf: n = 58; Merino: n = 156; Castellana: n = 56) and concluded that breed influenced significantly cervix morphology. According to them, Assaf and Churra breeds have cervix with large number of cervical rings and small diameter compared to Merino and Castellana that have thicker cervix and smaller number of cervical rings. These authors performed TCAI in all four above breeds and reported significant difference in the penetrability of the insemination pipette among them, and reported higher intrauterine deposition for the Merino and Castellana breeds.

The results of Paulenz et al. (2005) reflect the possibilities of the use of TCAI in sheep. These authors conducted experiments on 10 farms, with 543 Norwegian cross-bred females and six rams of the same breed and found significant difference between vaginal and cervical semen deposition among the farms and rams used. Cervical insemination resulted in significantly higher number of births compared to vaginal insemination (72.7% x 67.4%). The authors state that the fertility of the Norwegian breed and breeder affinity with TCAI favored high pregnancy rates. Also, different management and feeding among farms probably contributed to the differences in the results.

Another variable to be considered is animal age, which may influence insemination performance. In this experiment, sheep age varied between 10 months and 6 years old, which resulted in changing number of intrauterine deposition, younger and nulliparous females had an average 27.8%, while older and pluriparous females had an average 46.0% intrauterine deposition. Accordingly, Windsor (1994) observed that age of Merino ewes influenced significantly the number of intrauterine deposition and therefore, fertility, since younger females had lower rates (21.1%) of intrauterine deposition compared to older ones (41.2%). In addition, Kaabi et al. (2006) claimed to have found a correlation between animal age and cervix morphometry, and that older animals (> 3 years) have larger and thicker cervix, and fewer cervical rings as well.

Windsor (1994) also reports parturition order as a relevant factor in TCAI. Parturition order influenced significantly cervical penetration rate of Merino sheep, higher order pluriparous (3 or more) had 40% intrauterine deposition, followed by low order pluriparous,1 and 2, with 20 and 35%, respectively.

Degree of difficulty during cervical transposition

Although the degree of difficulty to perform TCAI was evaluated subjectively, this evaluation can demonstrate the relationship between the degree of difficulty that the technician faces during cervical handling and fertility. Table 3 shows the analysis of the results for this parameter using Chi-square test.

The degree of difficulty to transpose the cervix influenced significantly ($p = 0.011$) pregnancy rate. Only 20% of the females classified as high degree of difficulty were positive, while 52% of females classified as low degree of difficulty became pregnant. Moderate level of difficulty was not significantly different compared to high ($p>0.05$), although in case of pregnancy negative, the results were significantly different.

Several reports in the literature study the anatomy of the cervix and examine its influence on fertility

(HALBERT et al., 1990; DONOVAN et al., 2004; KAABI et al., 2006). Kershaw et al. (2005) observed that even among individuals of same breed and herd, some are more receptive to TCAI than others. Therefore, it is possible that a selection of individuals with favorable characteristics within a genetic improvement program entails in a flock more suited to the use of TCAI (DONOVAN et al., 2004).

The high degree of difficulty of cervical transposition implied in low fertility rate, while female with high intrauterine deposition had high fertility rate. On the other hand, high degree of difficulty resulted in superficial cervical deposition, where the semen was presumed deposited before the third cervical ring, the most difficult point for the passage of the applicator, according to Leethongdee et al. (2007).

CONCLUSIONS

Transcervical artificial insemination using frozen semen showed satisfactory success rate. The technique is feasible for Santa Ines sheep; however, the performance can be improved if the degree of difficulty of cervical transposition decreases and intrauterine semen deposition is achieved.

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