USE OF FLUNIXIN MEGLUMINE IN SANTA INÊS EWES SUBMITTED TO LAPAROSCOPIC AND TRANSCERVICAL INSEMINATION[1]

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ABSTRACT: The objective of this work was to evaluate the use of flunixim meglumine in Santa Ines ewes submitted to artificial insemination (AI). Forty-four Santa Inês ewes were synchronized and inseminated at fixed times, by the transcervical or laparoscopic route, between 52 and 58 hours after sponge removal. The ewes were split into two treatment groups, to receive intramuscular injections of 2 mL of saline (control treatment) or 2.2 mg/kg flunixin meglumine (FM treatment), twice a day between days 9 and 19 after AI. The pregnancy diagnosis was performed by ultrasound 30 days after the AI. The chi-square test was used to compare the pregnancy rate between the control and flunixin meglumine treatment and between type of insemination, while the t-test, at 5% probability was used to compare the average weight and body condition, using the SAS statistical software. Flunixin meglumine was not effect in increasing the pregnancy rate in Santa Ines ewes undergoing laparoscopic and transcervical insemination.

Keywords: estrus synchronization, ewes, luteolysis, prostaglandin.

USO DO FLUNIXIN MEGLUMINE EM OVELHAS SANTA INÊS SUBMETIDAS À INSEMINAÇÃO ARTIFICIAL TRANSCERVICAL E LAPAROSCÓPICA

RESUMO: O objetivo desse trabalho foi avaliar o uso do flunixin meglumine em ovelhas Santa Inês submetidas à inseminação artificial. Quarenta e quatro ovelhas da raça Santa Inês foram sincronizadas e inseminadas em tempo-fixo, por via transcervical ou laparoscópica, entre 52 e 58 horas após a retirada das esponjas. Após as inseminações, as ovelhas foram divididas em dois grupos para receberem entre os dias nove e 19 após esta, aplicações intramusculares de 2 mL de solução salina (tratamento controle) ou 2.2 mg/kg de flunixin meglumine (tratamento FM), duas vezes por dia. O diagnóstico de gestação foi realizado 30 dias após a IA, por ultrassonografia. A taxa de prenhez entre os tratamentos controle, flunixin e os tipos de inseminação foram comparados pelo teste chi-quadrado. As médias de peso e condição corporal foram comparadas pelo teste t a 5% de probabilidade, utilizando-se o programa estatístico SAS. O flunixin meglumine não foi eficaz em aumentar a taxa de prenhez em ovelhas da raça Santa Inês, submetidas à inseminação transcervical e laparoscópica.

Palavras-chave: sincronização do estro, ovelha, luteólise, prostaglandina.

INTRODUCTION

The achievement of good artificial insemination (AI) results with frozen semen in sheep requires the use of laparoscopic or via transcervical procedure due to the increased success when semen is deposited into the uterus (Rabassa *et al*., 2007; Prado *et al.*, 2013).

To obtain good pregnancy rates, knowledge of physiological aspects, embryonic development and the effects of external stimuli are critical. All actions are directed at understanding the strategies that allow the progression of pregnancy by stopping the luteolysis mechanism. Prostaglandin (PGF2)α is a key hormone involved in this process (Senger, 2003).

In pregnant ruminant females, luteolysis is the main function of prostaglandins. It also influences uterine contraction, transport of semen, uterine tube motility, embryo implantation and the ovulation mechanism (Schnitzer, 2001). Some authors report stress due to the conventional AI process in ewes, which requires cervical clamping or extensive manipulation of the uterus in cases of laparoscopic insemination (Pfeifer *et al*., 2010; Kaveh *et al*., 2011), is associated with signaling and prostaglandin release, with consequent premature luteolysis.

Although PGF2α is known to be the main luteolytic substance, its action on the corpus luteum (CL) is mediated by cytokines, peptide hormones, nitric oxide and tumor necrosis factor-alpha, which combined with decreased progesterone secretion, increase the intraluteal production of PGF2α, inducing cell death by apoptosis in the corpus luteum (Skarzynski *et al*., 2008).

Knowledge of the involvement of prostaglandins in luteolysis and the synthesis cascade and release of these substances has led to the development or improvement of several proposals. Despite extensive study in this area, many doubts still exist about ways to increase reproductive efficiency in ruminants. The use of cyclooxygenase inhibiting drugs (COX), particularly flunixin meglumine (FM), has been widely studied in cattle and goats (Salles *et al*., 1998; Guerra *et al*., 2011; Geary, 2012; Donalisio *et al*., 2012). However, few studies in sheep accurately report results corroborating the use of this drug.

According to Burns *et al.* (1997), in the cell model for biosynthesis of PGF2, free arachidonic acid is converted to prostaglandin H2 by the enzyme COX-2. Thus, non-steroidal antiinflammatory drugs such as FM can minimize the synthesis of PGF2α by inhibition of COX-2, promoting gains in pregnancy rates when administered after AI. Other studies, however, have not shown an increase in pregnancy rates (Pfeifer *et al*., 2010).

Since the extensive knowledge gained from research is sometimes contradictory, particularly in sheep, the complex mechanism of luteolysis and its inhibition are still commonly discussed at major meetings and in journals related to animal reproduction. Thus the use of non-steroidal antiinflammatory strategies as anti-luteolítics still needs answers. Therefore, the aim of this study was to evaluate the use of FM in Santa Ines ewes submitted to different AI procedures.

MATERIALS AND METHODS

Forty-four Santa Ines ewes were used in this experiment. They were maintained in paddocks of *Panicum maximum* cv. Aries and cv. Aruana during the day and gathered into the cote overnight, where they were offered sugarcane *ad libitum* and approximately 300g/animal of concentrated feed (16% crude protein) and water. Mineral salt for sheep was also provided *ad libitum*.

Initially, ultrasound examination (C40 NeuCristal) with a 7.5 MHz linear array transrectal transducer was performed to exclude pregnancy and identify any uterine pathologies. The body condition score (BCS) was determined by palpation of the lumbar region, to evaluate fat cover of the transverse and spinous processes, assigning a score of 1 to 5, where 1 corresponds to a very thin animal, 2 is thin, 3 is satisfactory, 4 is fat and 5 is obese (Sanudo and Sierra, 1986). The body weight was determined individually using an electronic scale (RUDD300, Coimma).

The second step was synchronization of the animals, which was achieved by insertion of vaginal sponges impregnated with 60 mg of medroxyprogesterone (MAP Progespon®, Intervet/ Schering-Plough) and intramuscular (i.m.) injection of 0.106 mg of cloprostenol (0.4 mL Ciosin®, Intervet/Schering-Plough) on a random day of the estrous cycle, considered as day 0 (D0). On day 12 of the protocol, the sponges were removed and the ewes were injected IM with 350UI of equine chorionic gonadotropin (eCG) (Novormon®, Intervet/Schering-Plough). The observations of estrus were made twice a day, between 12 and 54 hours after sponge removal, using a ram. The females were inseminated at fixed time (AI), by transcervical insemination (n=22) or laparoscopic

insemination (n=22), between 52 and 58 hours after sponge withdrawal (day 14).

The transcervical AI was performed in females while contained, with their hind region in a trestle, positioned at an angle of 45 degrees to the ground. The vulvar region was cleaned with a paper towel and a vaginal speculum was used to visualize the cervix. With the aid of Allis tweezers, the entry of the cervix was fixed and pulled to the vulvar opening. Subsequently, a mini-applicator suitable for sheep semen was used to overpass the rings and perform the cervical deposition of semen in the uterine body.

The AI was performed laparoscopically on fasting females, contained in a hammock in the supine position, positioned at an angle of 45 degrees to the ground. It was performed with an endoscopic device with trocars measuring 7 mm. Trocaterization was carried out at two points, where the sheep were anesthetized with 2% lidocaine, located caudally to the umbilicus and lateral to the midline. Through one of the trocars, an endoscope was inserted to view the uterus and ovaries, and an insemination pipette was introduced through which half of the dose of semen (0.125 mL) was deposited in each uterine horn. Both laparoscopic and transcervical inseminations were performed by the same person.

Between days 9 and 19 after AI, the sheep were divided and were given intramuscular injections of 2 mL of saline solution (control treatment) or 2.2 mg/kg flunexin meglumine (Banamine®, Intervet/Schering-Plough) (FM treatment), twice daily, with an interval of 12 hours between applications (7 a.m. and 7 p.m.) (Aké-López *et al.*, 2005). Pregnancy diagnosis was performed 30 days after AI by ultrasonography (C40 NeuCristal, with 7.5 MHz linear array transrectal transducer).

Body condition score (BCS), weight, time of estrus onset and pregnancy diagnosis were considered as the study variables. Analysis of variance was performed to determine the effect of treatment – Control or FM (GLM, SAS Inst., Inc., Cary, NC) – on pregnancy rate and the *t*-test for comparison of means was used to detect differences in BSC and weight. Finally, the chi-square test was used to compare the time of estrus onset and pregnancy diagnosis in relation to the treatments. We also tested the simple interactions between AI and PD effects, but the results were not significant (P>0.05).

RESULTS AND DISCUSSION

The results concerning estrus onset time after sponge removal and posterior application (FM) or not (Control) of flunixin meglumine are presented in Table 1. Note that of the 44 ewes, only one showed no signs of estrus (2.27%). Of the remaining animals, onset of estrus was identified to varying degrees at all times, with the highest frequency 36 hours after sponge removal (45.45%), however, without statistical difference when compared to other times (P>0.05).

Silva *et al.* (2010) reported that 100% of Santa Ines ewes entered estrus using two synchronization protocols, with means of 66.0 ± 2.5 h and 67.3 ± 2.8 h after sponge removal and estrus observation, respectively, for the protocols with prostaglandin only or progesterone associated with eCG. They found 2.6% (one animal) of the ewes entered estrus between 0 and 24 hours after sponge removal, the protocol of associated hormonal application, and 0% in the other protocol. Costa *et al.* (2006), working with five estrus synchronization protocols in Santa Ines ewes, also reported signs of estrus at different moments. However, in these reports there was no evidence showing ewes presenting estrus 12 hours after sponge removal.

The synchronization rate of 97.7% is similar to the one found by Marques Silva *et al.* (2010), who reported 100% of Santa Ines females entered estrus, and Santos *et al.* (2011), who observed rates between 73.5% and 100% in their experiment.

Table 1. Frequency (%) and number of Santa Ines ewes (in parentheses) in relation to estrus onset time after sponge removal and subsequent separation into treatments

Treatment	N	Time of estrus onset signs after sponge removal (hours)					
		No signs	12	24	36	48	54
FM[1]	21	4.76% (1)	0% (0)	23.81% (5)	42.86% (9)	28.57% (6)	0%(0)
Control	23	0% (0)	4.35 % (1)	34.78% (8)	47.83% (11)	8.70% (2)	4.35% (1)
Total	44	2.27% (1)	2.27% (1)	29.55% (13)	45.45% (20)	18.28 % (8)	2.27% (1)

[1]Flunixin meglumine.

The number of ewes with BCS 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 was, respectively, two, three, fourteen, seven, seven, seven and four. They were grouped with similar scores as with 1.5 and 2 BCS (G1 n = 5), 2.5 (G2 n = 14), 3 (G3 n = 7), 3.5 (G4 n = 7) and 4 and 4.5 (G5 n = 11). Only one sheep, G5 (BCS = 4.5), showed no signs of estrus (2.3%).

The BCS did not influence the response of estrus synchronization. However, due to the low cost and influence on ovulation rate, we recommend its use in breeding programs to identify sheep with BCS between 2.5 and 3.5, in order to improve reproductive efficiency. Table 2 presents the means and standard deviations of BCS and weight of sheep in relation to control or FM treatments, type of insemination and pregnancy diagnosis.

BCS and weight did not differ (P>0.05) in the groups with and without FM and pregnancy diagnosis. However, there was a difference (P<0.05) in BCS and weight for the sheep submitted to transcervical and laparoscopic insemination. It is possible to suggest that this is because all the ewe lambs were used in laparoscopic insemination, since it is not appropriate to use this category in transcervical insemination due to the increased occlusion of the cervix compared to sheep which have lambed. As the condition of the ewe lambs was higher than the sheep, an increase in the average BCS was found in this group.

As reported in Zielinski (2008), it is not recommended to use overweight animals in laparoscopic techniques since these animals have a higher percentage of fat in the intra-abdominal region, and in laparoscopic surgery the Trendelenburg position is used, which can cause pneumoperitoneum, reducing lung volume and generating hypoxia and negative influence on pregnancy rate.

Several studies report that the animal weight is closely related to fertility (Ribeiro *et al.*, 2003; Steinheim *et al.*, 2002). Reproductive efficiency is influenced by the weight, BCS, fertility and prolificacy of ewes, so the BCS of the sheep is a factor strongly related to its reproductive performance. When using reproductive biotechnologies, these factors are relevant. However, in this work, the weight and BCS did not affect the pregnancy rate.

The use of FM was not effective in increasing pregnancy rates, with no differences (P>0.05) between the control treatment (13.64% vs. 22.73%) (Table 3). Pfeifer *et al.* (2010) also found no differences in pregnancy rate with the use of FM.

Also, the pregnancy rate did not differ in relation to the methods used (P>0.05), with figures of 20.45% and 15.91% for laparoscopic and transcervical insemination, respectively (Table 3). Similar findings were presented by Windsor (1997) and Rabassa *et al.* (2007).

When using the laparoscopy technique, factors such as type of protocol synchronization, semen preservation method, climatic factors, time spent with each sheep at the time of insemination (Hill *et al.*, 1998) affect the final outcome. In this experiment, although it has shown accuracy on these variables, it took a long time to locate the uterine horns in laparoscopic AI, which may have influenced the final result. This difficulty was due to excess fat in the abdominal cavity of the animals, suggesting this as one of the causes of the low pregnancy rate found in this study.

In relation to the use of FM, since minimum handling or absence of endometrial tissue damage leads to a lower release of arachidonic acid, the effects of FM on the use of prostaglandin would be barely detectable. Rabassa *et al.* (2007) and Pfeifer *et al.* (2010) concluded that the use of highly trained

Table 2. Means and standard deviations of body condition score (BCS) and weight of Santa Inês ewes, according to the treatments, type of insemination and pregnancy diagnosis

		N	BCS	Weight (kg)
Treatments	FM[1]	21	2.98±0.8	51.38±7.0
	Control	23	3.17±0.8	52.28±7.7
Type of AI	Transcervical	22	2.45±0.5 a	49.36±5.2 a
	Laparoscopic	22	3.70±0.5 b	54.34±8.0 b
PD[2]	Positive	16	3.03±0.7	51.37±7.2
	Negative	28	3.11±0.9	52.12±7.5

[1]Flunixin meglumine. [2]Pregnancy diagnosis. Means followed by different letters in sub-groups of the same column differ statistically by the *t*- test (P<0.05).

Table 3. Pregnancy rate (%) and number of Santa Ines ewes (in parentheses) according to the treatments and type of insemination

		Pregnant	Not pregnant	Total
Treatments	FM	13.64% (6)	34.09% (15)	47.73% (21)
	Control	22.73% (10)	29.55% (13)	52.27% (23)
	Total	36.36% (16)	63.64% (28)	100%
Type of AI	Laparoscopic	15.91% (7)	29.55% (13)	45.46% (20)
	Transcervical	20.45% (9)	34.09% (15)	54.54% (24)
	Total	36.36% (16)	63.64% (28)	100%

technicians enables minimal cervical manipulation and the absence of differences in the comparisons made. In the present work, this conclusion was corroborated for transcervical insemination, but not for the laparoscopic procedure.

Regarding the dose of FM, we used 2.2 mg/kg twice daily (Aké-López *et al.*, 2002; 2005), following reports by other authors (Königsson *et al.*, 2003), indicating that this concentration is sufficient to inhibit luteolysis. Therefore, we believe this fact did not contribute to the low pregnancy rates achieved. Königsson *et al.* (2003) reported that the most appropriate time to initiate treatment with flunixin meglumine is on day 9 or 10 of the estrous cycle, before lysis of the corpus luteum begins, just before of the peak of PGF2 α, a fact also verified by Aké-López *et al.* (2002).

Although the use of FM as an anti-luteolytic is already known (Salles *et al.*, 1998; Pfeifer *et al.*, 2010), since it inhibits the formation of prostaglandins and thereby inhibit the regression of the corpus luteum, one possible explanation for results found in this experiment is the that meglumine inhibits the whole cascade of prostaglandin formation, not only PGF2α. This is a concern since PGF, PGE 2 and PGD2α have important roles in the early reproductive stages, influencing endometrial vascularization, hatching of the blastocyst, embryo implantation mechanisms and functionality of the corpus luteum (Sayre and Lewis, 1996).

Another factor we believe is more relevant to explain the low pregnancy rate is the stress level of the ewes, which were handled twice daily for ten days, the period considered the most critical for the establishment of pregnancy (20 days after conception). It is noteworthy that stress elevates levels of serum cortisol, causing the release of PGF2α from the endometrium, which leads to the regression of the corpus luteum (Gomes *et al.*, 2014). It is important to mention that embryonic survival depends not only on the functionality of the corpus luteum, but also on the ability of embryo to produce trophoblast proteins during the maternal recognition (Hafez and Hafez, 2003).

In any event, even though high pregnancy rates can be achieved using FM, we suggest carrying out an economic evaluation of actions linked to the use of the protocol and application of the technique to ratify the cost/benefit ratio drug-related increase of the pregnancy rate.

CONCLUSION

Flunixin meglumine was not effective in increasing the pregnancy rate in Santa Inês ewes in the flock studied, subjected to laparoscopic and transcervical insemination.

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