JENNIFFER HAUSCHILDT DIAS

STRATEGIES TO IMPROVE CERVICAL RELAXATION, OVARIAN RESPONSE, AND EMBRYO YIELD IN SHEEP UNDERGOING NON-SURGICAL EMBRYO RECOVERY

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae.*

Adviser: Jeferson Ferreira da Fonseca

Co-advisers: Maria Emília Franco Oliveira
Joanna Maria G. Souza-Fabjan

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Assent:

Jenniffer Hauschildt Dias
Author

Jeferson Ferreira da Fonseca
Adviser

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“É bom olhar pra trás e admirar a vida que soubemos fazer...
É bom olhar pra frente...”

(Nando Reis)

ABSTRACT

DIAS, Jenniffer Hauschildt, D.Sc., Universidade Federal de Viçosa, July, 2022. **Strategies to improve cervical relaxation, ovarian response, and embryo yield in sheep undergoing non-surgical embryo recovery.** Adviser: Jeferson Ferreira da Fonseca. Co-advisers: Maria Emília Franco Oliveira and Joanna Maria Gonçalves Souza-Fabjan.

Multiple ovulation and embryo transfer (MOET) play a key role in accelerating sheep farming, but it is still limited by obstacles such as embryo recovery technique and variability in the superovulatory (SOV) response. This study evaluates alternatives to these limitations, aiming to increase *in vivo* embryo production in sheep. The first chapter reviews some relevant points of non-surgical embryo recovery (NSER): ovine cervical anatomy, physiological mechanisms of cervical relaxation, and current cervical relaxation protocols (PRC). The second and third chapters evaluate the efficiency of PRC using zero (0.0mg), half (0.5mg), or full dose (1.0mg) of estradiol benzoate (EB) in synchronized and/or superovulated ewes and its effects on NSER feasibility and luteal function. Sheep were randomly assigned to receive 37.5 µg of d-cloprostenol i.m. and 0.0 mg (0EB group; n=12); 0.5 mg (0.5EB group; n=12) or 1.0 mg EB (1.0EB group, n=12) 16 h before and 50 IU oxytocin i.v. 20 minutes before the NSER. In synchronized sheep, NSER was performed in 91.7% (33/36) with a longer transposition duration with Hegar dilator and longer catheter-mandrel ($P<0.05$) in 0.0EB. The treatments did not differ ($P>0.05$) in the duration of uterine flushing or the rate of structure recovery. In superovulated ewes, there was no treatment or category effect ($P>0.05$) concerning NSER success. There was a reduction ($P<0.05$) in ovarian biometry and progesterone (P4) concentration and an increase in estradiol (E2) concentration on D17. The fourth chapter assesses the effectiveness of hCG administration after ovulation. Ovarian function, P4 profile, and embryo yield were evaluated. Superovulated ewes received 300 IU of hCG i.m. (GhCG, n=24) or not (GControl, n=25) 96 h after P4 removal. NSER efficiency, SOV response, and luteal tissue area were similar between groups ($P>0.05$). The hCG increased ($P<0.05$) P4 levels and tended to prevent structural luteolysis ($P=0.07$) but had no effect ($P>0.05$) on functional luteolysis. The fifth chapter studies the effect of 250, 333, or 400 IU of a commercial pFSH (1FSH: 1LH) on follicular growth, SOV response, and embryo yield.

Superovulated ewes with decreasing doses of 250 (G250), 333 (G333), or 400 IU (G400) of pFSH were submitted to NSER. Estrus response, estrus interval, CL and LUF counts and follicular growth did not differ (P>0.05) between groups. The treatments did not differ (P>0.05) in embryo yield, but G333 and G400 showed a tendency (P=0.06) to have a higher mean of recovered structures compared to G250. This study concludes that: 1) NSER can be successfully performed in different sheep breeds, regardless of category, using a cervical relaxation protocol without estradiol benzoate; 2) the use or not of EB affected the luteal function, interfering mainly in estradiol levels; 3) hCG treatment increased circulating P4 before NSER and may prevent early luteal regression; 4) protocols using 250, 333 and 400 IU of pFSH are efficient in promoting follicular growth and superovulatory responses, but 333 IU appears to be more efficient in increasing embryo yield.

Keywords: Biotechnologies. Embryos. MOET. Transcervical.

RESUMO

DIAS, Jenniffer Hauschildt, D.Sc., Universidade Federal de Viçosa, julho de 2022. **Estratégias para incrementar relaxamento cervical, resposta ovariana e produção de embriões em ovelhas submetidas a recuperação não-cirúrgica de embriões.** Orientador: Jeferson Ferreira da Fonseca. Coorientadores: Maria Emília Franco Oliveira e Joanna Maria Gonçalves Souza-Fabjan.

A múltipla ovulação e transferência de embriões (MOTE) desempenha papel fundamental na aceleração da ovinocultura, mas ainda está limitada à técnica de recuperação de embriões e a variabilidade na resposta superovulatória (SOV). Este estudo avalia alternativas a essas limitações, visando incrementar a produção de embriões *in vivo* em ovinos. O primeiro capítulo revisa alguns pontos relevantes da recuperação não cirúrgica de embriões (NSER): anatomia cervical ovina, mecanismos fisiológicos de relaxamento cervical e protocolos de relaxamento cervical atuais (PRC). O segundo e terceiro capítulos avaliam a eficiência de PRC com redução de benzoato de estradiol (BE) em ovelhas sincronizadas e/ou superovuladas e seus efeitos na exequibilidade da NSER e função luteal. As ovelhas foram aleatoriamente designadas para receber 37,5 µg de d-cloprostenol i.m. e 0,0 mg (0,0EB; n=12); 0,5 mg (0,5EB; n=12) ou 1,0 mg de EB (1,0EB, n=12) 16 h antes e 50 UI de ocitocina i.v. 20 minutos antes do NSER. Em ovelhas sincronizadas, a NSER foi realizada em 91,7% (33/36) com transposição cervical mais longa ($P<0,05$) em 0,0EB em comparação com outros grupos. Os tratamentos não diferiram ($P>0,05$) na duração da lavagem uterina nem na taxa de recuperação da estrutura. Em ovelhas superovuladas, não houve efeito de tratamento ou categoria ($P>0,05$) em relação ao sucesso de NSER. Houve redução ($P<0.05$) na biometria ovariana e concentração de progesterona (P4) e aumento da concentração de estradiol (E2) em D17. O quarto capítulo avalia o efeito da administração de hCG sob a função ovariana e concentração de P4 e a produção de embriões. Ovelhas superovuladas receberam 300 UI de hCG i.m. (GhCG, n=24) ou não (GControl, n=25) 96 h após a retirada de P4. A eficiência da NSER, resposta de superovulação e área de tecido lúteo foram semelhantes entre os grupos ($P>0,05$). A hCG aumentou ($P<0,05$) os níveis de P4 e tendeu a prevenir a luteólise estrutural ($P=0,07$), mas não teve efeito ($P>0,05$) sob a luteólise funcional. O quinto capítulo estuda o efeito de 250 (G250), 333 (G333) ou

400 UI (G400) de pFSH (1FSH:1LH) no crescimento folicular, resposta SOV e produção de embriões de ovelhas superovuladas submetidas a NSER. A resposta ao estro, o intervalo para o estro e as contagens de CL e LUF e crescimento folicular não diferiram (P>0,05) entre os grupos. Os tratamentos não diferiram (P>0,05) na produção de embriões, mas G333 e G400 mostraram uma tendência (P=0,06) a uma média de estruturas recuperados superior em relação ao G250. Este estudo conclui que: 1) NSER pode ser realizada com sucesso em diferentes raças de ovelhas, independentemente da categoria, utilizando um protocolo de relaxamento cervical sem benzoato de estradiol; 2) o uso ou não de EB afetou a função lútea, interferindo principalmente nos níveis de estradiol; 3) o tratamento com hCG aumentou o P4 circulante antes da NSER e pode prevenir a regressão luteal precoce; 4) protocolos usando 250, 333 e 400 UI de pFSH são eficientes na promoção do crescimento folicular e respostas superovulatórias, mas 333 UI parece ser mais eficiente no aumento população embrionária.

Palavras-chave: Biotecnologias. Embriões. MOTE. Transcervical.

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SUMMARY

1. INTRODUCTION:

Sheep (*Ovis aries*) breeding is directly associated with human culture and development. It was one of the first domesticated species, mainly for obtaining food (meat and milk) and protection (wool and skins) (FAO, 2022). The great adaptation of the species to different climates, reliefs, and vegetation justifies its widespread presence on all continents (VIANA, 2008). In Brazil, it is an excellent alternative to small, medium, and large producers, specialized or not (VIANA; REVILLION; SILVEIRA, 2013). In recent years, sheep farming has shown a significant rise in the country, stimulated mainly by the increase in consumption by the urban population. In addition, the species has zootechnical indices that encourage its creation, such as a reduced need for investment in the implementation and maintenance of production (SANTOS; BORGES, 2019), financial return, and profitability superior to that obtained with cattle (EMBRAPA CAPRINOS E OVINOS, 2022). Also, sheep farming has an important socioeconomic role, both due to the high adaptability and dual aptitude of most breeds and the possibility of sustainable development (FAO, 2022).

Unfortunately, sheep production in Brazil is still insignificant when compared to cattle. According to data from the Brazilian Institute of Geography and Statistics, in 2020 the national sheep herd contained approximately 20.6 million heads, which represents about 10% of cattle production in the same year (IBGE, 2020). On a world scale, we are behind Australia and Uruguay which, although they do not have the largest sheep population, have the largest production of the species (FAO, 2022). The wool market crisis between the 80s and 90s (SILVA *et al.*, 2013) and the search for more attractive production systems, weakened the system and drastically reduced the sheep herd. It should be added that the Brazilian sheep production chain is still unstructured, whether due to reproductive limitations, theft or the low number of specialized slaughterhouses (VIANA; REVILLION; SILVEIRA, 2013), or the competitiveness of the market that favors the importation of more technified countries, such as Uruguay (VIANA, 2008). In this context, the implementation and adaptation of reproductive biotechnologies would optimize and benefit the production system (MALHEIROS; HÖFLER; PATIAS, 2017).

Among *in vivo* biotechnologies, multiple ovulation and embryo transfer (MOET) stands out as a major contributor to sheep multiplication (MENCHACA *et al.*, 2010), since it allows the maximization of the female's reproductive potential, the reduction of

the calving interval, and the improvement herd genetics. With MOET, it is possible to stimulate the growth and ovulation of a greater number of follicles per estrous cycle, through the use of follicular growth stimulating hormones (GONŹALEZ-BULNES *et al.*, 2004). It consists of selecting donors of genetic or productive merit, submitting them to a superovulation protocol and subsequent fertilization by natural mating or artificial insemination, and embryo recovery about 7 days after estrus behavior (COGNIE, 1999). However, several factors limit the wide spread of MOET in sheep, including the variability of the superovulatory response and the embryo recovery technique in sheep.

In ewes, superovulation is performed after estrus induction-protocol containing intravaginal progesterone device (P4) for 5-14 days with or without injections of prostaglandin F2 α (PGF) (OLIVEIRA *et al.*, 2014). Superovulation treatments can start 3-4 days before P4 withdrawal, with decreasing doses of porcine or ovine follicle-stimulating hormone (pFSH or oFSH) associated or not with equine chorionic gonadotrophin (eCG) (BARTLEWSKI *et al.*, 2016). Even though it seems simple, the SOV protocol involves several intrinsic and extrinsic factors that justify its variability. Currently, the reduction in P4 exposure time associated with PGF administration reversed the negative effects on protocol response and embryo quality caused by low P4 concentration at the end of treatment (Kafi and McGowan, 1997). But it still was not enough to prevent a common phenomenon that occurs in sheep and goats: the early regression of corpus luteum (ERCL) (ROCHA *et al.*, 2022).

The causes of ERCL are not fully elucidated, but are related to a drop in P4 concentrations (SAHARREA *et al.*, 1998) and inadequate follicle luteinization, leading to a consistently high estradiol (E2) concentration (OKADA *et al.*, 2000). It is more frequent in superovulated animals, probably due to the greater number of developing follicles (Kafi and McGowan, 1997), affecting part or all CLs (Oliveira et al., 2018). For SOV programs, the ERCL is a reason of concern, since it has as a consequence an abnormal ova transport and recovery of poorer quality embryos (Ishwar and Memon, 1996a; Kafi and McGowan, 1997). Alternatives to prolong the luteal phase and prevent ERCL were performed using luteotrophic gonadotropins (such as GnRH or hCG) (BRASIL *et al.*, 2016; SAHARREA *et al.*, 1998) or non-steroidal anti-inflammatory drugs (NSAIDs; Odensvik et al., 1998), but still with no satisfactory results.

Another concern regarding superovulatory success is the stimulatory gonadotropin used. In sheep, the first superovulations used eCG, which was discontinued due to the high incidence of persistent follicles and high E2

concentrations (BARTLEWSKI *et al.*, 2016) and the deleterious effects on embryo fertilization (EVANS; ARMSTRONG, 1984) and quality (MENCHACA *et al.*, 2009). The substitution for porcine (pFSH) and ovine (oFSH) FSH pituitary extracts resulted in increased efficiency, especially when administered in decreasing doses (COGNIE, 1999), but still with variability, closely related to the amount of FSH and LH in the formulation (GONZÁLEZ-BULNES *et al.*, 2004) and its interaction with physiological hormones (BARTLEWSKI *et al.*, 2009; BETTENCOURT *et al.*, 2008). Currently, the market has commercial pFSH formulations containing a low or equivalent amount of LH in the LH: FSH ratio, and both appear to be effective in promoting superovulatory response and recovery of viable embryos (MIKKOLA; TAPONEN, 2017). In general, superovulations in sheep are performed with FSH containing a low concentration of LH, and the impacts of a different LH: FSH ratio are not fully elucidated in this species.

The surgical technique of embryo collection also does not seem attractive for the implementation of MOET in sheep. The reduced repeatability of the technique and the female's reproductive life and the occurrence of post-surgical adhesions impact the concepts of animal welfare, in addition to making the technique more expensive (ANDRIOLI *et al.*, 1999; HOLTZ, 2005). In this environment, the transcervical technique, also known as non-surgical embryo recovery (NSER) appears, showing excellent collection results, equivalent to the surgical technique and without the same impacts on the animal's comfort (FONSECA *et al.*, 2016). The main limitation of the NSER is the anatomical structure of the sheep, which has a long and tortuous cervix (KERSHAW *et al.*, 2005), with or not vestibulo-vaginal stenosis that, in many cases, hampers or prevents the procedure execution (Fonseca et al., 2018). For this, a protocol was created based on the physiological mechanism of cervical dilatation that assists in transposition and allows access to the uterus without the need for surgery or sedation, containing the administration of PGF and estradiol benzoate (EB) and oxytocin (OT) before the start of NSER (FONSECA *et al.*, 2016). Updates to the protocol have been made by changing drugs (Fonseca et al., 2019a) or routes (FONSECA *et al.*, 2019b; PRELLWITZ *et al.*, 2019) of administration and withdrawing or reducing the amount of EB (DIAS *et al.*, 2020) as the impacts of this protocol on luteal function and embryonic viability are unknown.

Knowing the need to technify sheep production in Brazil and that MOET can play a fundamental role in the acceleration of sheep farming, this work aimed to study alternatives to the main limitations of the technique in sheep, such as the variability of

SOV response and ERCL and the effect of the cervical relaxation protocol on luteal function and embryo recovery, to increase, in number and quality, the embryos produced *in vivo*.

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2. Chapter 1 – Critical points and updates for non-surgical embryo recovery (NSER): a review

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Critical points and updates of cervical penetration in sheep: a review

Jenniffer Hauschildt Dias[a]; Mariana Machado Neves[a]; Joedson Dantas Gonçalves[b]; Gabriel Brun Vergani[b;] Ribrio Ivan Tavares Pereira Batista[c]; Joanna Maria Gonçalves Souza-Fabjan[c]; Maria Emília Franco Oliveira[b]; Jeferson Ferreira da Fonseca[d*]

[a]Universidade Federal de Viçosa, Viçosa, MG, Brazil

[b]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brazil

[c]Universidade Federal Fluminense, Niterói, RJ, Brazil

[d]Embrapa Caprinos e Ovinos, Sobral, CE, Brazil

*Correspondence: Jeferson Ferreira da Fonseca, Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, Km 4, Cep 62011000, Sobral-CE, Brazil. Email: jeferson.fonseca@embrapa.br

ABSTRACT

Success of non-surgical embryo recovery in sheep is linked to efficient access to the uterus through the cervix, which in sheep is restricted by anatomical peculiarities. This limitation not only compromises the dissemination of the technique in the species, but also limits the use of reproductive biotechnologies such as multiple ovulation and embryo transfer. Adaptations and improvements in the technique, especially regarding the cervical relaxation protocol, allowed an increase from 27 to 94% in cervical transposition rates and the possibility of performing the procedure in different breeds and animal categories. In addition, they proved that NSER is more advantageous as it causes less harm to animals, corroborating with current animal welfare standards. This review present some critical and relevant points of the NSER procedure and brings updates and perspectives of the technique aiming at the replacement of the surgical technique and the diffusion of NSER in MOET programs in sheep around the world.

Keywords: cervical relaxation, ewes, NSER, transcervical, TCAI.

1. Introduction

The uterine access via the transcervical route is an excellent approach for artificial insemination and embryo recovery and transfer, replacing the surgical technique that demands veterinary expertise and higher procedural costs. In some species, such as in cattle, transcervical penetration is a reality in the routine of reproductive management, which does not occur in sheep. The anatomical structure of sheep cervix does not allow rectal access for cervical fixation during transposition, as in cattle, combined with the structural characteristics of the cervix that hampers and/or prevent transposition due to misalignment of the cervical rings (Kershaw et al., 2005). Thus, most assisted reproductive technologies (ARTs) in sheep use surgical or semi-surgical approaches, which are already well established in small ruminants (Holtz, 2005).

New attempts to use non-surgical ARTs have been evaluated in sheep, mainly focusing on embryo collection and transfer. Non-surgical embryo recovery (NSER) is an efficient technique for application in multiple ovulation and embryo transfer (MOET) programs in small ruminants (Fonseca et al., 2018) and has demonstrated encouraging results. Firstly, because it is less expensive and promotes greater comfort to the animal compared to the surgical technique (Pereira et al., 1998; Fonseca et al., 2016). In addition, recent studies have shown favorable NSER repeatability in different breeds (Arrais et al., 2021; Dias et al., 2020; Figueira et al., 2020; Prellwitz et al., 2019) and categories (Dias et al., 2022). The objective of this review is to present some critical and relevant points of the NSER procedure, bringing updates and perspectives of the technique aiming at the replacement of the surgical technique and the diffusion of NSER in MOET programs in sheep around the world.

2. The cervix:

Gross anatomy:

The ovine cervix is a long organ with a tubular and narrow lumen, through which annular folds are formed, represented by protrusions and depressions, playing the role of uterine protection (Kershaw et al., 2005). Its length varies according to the sheep breed, age, and number of parturitions, with an average of 4 to 5 cm and 5 to 7 cervical rings (Halbert et al., 1990; Naqvi et al., 2005; Cruz Júnior et al., 2014). The external

cervical os has a lumen closed by prominences and depressions of the mucous membrane, projecting itself cranially to the vagina by one or more folds of fibrous tissue (Dun, 1955), generating individual and racial characteristics, variable frequency, and can be classified as flap, duckbill, smooth , spiral, rosette and papilla (Halbert et al., 1990b; Kershaw et al., 2005). This is the first barrier encountered in insemination and/or embryo collection procedures in sheep, as it makes transcervical transposition difficult.

The cervical lumen is tubular and constricted, and with multiple circular projections and depressions protruding into the cervical canal, forming a corkscrew-like shape (Senger, 2003). The projections are called cervical rings and are organized with variable diameter, height and interval, with an asymmetrical funnel-shaped opening (Halbert et al., 1990b; Moré, 1984). The depressions form folds that are also known as the cervical sack bottom (Moré, 1984). This non-uniformity gives the tortuous characteristic of the sheep cervix. Furthermore, there is a degree of misalignment, mainly referring to the second cervical ring which is more eccentric compared to the others (Naqvi et al., 2005). Studies show that the diameter of the cervical rings is greater at the cervical ends (Franco et al., 2014), corroborating the asymmetric funnel shape described by Halbert et al. (1990). The lumen is also correlated with factors such as age, race, phase of the estrous cycle and number of births (EL Khalil et al., 2018) and can be characterized as the second barrier, as it hampers the transcervical penetration (Kershaw et al., 2005). This difficulty in the cervix transpositions implies in longer cervical manipulation and consequent deleterious effects on embryonic recovery (Wulster-Radcliffe et al., 1999).

Histology:

The cervix differs histologically from the uterus due to its conformation with a predominance of dense connective tissue (85% of the composition) and few bundles of muscle fibers (Junqueira & Carneiro, 2004).

The mucosa or cervical epithelium, which provides the characteristic protrusions and depressions in the cervical lumen, is composed of a non-keratinized stratified squamous epithelium containing secretory cells, mainly mucigenous cells, including goblet cells. (Ferenczy, 1977; Moré, 1984). The epithelium is supported by fibrous connective tissue, with cellular component and extracellular matrix, which is rich in collagen fibrils, and amorphous substance. The extracellular matrix has a stroma

composed basically of fibrous constituents and cellular elements organized in bundles and fibers, which play an important role in the cervical maintenance and conformation, and undergo stimuli in order to maintain rigidity and/or promote cervical relaxation (Halbert et al., 1990b).

The muscle layer consists of smooth muscle layers in a circular and/or longitudinal arrangement and elastic fibers incorporated into dense connective tissue fibers. The muscular tunic of the cervix is continuous with the vagina and is important in postpartum structural reconstruction. This layer differs between species, and is involved in the conformation of thickening and unfolding in the region of the cervical rings observed in sheep (Moré, 1984).The cervical serosa consists of loose connective tissue covered by mesothelium cells and the presence of longitudinal duct of the epoophoron (Gärtner's duct) (Eurell & Frappier, 2006). The cervical rings are covered by a surface similar to the epithelial and muscular layers, covered by dense collagen and reticular fibers organized towards the opening of the ring. (Moré, 1984).

Extracellular matrix components:

The extracellular matrix is located in the connective tissue of the cervical epithelium and consists of several cellular components, among which collagens, mast cells, glycosaminoglicans and glycoproteins stand out due to their action in the conformation and maintenance of the epithelium structure (El-Banna & Hafez, 1972). Changes in its composition occurs during all reproductive life, mainly by hormonal response and it is regulated by estradiol-17β (E2) and progesterone (P4; Ferenczy, 1977).

The collagen is the main component of the cervical structure, mainly types-I and III, and it is responsible for providing textile strength and elasticity (Robinson et al., 2011). The concentration and quality of collagen in the extracellular matrix change during reproductive life, especially during pregnancy, in response to increased prepartum collagenolysis (Riskin-mashiah & Wilkins, 1999). The maintenance of tension between collagen fibrils is due to the action of glycosaminoglycans, which prevent or allow loosening between the fibrils. (Fitzpatrick & Dobson, 1979).

The arrangement composed of sulfated glycosaminoglycans become proteoglycans that not only act as a cellular component of the extracellular matrix but also allow binding to amino acid residues present in collagen or to other cellular components, such as mast cells or growth factors (Junqueira & Carneiro, 2004).

Proteoglycans play an important role mainly in the hydration of the extracellular matrix, since the control and/or retention of water flow in the extracellular matrix of the cervix by chondroitin sulfate, heparin sulfate, dermal sulfate, keratin sulfate and hyaluronic acid has already been reported (Scott, 1986).

Other cells of structural importance are glycoproteins, such as fibronectin and laminin, which act in the interaction and adhesion between cells and their substrates through integrins (Junqueira & Carneiro, 2004). Presence of inflammatory cells, such as mast cells and interleukins, and proteinases were also observed and correlated with the process of extracellular matrix remodeling through collagen degeneration by a mechanism not fully understood (Uldbjerg & Ulmsten, 1990; Leppert, 1992)

3. Physiological cervical relaxation

3.1. Hormonal mechanism:

Cervical relaxation occurs through different execution pathways that complement each other. Changes in endocrine patterns during the female's reproductive life regulate the biochemical interaction of extracellular matrix components resulting in the relaxation mechanism. Peak secretion of follicle-stimulating (FSH), luteinizing (LH) and estradiol (E2) hormones regulate the expression of their own receptors and oxytocin receptors (OTr), culminating in the dispersion of collagen from the extracellular matrix (Kershaw et al., 2007) and reduction in the tone of the smooth muscle (Pereira et al., 2007) of the cervix, allowing cervical dilation.

Both FSH (Mizrachi & Shemesh, 1999) and LH (Shemesh et al., 2001) receptors were detected in bovine (Mizrachi & Shemesh, 1999; Shemesh et al., 2001) and ovine (Leethongdee et al., 2010) cervix during estrus phase, suggesting an action of these hormones on cervical relaxation. There is a correlation between the secretion of these hormones and the increase in available COX-2, which consequently increases PGE2 (Shemesh et al., 2001). Leethongdee et al. (2016) demonstrated an effect of FSH on the stroma and smooth muscle while LH acts only on smooth muscle in the sheep cervix. Furthermore, exogenous applications of PGE2 increased the expression of LHR, FSHR and COX-2 mRNA, which suggests a positive feedback loop between PGs and gonadotropins with pro-relaxation action (Leethongdee et al., 2010).

The hormonal mechanism of cervical relaxation that occurs during estrus or peripartum is similar and is triggered by an increase in E2 levels of follicular or fetal

cortisol origin. Thus, E2 plays a fundamental role in the dilation process of the cervix canal, acting indirectly through several pathways (Windsor, 1995). The elevated E2 concentration leads to an increase in oxytocin (OT) receptors (OTr; Arrowsmith and Wray, 2014) and OT-OTr binding results in the opening of endoplasmic reticulum Ca^{2+} channels, thereby increasing intracellular Ca^{2+} levels, which is required to activate the enzyme cytosolic phospholipase A_2 ($cPLA_2$) (Asselin et al., 1997). When $cPLA_2$ is phosphorylated, it mobilizes arachidonic acid (AA) which is a substrate for cyclooxygenase-2 (COX-2), which converts AA into prostaglandin (PG) H, which is a substrate for conversion into other PGs, such as PGE2, the main hormone that acts on cervical relaxation (Asselin et al., 1997; Falchi & Scaramuzzi, 2015).

The PGE2 acts mainly on its specific EP2 and EP4 receptors, present mainly in the smooth muscle of the cervix, and which induce smooth muscle relaxation (Kershaw-Young et al., 2009). In addition, PGE2 acts on EP4 receptors in the epithelium, increasing the synthesis of glycosaminoglycans, such as hyaluronic acid, promoting the dispersion of collagen fibers from the extracellular matrix (Schmitz et al., 2001). The PGE2 promotes collagenolysis through a proinflammatory action, in synergism with interleukins (Kelly, 1996), in addition to having a positive feedback with oxytocins, increasing the number of available OTr (Weems et al., 2006). PGE2 receptors are regulated by oestrogen receptors, which are present in medial and caudal zone of the cervix, while OTr are more common in the cranial zone (Falchi & Scaramuzzi, 2013; Rodríguez-Piñón et al., 2014). Inflammatory cell infiltration (Muñoz-de-Toro et al., 2003) and collagenase action (Rajabi et al., 1991) will also be mediated by E2 and relaxins.

3.2. Cervical remodeling:

The cervical remodeling process is initiated by an increase in the concentration of heavier sulfate polymers (Taverne, 1992) and in hyaluronic acid synthesis that will attract water to the extracellular matrix (Cabrol et al., 1987; Feltovich et al., 2005). The greater hydration of the extracellular matrix will lead to a dispersion of collagens fibers, decreasing its tension and promoting a greater flexibility of the cervix (Takemura et al., 2005). Hyaluronic acid also stimulates the migration of inflammatory cells such as neutrophils and eosinophils to the epithelium by increasing the production of inflammatory cytokines, such as interleukins (Junqueira et al., 1980). Among the interleukins (IL), IL-8 stands out, which increases leukocyte infiltration and the concentration of matrix metalloproteinases (MMPs), having a direct effect on dilation

when administered vaginally (Maradny et al., 1997; Winkler et al., 1999) and IL-4 and IL-10, which are strongly involved in stimulating the release of PGs (Gahlot et al., 2017). MMPs will act in collagenolysis, promoting the cleavage of collagen fibers types I, III and IV (Hulboy et al., 1997; Nagaset & Woessner, 1999). The MMPs act in collagenolysis, by cleaving collagen fibers types I, III and IV. It was also observed that during estrus there is a lower concentration of dermatan sulfate, responsible for keeping the cross-links of collagen bundles intact, increasing the cervical relaxation mechanism (Kershaw-Young et al., 2009).

Cervical lubrication by cervical mucus also acts on cervical relaxation. Studies evaluating the production of cervical mucus during the menstrual cycle of women have shown that under the influence of estrogen in the follicular phase (Gipson, 2001), cervical mucus becomes more hydrated and there is an increase in permeability (Gorodeski, 2000) and volume of cervical cells (Arslan et al., 2015), culminating in a greater ease of cervical penetration. In sheep, the identification of different O-glycans allows to correlate the hydration promoted by the cervical mucus and the greater degree of cervical penetration by the semen (Abril-Parreño et al., 2022). O-glycans are the main constituent of mucin glycoproteins present in the cervical epithelium, which promote mucus hydration (Gipson, 2001).

4. Transcervical uterine access:

The intrauterine access via the transcervical route in sheep is mainly affected by the anatomical structure of the sheep, which does not allow cervical manipulation through the rectum, as is done in cows, as well as the variable number of cervical rings and the narrow and tortuous lumen (Halbert et al., 1990; Kershaw et al., 2005; Kaabi et al., 2006). In recent decades, several studies aiming expanding artificial insemination (AI; Buckrell et al., 1994; Halbert et al., 1990a) and embryo recovery (Fonseca et al., 2016; Robinson et al., 2011) by transcervical route in sheep have been performed. The knowledge of the physiological mechanism of cervical dilatation was essential for the improvement of AI and embryo recovery techniques, which require cervical transposition for intrauterine access. Cervical transposings after cervical relaxation hormonal protocols becomes more attractive than mechanical dilations for several reasons. Firstly, it reduces the need for several specific curved catheters, since penetration is variable and unpredictable due to different morphological characteristics among sheep species (Alvarez et al., 2019). Second, because the time required is

relatively long and the penetration success is also variable (2.0-5.2 min; 9.6-66.7%; Windsor, 1995; Casali et al., 2017), which compromises its commercial application and impairs the AI technique, reducing AI fertility rates. Finally, although cervical penetration does not induce an inflammatory response (Alvares et al., 2016), repeated attempts to cross the narrow canal can cause epithelial damage in the most sensitive areas of the cervix (Alvarez et al., 2019).

Majority of cervix relaxation protocols aim to dilate cervix by the same physiological mechanisms, using OT (Khalifa et al., 1992), interleukins (Croy et al., 1999), α/β-adrenoreceptor antagonist (Gündüz et al., 2010) and PGs combined (Fonseca et al., 2019) or not (Gusmão et al., 2007) with oestrogens. First works on cervical relaxation, focusing on AI, used oxytocin based on its dilating capacity. An overall average cervical penetration of 5.8 cm and a transposition rate of 77% were obtained after 200-600 IU of oxytocin injection immediately before penetration procedure (Khalifa et al., 1992). OT-treatment, however, does not induce sufficient cervical dilatation for intrauterine access, probably due to the dose or timing of administration (Falchi et al., 2012). Even so, its use can be advantageous, not only for allowing cervical dilation but also for sperm and/or ovum transport during AI/embryo retrieval procedures. Studies show that oxytocin-triggered uterine contraction stimuli have a positive effect on sperm transport, bringing little or no damage to pregnancy responses (Khalifa et al., 1992; King et al., 2004). Higher oxytocin doses and/or associated with other drugs are necessary when the focus is on embryo recovery, which is performed at the beginning of the luteal phase when there is little expression of available oxytocin receptors (Falchi et al., 2012).

PGE analogues (PGE1 - Misoprostol®; PGE2 - Dinoprostone, Cervidil®) were used and showed effectiveness in cervical transposition, with an average of 2.8 to 3.6 cm depth of penetration (Falchi et al., 2012; Perry et al., 2010), 1-3 min of procedure (Candappa & Bartlewski, 2014) and transposing rates ranging from 63.0 to 94.8% in Santa Inês (Gusmão et al., 2007; Leite et .al., 2018), Dorper (Gusmão et al., 2009) and crossbred (Bartlewski & Candappa, 2015; Candappa & Bartlewski, 2014) ewes. This drug is available in several presentations (intravaginal device, gel, liquid infusion), but in some countries its use is restricted to hospitals due to its abortifacient effects. Its success rates, however, are almost always associated with combination with other dilating drugs or mechanical systems developed for sheep, such as the Guelph system and curved catheter (Alvarez et al., 2019). Thus, a replacement for PGF2α analogues

occurred, also because cervical relaxation protocols protocols with PGF2α in showed transposition rates equivalent to those obtained with PGE_2 (Gusmão et al., 2007; Leite et al., 2018).

Fonseca et al. (2016) developed the EMBRAPA's cervical relaxation protocol for non-surgical embryo recovery (i.e. transcervical embryo recovery; NSER). It has already been tested in Santa Inês (Fonseca et al., 2019; Fonseca et al., 2019), Dorper (Dias et al., 2020), Lacaune (Lucas Machado Figueira et al., 2020), Morada Nova (Arrais et al., 2021) and crossbred (Dias et al., 2022) ewes and its cervical transposition success rates and procedure time are described in Table 1. This protocol is based on the administration of 37.5µg of PGF2α and 1mg of EB i.m. 16h before NSER plus a dose of 50IU i.v. of OT 20 min before starting the procedure. The start of treatment is related to the time needed to increase the availability of oxytocin receptors, which is more effective 12 or 16h before procedure (Khalifa et al., 1992; Fonseca et al., 2016). Although there are no significant differences between the route of administration of hormones, the intramuscular and intravenous routes were chosen for greater practicality of application (Fonseca et al., 2019; Prellwitz et al., 2019). Although the management and the amount of drugs needed do not seem so attractive, this protocol has shown execution and success rates equal or superior when compared to PGE2 analogues, especially considering female embryo donors. Locally acting hormones, such as the one developed by Bartlewski & Candappa (2015) may be more attractive, as they do not trigger general effects to the animal that can be negative on ovarian function and embryonic development. However, they may be insufficient to promote adequate relaxation, since, in the pre-NSER moment, embryo donor sheep are in a high progesterone phase, which inhibits the expression of receptors that act in cervical dilatation.

Table 1: Time of procedure and rates of success of cervical penetration in ewes for transcervical artificial insemination (TCAI) or non-surgical embryo recovery (NSER).

Reference	Breed	Drugs of the relaxation protocol	Start of relaxation treatment[1]	Transposition success rate (%)	Duration of procedure (min)
Khalifa et al. (1992)[a]	Crossbred[2]	OT	Immediatelly	77.0	7.0*
Khalifa et al. (1992)[a]	Crossbred[2]	E2 + OT	6 h	41.7	8.0*
Khalifa et al. (1992)[a]	Crossbred[2]	E2 + OT	12 h	83.3	6.6*
Gusmão et al. (2007)[b]	SI	PGF2α	12 h	59.0	27.30
Gusmão et al. (2007)[b]	SI	PGE_1	5 h	63.0	32.75
Gusmão et al. (2007)[b]	DP	PGE_1	5 h	94.8	-
Candappa & Bartlewski, (2014)[a]	Crossbred[3]	PGE	12 h	40.0	2.80*
Candappa & Bartlewski, (2014)[a]	Crossbred[3]	PGE	24 h	67	1.83*
Fonseca et al. (2015)[a]	MN	PGF2α + BE + OT	18 h	96.9	-

Figueira et al. (2018a)[b]	LA	PGF2α + BE + OT	16 h	85.7	25.15
Figueira et al. (2018b)[a]	LA	PGF2α + BE + OT	16 h	91.0	29.25
Leite et al. (2018)[a]	SI	BE + OT	12 h	90.0	-
Leite et al. (2018)[a]	SI	PGE_1 + BE + OT	12 h	83.3	-
Souza-Fabjan et al. (2018)[b]	LA	PGF2α + BE + OT	16h	100.0	30.0
Figueira et al. (2020)[a]	LA	PGF2α + BE + OT	16 h	88.9	23.8
Fonseca et al. (2019)[a]	SI	PGF2α + BE + OT	16 h	77.8	35.6
Fonseca et al. (2019)[a]	SI	PGF2α + EC + OT	16 h	44.4	28.0
Prellwitz et al. (2019)[a]	SI	PGF2α + EC + OT	16 h	57.14	29.4
Prellwitz et al. (2019)[a]	SI	PGF2α + EC + OT	16 h	57.14	26.5
dos Santos et al. (2020)[a]	SI	BE + OT	12 h	88.0	-
dos Santos et al. (2020)[a]	SI	OT	12 h	84.0	-
Arrais et al. (2021)[a]	MN	PGF2α + BE + OT	16 h	83.3	33.6
Arrais et al. (2021)[a]	MN	PGF2α + BE + OT	16 h	100.0	37.3
Dias et al. (2020)[a]	Dorper	PGF2α + BE + OT	16 h	91.7	24.0
Dias et al. (2020)[a]	Dorper	PGF2α + BE + OT	16 h	100.0	21.6

Dias et al. (2022)[b]	SI x LA	PGF2α + BE + OT	16 h	83.3	25.9
Dias et al. (2022)[b]	SI x LA	PGF2α + BE + OT	16 h	64.3	22.2

[a]Oestrus induced ewes. [b]Superovulated ewes. [1]Time from initiation of cervical relaxation treatment (i.e. administration of dilating drugs) to initiation of cervical penetration procedure. [2]Dorset, Rambouillet, Hampshire, and Suffolk Crossbred ewes. [3]Santa Inês x Lacaune Crossbred ewes. *Time to cervical penetration attempt

5. New approaches and perspectives

After more than 30 years of studies, the uterine access via the transcervical route for embryo recovery (NSER) is now a feasible reality. The repeatability of NSER in different breeds of sheep proves its effectiveness in embryo recovery technology. Recent studies show that, with prior preparation of the animals through the cervical relaxation protocol, it is possible to perform the NSER even in those with limiting factors (Dias et al., 2022) such as breed and category (Kaabi et al., 2006). For the greatest success of the technique and to avoid damages in the MOET programs, it is necessary to select donors according to the reproductive history and cervical anatomy. Fonseca et al. (2018) suggest a visual inspection to note anatomic abnormalities, which limit the performance of the procedure (Prellwitz et al., 2019) and are not resolved with the action of the relaxation protocol. The selection should also include animals with a recent calving history, as the long time cervical closure can lead to NSER failure (Fonseca et al., 2021; Dias et al., 2022).

Comparisons between surgical and transcervical techniques demonstrate that both are efficient in embryo recovery (Santos et al., 2020), with similar (Oliveira et al., 2018) or even reduced (Gusmão et al., 2007; Santos et al., 2020) durations of execution and expressing the same inflammatory markers (Oliveira et al., 2018). NSER is more advantageous as it is a less invasive and simplified approach. According to Fonseca et al., (2018), the low risk of sequelae to the reproductive tract allows a quick recovery and increases the number of times that the animal can be subjected to the procedure (Oliveira et al., 2020). Cervix manipulation causes a transient increase in inflammatory markers (Santos et al., 2020), but this does not seem to affect reproduction in the short term as the return to estrus and fertilization of Dorper ewes after NSER has already been observed (Dias et al., 2020). Nonetheless, Oliveira et al. (2018) suggest that studies evaluating the repeatability of the NSER technique should be done in sheep.

Aiming to reduce hormonal residues in the environment non- or reduced-hormonal cervical relaxation alternatives are being tested in sheep in order to promote higher fertility rates in FTAI. Three different doses of EB were tested in estrus-induced Dorper sheep associated with PGF and OT and interestingly, the PGF-OT protocol achieved similar procedural durations and transposition success compared with EB treatments (Dias et al., 2020). The same responses occurred in superovulated crossbreed and White Dorper (Dias et al., 2022; unpublished data) and Santa Inês

(Fonseca et al., 2019) ewes, but in the latter, with lower transposition rates (27% *versus* 83.3%). In fact, the withdrawal of EB from the cervical relaxation protocol is of great importance in NSER because i) it is a hormone of restricted use in some countries (*Hormones in meat*, n.d.); ii) allows the worldwide diffusion of the technique (Dias et al., 2020); iii) estradiol residues are altering the fish population growth profile (Jackson & Klerks, 2020).

Horta et al., (2010) tested the association of 1mg of misoprostol and 5mg of terbutaline sulfate 6h before AI and observed an increase of approximately 10% in fertility compared to untreated animals and vaginally inseminated animals. The α- and β- adrenergic agonist receptors are expressed in the ovine uterus and are associated with increased uterine contractility and promote smooth muscles relaxation (Gündüz et al., 2010; Hawk & Conley, 1985). Local administration of the maximum dose of prazosin or tamsulosin alone (α1-adrenergic receptor antagonist agents) promoted an increase in cervical transposition rates, without causing deleterious effects on hemodynamics (Padilha-Nakaghi et al., 2020). By increasing contractility, these drugs could also have a beneficial effect on embryo recovery, facilitating uterine flushing. However higher fertility rates, which would be obtained when cervical transposition is facilitated by a greater degree of relaxation, are better obtained when associated α- or β- adrenergic agonist with PGs (Horta et al., 2010).

Transient effects of cervical relaxation protocol on luteal function and embryonic quality were recently studied. Treatment (EB+PGF2α+OT) decreased luteal perfusion and P4 concentration at baseline levels up to 12h after treatment (J. Santos et al., 2022), although when only PGF2α+OT was used P4 remained above baseline (Dias et al. unpublished data). It is important to elucidate that the treatment changed the E2:P4 ratio to a 2x greater amount of E2 16h after the application of PGF2α or PGF2α-EB, which may be critical at this stage of early embryo development (unpublished data). Santos et al. (2022) showed reduced expression of NANOG and OCT4 genes, which are related to embryonic differentiation, compared to untreated animals. The protocol does not seem to affect embryo viability in goats (Pereira et al., 1998) and pregnancy rates after embryo transfer in ewes (Lucas Machado Figueira et al., 2019), but more studies need to be done in order to clarify its short- and long-term effect on the reproductive life of donor females and embryos.

6. Concluding remarks:

The transcervical route for embryo recovery and transfer is widely used in cattle, goats and horses, and the recent results in different sheep breeds encourage the commercial possibility of this technique in this species as well. The progress obtained with the cervical relaxation protocols associated with the correct previous selection allowed the execution of NSER even in animals of limited breed or categories. This makes the NSER possible for use in different herds or managements. In addition, it positively agrees with current proposals for animal comfort and welfare as it is a simpler, less invasive technique, with fewer adverse effects, appearing as an excellent replacement for surgical techniques, mainly because it allows repeatability and a faster return. the reproductive life.

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Chapter 2 - Successful transcervical uterine flushing can be performed without or reduced dose of estradiol benzoate in cervical relaxation protocol in Dorper ewes

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Successful transcervical uterine flushing can be performed without or reduced dose of estradiol benzoate in cervical relaxation protocol in Dorper ewes

Jenniffer Hauschildt Dias[a]; Maria Amélia Pupin[b]; Gabriela Saloni Duarte[b]; Viviane Lopes Brair[c]; Cleber Jonas Paula[c]; Marco Antonio Paula de Sousa[d]; Ribrio Ivan Tavares Pereira Batista[c]; Joanna Maria Gonçalves Souza-Fabjan[c]; Maria Emília Franco Oliveira, [b]; Jeferson Ferreira da Fonseca[e*]

[a]Universidade Federal de Viçosa, Viçosa, MG, Brazil

[b]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brazil

[c]Universidade Federal Fluminense, Niterói, RJ, Brazil

[d]Universidade Federal do Pará, Castanhal, PA, Brazil

[e]Embrapa Caprinos e Ovinos, Sobral, CE, Brazil

*Correspondence: Jeferson Ferreira da Fonseca, Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, Km 4, CP D 10, Cep 62011000, Sobral-CE, Brazil. Email: jeferson.fonseca@embrapa.br

ABSTRACT

This study assessed the efficiency of cervical relaxation protocol using none, half, or full dose (1.0 mg) of estradiol benzoate in Dorper ewes subjected to non-surgical embryo recovery (NSER). Thirty-six pluriparous ewes received progestogen sponge (60 mg) for nine days plus eCG administration (300 IU i.m.) 24 h before sponge removal. Ewes were not mated and were randomly assigned to receive at 16 h before NSER 37.5 µg d-cloprostenol i.m. and different doses of estradiol benzoate: 0.0 mg (0EB group; n=12); 0.5 mg (0.5EB group; n=12) or 1.0 mg of estradiol (1.0EB group, n=12). All ewes received oxytocin (50 IU) i.v. 20 min before NSER, which was performed eight days after sponge removal. Corpora lutea were counted by transrectal ultrasonography 24 h before NSER. After procedure, the ewes were kept in natural breeding period to check their post-NSER fertility. NSER was performed in 91.7% (33/36) of the animals with overall fluid recovery efficiency over 97% ($P>0.05$). The cervical transposing with Hegar dilator was longer ($P<0.05$) in 0EB (4.2±0.3 min) compared to 0.5EB (1.7±0.3 min) and 1.0EB group (1.5±0.3 min). The cervical

transposing with mandrel/catheter was longer (P<0.05) in 0EB (2.4±0.5 min) than 1.0EB group (1.3±0.5 min). Overall duration of uterine flushing was 25.4 min with structure recovery rate of 43.5%, with no difference among groups (P>0.05). The post-NSER fertility was higher (P<0.05) in 0.0EB (90%) than 0.5EB group (36.4%). In conclusion, NSER can be successfully performed in Dorper ewes by using a cervical relaxation protocol without estradiol benzoate.

KEYWORDS: Cervical dilation, NSER, post-NSER fertility, Sheep, Transcervical embryo recovery.

1. INTRODUCTION

Successful non-surgical embryo recovery (NSER) has been proposed as an efficient choice for *in vivo* embryo production in goats and sheep (Fonseca et al., 2016, 2018) The success of NSER depends in first instance on the efficiency of cervical transposing (Candappa & Bartlewski, 2012). Although in goats cervix can be transposed during diestrus with only cloprostenol (Fonseca et al., 2013; Pereira et al., 1998) or without drugs (Fonseca et al., 2014), in sheep a more complex combination of hormones are needed for allowing relaxation and transposing of the cervix, which is a narrow, long and tortuous organ (Candappa & Bartlewski, 2012). There is also great variability of types of cervical os (Kershaw et al., 2005) and number of cervical rings between breeds (Kaabi et al., 2006) that may affect successful NSER rates. Some strategies, as a pre-selection of animals using a score of transposing difficulty with Hegar dilator (Fonseca et al., 2019) or a cervical map for guiding the mandrel/catheter transposing (Fonseca et al., 2018) appear to be helpful and easy tools during the procedure which can increase NSER successful rates.

Important advances related to successful cervical transposing and uterine flushing in diestrus phase were obtained in Santa Inês ewes, testing different combination of drugs (Fonseca et al., 2019; Leite et al., 2018), different routes of estradiol (Fonseca et al., 2019) and oxytocin (Prellwitz et al., 2019) administration as well as strategies to select and classify ewes according to degree of difficulty for cervical transposing (J. F. Fonseca et al., 2018). Results of those studies showed near to 80% of cervical transposing in Santa Inês ewes using estradiol benzoate-d-cloprostenol-oxytocin based protocol to NSER. Interestingly, the same protocol has

showed greater success (near to 100%) in Lacaune ewes (Figueira et al., 2020). Cervical transposing was possible in almost 95% in Dorper ewes using misoprostol (prostaglandin-E analogue) as cervical dilator (Gusmão et al., 2009) but there is no data in this breed using others mentioned protocols.

In order to apply the NSER worldwide, it is important to evaluate the efficiency and adapt this technique in other commercial breeds of sheep. Dorper sheep has a great economic importance in ovine meat production system. In addition, the hormonal combination used for cervical dilation should consider the use of drugs available worldwide. In Europe and Oceania, the sale and use of estrogen analogs are restricted or prohibited by environmental waste and residues in animal meat (Directive 81/602/EEC) once that data available confirmed oestradiol-17β as a carcinogen agent (*Hormones in meat*, n.d.). In sheep, the use of estrogen in the diestrus period did not affect embryonic implantation and pregnancy rate (Lewis, 2010). Its effect on embryos *in vitro* has not been evaluated yet but there is some successful data on lambing rate in animals undergoing embryo transfer after NSER procedure (Figueira et al., 2020). Even though in South America there is still no restriction on the use of this drug, it is possible that in the future it will be replaced by potentially less harmful hormones.

Taking into account that in goats the cervical relaxation for NSER is done only with d-cloprostenol (Fonseca et al., 2013) and that there was a high degree of facility of transposition in Lacaune ewes submitted to the standard protocol (Figueira et al., 2020), we hypothesized that in sheep it would be possible to perform the NSER without or with reduced estrogen dose. Thus, the aim of this study was to evaluate the efficiency of cervical relaxation protocol using none, half or full dose of estrogen analogue for NSER success in Dorper ewes.

2. MATERIAL AND METHODS:

2.1. Animals:

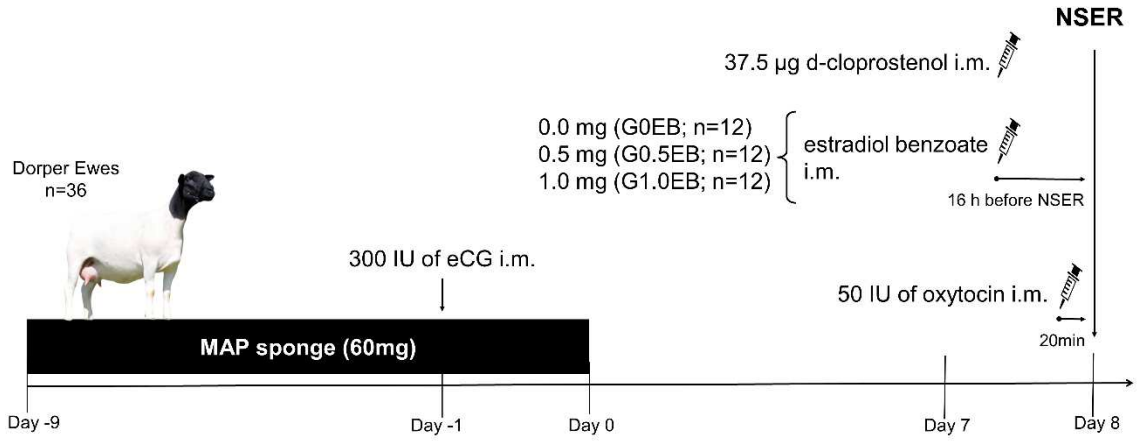
This study was conducted after the approval of the Ethics in Animal Care Committee of the Embrapa Dairy Cattle (process 2512100516). The experiment was carried out from August to September at a commercial farm, located in Juiz de Fora city, Minas Gerais state, Brazil (latitude 45′ 51″ S, longitude 43° 20′ 59″ W). A total of 36 pluriparous Dorper ewes, with average of 4.3 ± 0.2 years old (mean ± SE), 46.9 ±

1.1 kg of body weight and 2.9 ± 0.1 of body condition score (scale from 1 to 5, when 1 = emaciated and 5 = obese; Suiter, 1994) and with 100 to 120 days post-partum (weaning at 80 to 90 days) was used. The animals were kept in semi-confinement system, maintained in native pasture (*Brachiaria decubens*) and supplemented with corn silage according to demands (NRC, 2007), and had *ad libitum* access to fresh water and mineral salt.

2.2. Experimental design:

All ewes received an intravaginal sponge containing 60 mg of medroxyprogesterone acetate (MAP, PROGESPON®, Zoetis, São Paulo, Brazil) maintained for nine days, plus an administration of 300 IU of eCG (Novormon®, Zoetis, São Paulo, Brazil) i.m. 24 h before sponge removal. Ewes in estrus were not mated and were randomly assigned to receive at 16 h before NSER intramuscularly d-cloprostenol (37.5 µg; Prolise®, Agener União Saúde Animal, Taboão da Serra, Brazil) and different doses of estradiol benzoate: 0.0 mg (0EB group; n=12); 0.5 mg (0.5EB group; n=12) or 1.0 mg (1.0EB group, n=12) of estradiol benzoate (RIC-BE®, Agener União, São Paulo, Brazil). All ewes received oxytocin 50 IU, (Ocitocina Forte UCB®, Jaboticabal, Brazil) i.v. 20 min before NSER. The experimental design is shown in Fig. 1.

Figure 1: Schematic representation of the experimental procedures used to assess in Dorper ewes the effect of cervical relaxation protocols containing none (0.0), half (0.5) or full dose (1.0) of estradiol benzoate in estrous-induced ewes subjected to non-surgical embryo recovery (NSER). MAP: medroxyprogesterone acetate; eCG: equine chorionic gonadotropin; i.m.: intramuscular.



2.3. Cervical manipulation and uterine flushing:

Cervical penetration and uterine flushing were attempt eight days after the sponge removal. Animals in a standing position were restrained in a cart and received acepromazine maleate (1 mg/kg; Acepromazin®; Vencofarma, Londrina, Paraná, Brazil) i.m. and 10 mL (5mL i.v. and 5mL i.m.) of dipyrone and n-butyl hyoscine bromide solution (Buscofin Composto®, Agener União, Taboão da Serra, Brazil), both at 20 min before procedure. A lidocaine epidural block (S5-C1) using 2 mL of 2% lidocaine (BLOC®, J.A. Saúde Animal, São Paulo, Brazil) was performed immediately before insertion of a vaginal speculum. The cervical penetration and uterine flushing approach used in this study was identical to that previously described for ewes (Figueira et al., 2020). After vaginal speculum insertion, cervical os was classified according to Kershaw et al. (2005) modificated and anatomical features of the uterine cervices (i.e., number and relative position of consecutive cervical rings, depth of penetration) were recorded for each donor animal to create the "cervical map" (Fonseca et al., 2018). The time taken to traverse the cervix with Hegar dilator and mandrel/catheter and the duration of flushing were recorded. All ewes were classified based on time required for cervical penetration and successful penetration rates into the five following categories or grades: Grade 1 (very easy; cervical penetration achieved in less than 1 min; Grade 2 (easy; between 1 and 3 min); Grade 3 (moderate difficulty; between 3 and 7 min); Grade 4 (difficult; between 7 and 10 min); and Grade 5 (impossible to penetrate the cervix or a lack of complete cervical passage) as previously described (Fonseca et al., 2019C). All recovered structures were classified according to the International Embryo Society Transfer manual (Stringfellow & Seidel, 1999).

2.4. Ultrasound assessment:

Ultrasound evaluation was performed 24h before NSER in all ewes by B-mode Ultrasound (Mindray M5Vet®, Shenzen, China) equipped with a 8.0 MHz transrectal linear probe, fitted with a rigid protractor. Corpora lutea (CL) was counted and animals presenting absence of CL was not considered for structure recovery rate.

2.5. Post-NSER fertility:

Ten days after NSER, all ewes received 37.5 µg d-cloprostenol i.m. and were kept in continuous natural breeding for seven weeks with fertile rams previously

attested by andrological evaluation, in order to check fertility of females after NSER procedure. Pregnancy rates were assessed 60 days after the NSER by the same ultrasound.

2.6. Statistical analyses:

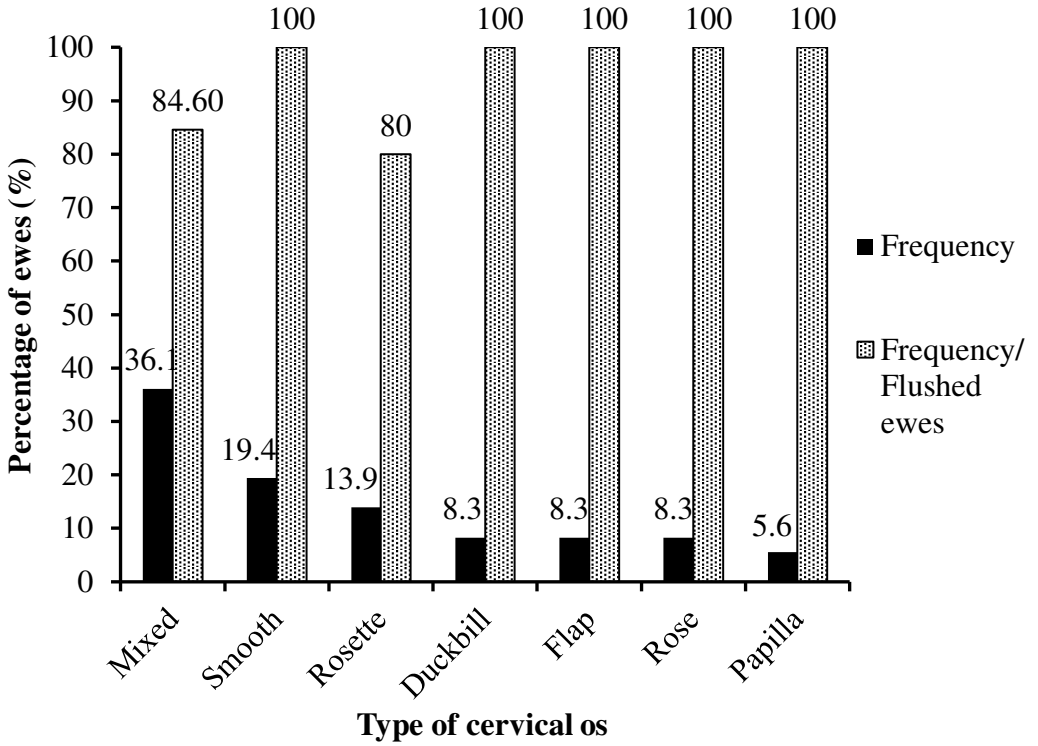
The following data were recorded for all ewes studied: mean number of corpora lutea per ewe; type of cervical os; Hegar transposing success rate (number of ewes successful transposed with Hegar/total number of ewes of the group x 100); number of cervical rings; duration of Hegar transposing (in minutes, time from Hegar dilator insertion to removal); degree of difficulty performing Hegar penetration procedure; duration of mandrel/catheter transposing (in minutes, time from mandrel/catheter insertion to removal); degree of difficulty performing mandrel/catheter penetration procedure; duration of flushing (in minutes, time from the first 5Ml injection of PBS solution to flushing catheter removal); NSER success rate (number of ewes successfully flushed/total number of ewes x 100); fluid recovery efficiency rate (percentage of fluid recovered post-infusion: volume retrieved/400Ml x 100); number and type of structures recovered (unfertilized eggs); ewes with at least one structure recovered rate (number of ewes with one or more structures recovered /number of ewes successfully flushed x 100); average structures recovered (mean number of structures recovered); structure recovery rate (considering animals presenting at least one corpora lutea: number of structures recovered/number of corpora lutea x 100); post-flushing fertility rate (number of pregnant ewes/number of successfully flushed ewes x 100).

Data analysis was performed using the libraries of the “car”, “stats”, “agricolae” and “emmeans” packages of the R software (version 3.6.1, The R foundation for Statistical Computing). To assess the normality of the residues, the Shapiro-Wilk test was used, and the Levene test for the assumption of homogeneity of variance of the treatments. The Box-Cox transformation was performed when necessary, and the data was returned to the original condition for presentation of the results. The Kruskal-Wallis test and Fisher's Exact test were used for non-parametric analyses, depending on the variable. Parametric data was analyzed by analysis of variance (ANOVA) followed by Tukey test, for mean comparisons. Values are presented as mean ± standard error. The level of significance used for all analyses was 5%.

3. RESULTS:

Regardless of the treatment, the efficiency of NSER according to cervical os presentation was described in Fig. 2 (P>0.05). Overall Hegar transposing success and uterine flushing success were reached in 97.2% (35/36) and 94.3% (33/35) of ewes, respectively. Overall, the NSER could not be performed in 8.3% of ewes (3/36; 0.0EB = 2 and 0.5EB = 1), due to anatomical problems.

Figure 2: Frequency of different types of cervical os observed in Dorper ewes at the time of non-surgical embryo recovery (NSER) and their influence on the efficiency of the technique (P>0.05).



The average number of cervical rings was 7.4 ± 0.18 (5 to 9 range). The total procedure duration averaged 25.4 min (range: 14 to 33 min). A total of 27 structures were recovered and classified as unfertilized eggs. The overall structure recovery rate was 43.5% (27/62), considering only ewes successful flushed. One ewe successfully flushed from 0.5EB group presented no CL and was not considered for data related to structures recovered. Data regarding to the efficiency of transcervical embryo recovery according to treatment groups are presented in Table 1.

Table 1: Summary (mean ± SEM or %) of the cervical penetration attempts and embryo recovery procedures in pluriparous Dorper ewes who received different concentrations of estradiol benzoate 16 h before non-surgical embryo recovery (NSER) in a cervical relaxation induction protocol.

End Points	Estradiol benzoate dose (mg)		
	0.0	0.5	1.0
Number of animals	12	12	12
Mean number of corpora lutea/ewe*	2.0 ± 0.3 (19)	2.1 ± 0.3 (21)	1.7 ± 0.2 (20)
Hegar transposing successful (%)	100.0 (12/12)	91.7 (11/12)	100.0 (12/12)
Duration of Hegar dilator transposing (min)	4.2 ± 0.3[a]	1.7 ± 0.3[b]	1.6 ± 0.3[b]
Number of cervical rings transposed	7.3 ± 0.3	7.4 ± 0.3	7.7 ± 0.4
Duration of mandrel/catheter transposing (min)	2.4 ± 0.5[a]	1.6 ± 0.4[ab]	1.3 ± 0.5[b]
Duration of flushing (min)	19.2 ± 1.2	21.4 ± 1.4	18.6 ± 1.1
Total duration of procedure (min)	25.4 ± 1.6	24.0 ± 1.6	21.6 ± 1.2
NSER Success (%)	83.3 (10/12)	91.7 (11/12)	100.0 (12/12)
Fluid recovery efficiency (%)	98.0 ± 2.0	99.0 ± 1.0	97.0 ± 1.6
Ewes with at least one structure recovered (%)*	70.0 (7/10)	60.0 (6/10)	58.3 (7/12)
Average structures recovered*	1.0 ± 0.3	0.9 ± 0.3	0.7 ± 0.2

Structure recovery (%)	52.6 (10/19)	39.1 (9/23)	40.0 (8/20)
Post-flushing fertility (%)	90.0 (9/10)^{A}	36.4 (4/11)^{B}	58.3 (7/12)^{AB}

^{a,b} Means with different superscripts within rows differed (Tukey test; P<0.05).

^{A,B} Means with different superscripts within rows differed (Fisher exact test; P<0.05).

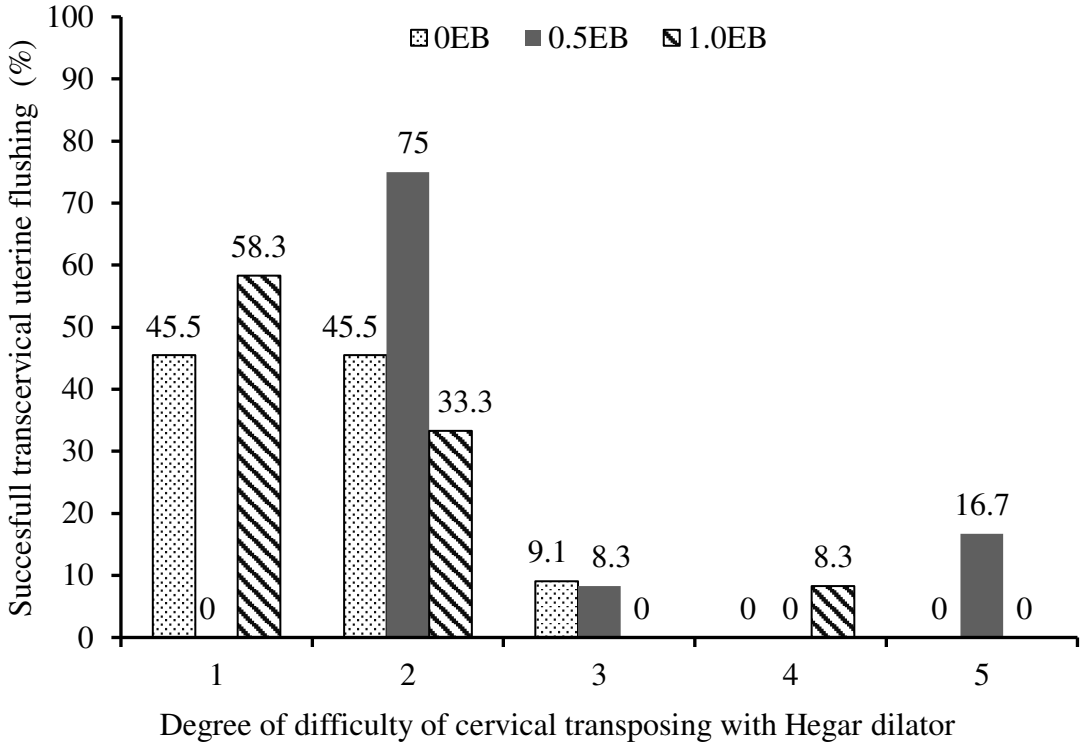
( ) number of corpora lutea, animals or structures.

* Only ewes successfully flushed and with at least one CL counted.

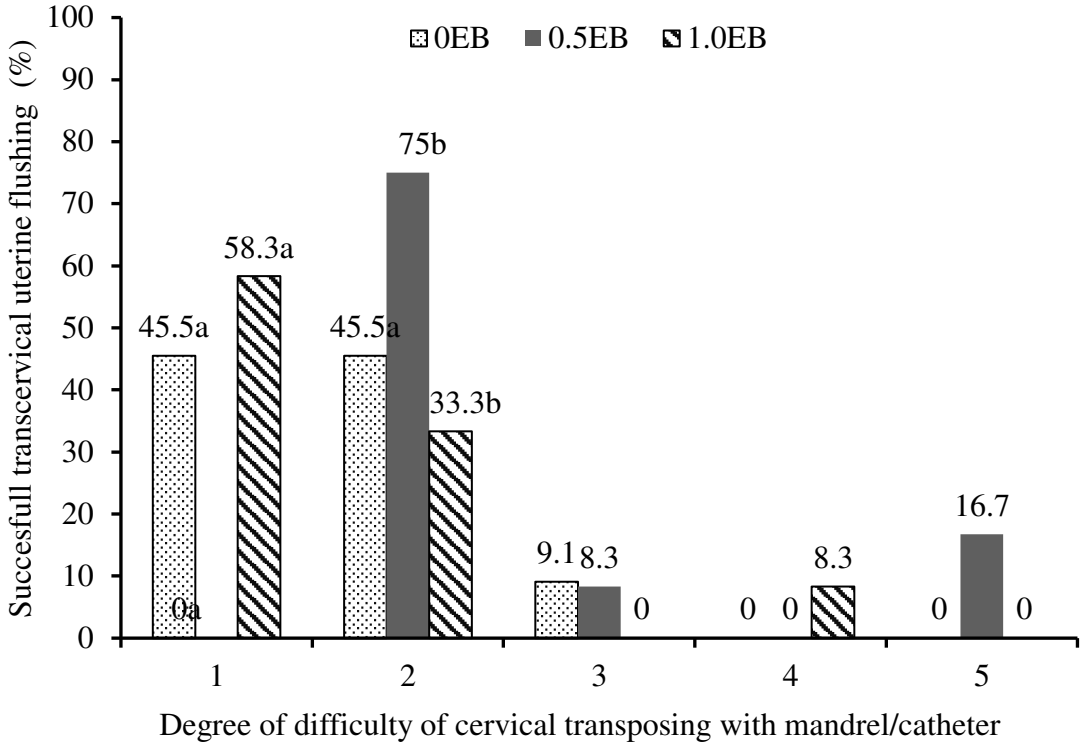
The degree of difficulty of cervical transposing with Hegar dilator and mandrel/catheter are presented in Fig. 3 A and B, respectively.

Figure 3: Number of Dorper ewes successfully submitted to non-surgical embryo recovery (NSER; y-axis). The x-axis means the degree of difficulty in transposing the cervix with Hegar dilator (3A) and mandrel and catheter equipment (3B). Scale 1–5 where: 1) very easy, cervical penetration achieved in <1 min; 2) easy, between 1 and 3 min; 3) moderate difficulty, between 3 and 7 min; 4) difficult; between 7 and 10 min; and 5) impossible to penetrate the cervix with dilator or a lack of complete cervical passage. [a,b] Values with different letters differ between grades (Fisher exact test; $P<0.05$).

A



B



4. DISCUSSION

Considering that the objective of the present study was to evaluate cervical relaxation protocols using different doses of estrogen, it is possible to infer that NSER can be efficiently performed with no or reduced estrogen dose without affecting the feasibility of the whole technique. Our results showed a transposition rate with Hegar dilator close to 100%, regardless of the protocol used, and a success rate of NSER with a linear, but not significant, increase when using doses of 0.0 (83.0%), 0.5 (91.7%) and 1.0 (100.0%) mg of estradiol benzoate in Dorper ewes subjected to cervical relaxation. Moreover, it should be highlighted that the three ewes not flushed had anatomical problems detected at the time of the procedure. Applying screening tests to identify and avoid ewes with such problems (Prellwitz et al., 2019) may increase the NSER success in Dorper ewes. In fact, the design used in the present study can also be an efficient, field applied and no expensive way to test NSER success in any breed before superovulation.

The NSER success (92 to 100%) in Dorper ewes of the present study using an association of prostaglandin F2α and oxytocin (PGF-OT) and estradiol benzoate protocol for cervical relaxation showed superior results than those reported in Santa Inês ewes (range 44 to 81.8%) (Fonseca et al., 2019; Fonseca et al., 2019; Prellwitz et al., 2019). Interestingly, the same misoprostol (PGE2) based protocol for cervical relaxation revealed successful transcervical uterine flushing rates of 94.8% (55/58) in pluriparous Dorper ewes (Gusmão et al., 2009) and 63.1% (12/19) in pluriparous Santa Inês ewes (Gusmão et al., 2007). These findings suggest that the same protocol could reach variable efficiencies when using different sheep breeds.

The NSER success ranged from 83 to 92% in the protocols using estradiol benzoate dose reduction combined with PGF-OT, and were superior than another report that used the same protocol in Santa Inês ewes (27.3%; Fonseca et al., 2019b). Our data show that the removal of estrogen in the relaxation protocol does not affect the overall success of the technique, as it does not affect successful NSER rate, fluid recovery rate and structure recovery rate, as already demonstrated in other studies (Fonseca et al., 2019b). It means that in the study conditions the use estradiol benzoate in protocols for cervical relaxation and NSER can not only reduced but also be abolished in Dorper ewes and possibly in other sheep breeds. Therefore, the results of this study can directly affect the wide dissemination of the transcervical embryo recovery technique, since there are countries where the use of estrogen is not allowed. In addition, the present results can also promote a reduction in the amount of hormones needed in protocols for the embryo recovery in sheep and decreasing hormonal residues in the environment. Abolishing or reducing estradiol benzoate use are in accordance of the principles of “Clean and Green” techniques recommended for control reproduction in small ruminants (Martin & Kadokawa, 2006).

Our results showed that only one animal from 0.5EB group could not be transposed with Hegar dilator, while two animals from 0.0EB could not be successfully flushed (mandrel/catheter transposition). This result is better than that obtained in Santa Inês ewes using estradiol benzoate (Prellwitz et al., 2019) and shows that NSER can be spread in different breeds. As we know, each ewe has an individual cervical characteristic of os, cervix alignment and number or thickness of cervical rings (Kershaw et al., 2005), which may vary according to age, breed and lambing (Kaabi et al., 2006). In the present study it was not observed significant difference among groups

related to the type of cervical os or interference of the type of cervical os and successful flushing. Fonseca et al. (2019a) suggest a “cervical map”, which is a sketch of cervical alignment made during cervical transposition with Hegar dilator, and serves as a guide for AI or NSER. However, transposition is not always possible with the mandrel/catheter as they are more flexible compared to Hegar, which justifies the difference found between the two transpositions in these studies. It is also worth remembering that the animals in this study were not submitted to previous evaluation of cervical anatomy when they were selected to this study.

Hegar dilator and mandrel/catheter transposing duration differed among treatments, in which the cervical passage time was longer in 0.0EB group compared to the 1.0EB group. However, this longer time of transposing of the 0.0EB group did not affect the duration of flushing or the fluid recovery efficiency and structure recovery parameters, since for these variables there was no statistically significant difference, corroborating that it is possible to successfully perform NSER using a PGF-OT based protocol.

The reduction or non-use of estradiol benzoate in sheep for cervical relaxation was not detrimental to the safety and/or the level of difficulty of the procedure, nor to the comfort of the animal. In addition, our data on the duration of transposition and the total duration of the procedure were even lower than those found in other studies (Fonseca et al., 2019; Prellwitz et al., 2019), but the breed factor can interfere with the durability of the procedure (Kaabi et al., 2006), explaining this difference. The mechanism of estradiol on cervical relaxation is through the remodeling of the cervical extracellular matrix, acting by increasing the action of hyaluronan on collagen fibers (Robinson et al., 2011). This consequent softening that occurs allows a deeper penetration, facilitating intrauterine access through the cervix, which is desired when it comes to transcervical AI or embryo recovery and justifies the faster penetration of Hegar and mandril dilators in the 1.0EB treated group of this study.

According to Fonseca et al., (2018) the successful NSER can be classified according to duration of cervical transposing, in a 1-5 score, where 1 is equivalent to very easy transposing and 5 is equivalent to impossible transposing. In our study, there was no statistical difference between the degrees of the Hegar dilator, but there was a difference between the degrees of the mandrel/catheter. This means that there is a preference for the cervical passage to become easier with the knowledge of the

cervical map, since the number of animals classified as degrees three and four decreased in comparison to the two transpositions, although there is no significant difference. Even though it has already been observed in other experiments of our group, more studies need to be carried out to prove the efficiency of mapping the cervical alignment in order to facilitate the transposition. Besides that, scoring duration of Hegar or mandril penetration also can works as a selection method of donor ewes, since females classified as grade four during natural estrous could not be traversed after 6-7 days for NSER (Fonseca et al., 2019b) or even when classified as grade four in the Hegar, the animal could not be transposed with mandrel/catheter during the same procedure, as observed in this study. The animal classified as grade five in this study for Hegar dilator could not be transposed, corroborating with Prellwitz et al. (2019) and Fonseca et al. (2019).

Even though it is a low-cost and easily acquired hormone, estrogen has had limited use in countries in Europe and Oceania due to residues in animal products and the environment, which becomes a barrier to the expansion of the NSER technique. Moreover, considering the need to recover optimal quality embryos to survive the embryo cryopreservation and transfer process, wouldn't it be possible that these high levels of estradiol on early luteal phase cannot be affecting the embryo in terms of survival and implantation genes? Some studies in cows (Madsen et al., 2015) and sheep (Lewis, 2010) shows that supraphysiological levels of estradiol in the embryonic implantation period after ET or AI do not cause harmful effects to the embryo's survival *in vivo*. However, we found no studies showing these effects on embryo's survival *in vitro*.

Besides the success of uterine access, high fluid recovery rate and relatively little time required to perform NSER in Dorper ewes appear to be attractive. As well as reported for Lacaune ewes (Figueira et al., 2020), more than 50% of ewes flushed showed at least one structure and the structure recovery rates varied from 40 to 53% even considering low CL count. These findings suggest not only the efficiency but the relatively good precision of the NSER technique even when CL count is lower, opening the opportunity to apply NSER in ewes with low superovulatory response or in non superovulated animals (Figueira et al., 2020).

From the best of our knowledge, pregnancy rates in ewes after embryo collection by any technique were not reported. In the present study not only pregnancy

after NSER were reported but fertility after this procedure was affected by treatment groups favoring 0.0EB group. Thus, NSER technique associated with no use of estradiol benzoate apparently preserve ewe fertility, benefiting the production system by further maximizing the use of the animal. This finding must be confirmed in future and large-scale study.

Finally, despite the recent and significant increases in NSER success in sheep were done using Santa Inês sheep as model, the interest and dispersion of this breed are mostly restricted to Brazil and in some countries of South and Central America. Recently, NSER was encouraged to Lacaune sheep (Figueira et al., 2020), one of the most important dairy sheep breeds in the world. Results of the present study now encourage the use of NSER in Dorper, one of the most important breeds for lamb production in in the world. Considering NSER as more “ethical” technique for embryo recovery than surgical procedures as proposed earlier (Martin, 1995; Martin & Kadokawa, 2006) the whole results of this study can reach the status of “Clean, Green and Ethical” way for NSER of ovine *in vivo* produced embryos.

5. CONCLUSIONS

Based on the data obtained in this study, the estradiol benzoate can be either removed or reduced in protocols for cervical relaxation in Dorper ewes without any effect in the efficiency of the whole NSER process. Now tested efficiently in Dorper sheep, this design appears to be promising also in other breeds for NSER procedure, although it needs to be evaluated due to anatomical differences.

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CONFLICTS OF INTERESTS

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

AUTHORS CONTRIBUTIONS

JFF elaborated the hypothesis, discussed the experimental design. JHD collected the data from the animals. JHD analyzed the data and wrote the first version of the manuscript. GSD, VLB, CJP and RITPB assisted with the animals and helped collecting and analyzing the data. JFF, RITPB, MEFO and JMGS-F discussed the design of the experiment and analyzed the data. MAPS elaborated and worked on the statistics. JFF, MEFO and JMGS-F approved the final version of the manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 3 - Effects of different doses of estradiol benzoate used in a cervical relaxation protocol on the success of non-surgical embryo recovery and luteal function in superovulated ewes

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Effects of different doses of estradiol benzoate used in a cervical relaxation protocol on the success of non-surgical embryo recovery and luteal function in superovulated ewes

Dias, JH[1]; Gonçalves, JD[2]; Arrais, AM[3]; Souza-Fabjan, JMG[4]; Bastos, R[5]; Batista, RITP[4]; Siqueira, LGB[6]; Oliveira, MEF[2]; Fonseca, JF[7]

[1]Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, 36570-000, Viçosa, MG, Brazil

[2]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de acesso Prof. Paulo Donato Castellane, s/n, 14884-900, Jaboticabal, SP, Brazil

[3]Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465, Km7, s/n, 23890-000, Seropédica, RJ, Brazil

[4]Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, 24230-340, Niterói, RJ, Brazil

[5]Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego, n° 2000, 28013-642, Campos dos Goytacazes, RJ, Brazil

[6]Embrapa Gado de Leite, Av. Eugênio do Nascimento, 610, 36038-330, Juiz de Fora, MG, Brazil

[7]Embrapa Caprinos e Ovinos, Três Lagoas Farm, Road Sobral –Groaíras, km 04, CP 145, 62010-970, Sobral, CE, Brazil

*Corresponding author: J.F. Fonseca, Tel.: +55 32 3311-7662, Três Lagoas Farm, Road Sobral –Groaíras, km 04, CP 145, 62010-970, Sobral, CE, Brazil

E-mail address: jeferson.fonseca@embrapa.br

ABSTRACT

This study investigated the effectiveness of different doses of estradiol benzoate (EB) to promote cervical relaxation and their effects on luteal function and outcomes of non-surgical embryo recovery (NSER) in sheep. Multiparous (MULT) and nulliparous (NULL) crossbred Lacaune X Santa Inês ewes were superovulated and naturally bred. Seven days after progesterone withdrawal , females were randomly assigned to one of three distinct cervical relaxation protocols, consisting of i.m. treatment with 37.5 µg d-cloprostenol and different doses of EB: 0.0 mg (Control group; n = 3 NULL and 14 MULT); 0.5 mg (0.5EB group; n = 4 NULL and 12 MULT) or 1.0 mg (1.0EB group, n = 6 NULL and 11 MULT) 16 h before NSER. All ewes received 50 IU of oxytocin 20 min

before NSER (D17). Blood samples were collected and ultrasound exams (B-mode and color Doppler) were performed at two timepoints: immediately before d-cloprostenol and EB treatments and prior to NSER. Estrous behavior, corpora lutea count and NSER success outcomes were not affected by EB treatments nor parity (P>0.05). Embryo recovery rate was greater for ewes in the 0.5EB group and in the NULL ewes (P<0.05). Ovarian biometrics differed between the two evaluation timepoints in all groups (P<0.05). Plasma estradiol increased over time, reaching a significant greater level in 1.0EB ewes compared to controls on D17 (P<0.05), whereas progesterone concentrations decreased over time in all groups (P>0.05). In conclusion, EB treatment did not affect neither the NSER procedure success nor luteal function. Therefore, the NSER technique can be successfully performed in ewes with or without prior treatment with EB.
Keywords: cervical dilation; NSER; progesterone; sheep.

1. Introduction

Non-surgical embryo recovery (NSER) has been a great advance for the sheep embryo industry and animal welfare because it allows successive collections and the use of simpler anesthesia protocols (FONSECA *et al.*, 2016). Nevertheless, one of the major obstacles in popularizing the use of NSER in sheep is the need for a cervical relaxation treatment before embryo recovery. Several studies (CANDAPPA; BARTLEWSKI, 2012; FONSECA *et al.*, 2016) have evaluated different treatment timing, routes, and drug dosage in an effort to develop a protocol that mimics the physiological cervical dilation mechanism, which naturally occurs during estrus and/or parturition (CANDAPPA; BARTLEWSKI, 2012). This physiological mechanism involves the induction of changes in hormonal releasing patterns, mainly estradiol (E2) and prostaglandin E2 (PGE2) (CANDAPPA; BARTLEWSKI, 2014; KERSHAW-YOUNG *et al.*, 2009) which actions induce the lysis of the corpus luteum (CL) and, finally, the relaxation of the cervical tissue (LEETHONGDEE, 2011). These two hormones act through a positive feedback mechanism, remodeling the cervical extracellular matrix and activating uterine oxytocin (OT) receptors, leading to a subsequent increase in uterine motility and contractility (CANDAPPA; BARTLEWSKI, 2012).

Considering the known effects of exogenous prostaglandin F2α (PGF) and estradiol benzoate (EB) on the mechanisms of cervical relaxation, it has been

established a protocol based on treatment with these hormones 16 h before the NSER procedure attempting to activate OT receptors and induce cervical relaxation (FONSECA *et al.*, 2016). Thus, the combination of PGF2α and EB administered 16 h prior to and OT treatment 20 min before NSER has shown to benefit cervical transposition rates in goats and sheep (CANDAPPA; BARTLEWSKI, 2012; FONSECA *et al.*, 2016; PEREIRA; SOHNREY; HOLTZ, 1998a). Despite this convincing improve on NSER efficiency in sheep, transitory negative effects of EB on embryos recovered from superovulated sheep have been reported (SANTOS *et al.*, 2021). In addition, restrictions for the use of EB have increased in some countries (HORMONES IN MEAT, *[s. d.]*). Together, these limitations could prevent the adoption and expansion of the NSER technique worldwide.

In goats, EB treatment prior to NSER is not required and recent studies have shown up to 100% NSER success in this specie (FONSECA *et al.*, 2021b; MAIA *et al.*, 2020). Interestingly, treatment with a PGF analogue (d-cloprostenol) 16 h prior to NSER in superovulated goats did not negatively affect pregnancy rates of fresh and frozen-thawed embryos transferred into recipient goats by semi-laparoscopy (FONSECA *et al.*, 2018a) or transcervically (MORAIS *et al.*, 2020). Taken that into account, our group previously tested and demonstrated the efficiency of no EB treatment for cervical relaxation in estrus-induced Dorper ewes, and reported interesting results that favors the reduction or exclusion of EB from the NSER protocol (DIAS *et al.*, 2020). Nonetheless, as for other developing technologies, that previous study still needs to be replicated in superovulated donors. Another study reported a 10-fold increase in plasma E2 after EB treatment 16 h prior to NSER (OLIVEIRA *et al.*, 2020), but it is not known whether this increase was associated only to exogenous EB treatment or if PGF could also play a role in this E2 increase as well. The effects of different EB dosage in association with PGF in the cervical relaxation protocol prior to NSER, however, has not been determined in sheep. In particular, their impact on P4 and E2 profiles in superovulated ewes not receiving EB prior to NSER remains to be elucidated.

Considering the lack of knowledge about the effects of the EB-PGF based cervical relaxation protocol on ovarian function and circulating hormonal profile of superovulated ewes, the present study evaluated the feasibility of NSER in multiparous and nulliparous Lacaune x Santa Inês crossbred superovulated ewes subjected to a cervical relaxation treatment in the presence or absence of EB. Moreover, we tested

whether a dose reduction and/or removal of EB from the protocol affect luteal function and the outcomes of the NSER technique.

2. Material and methods

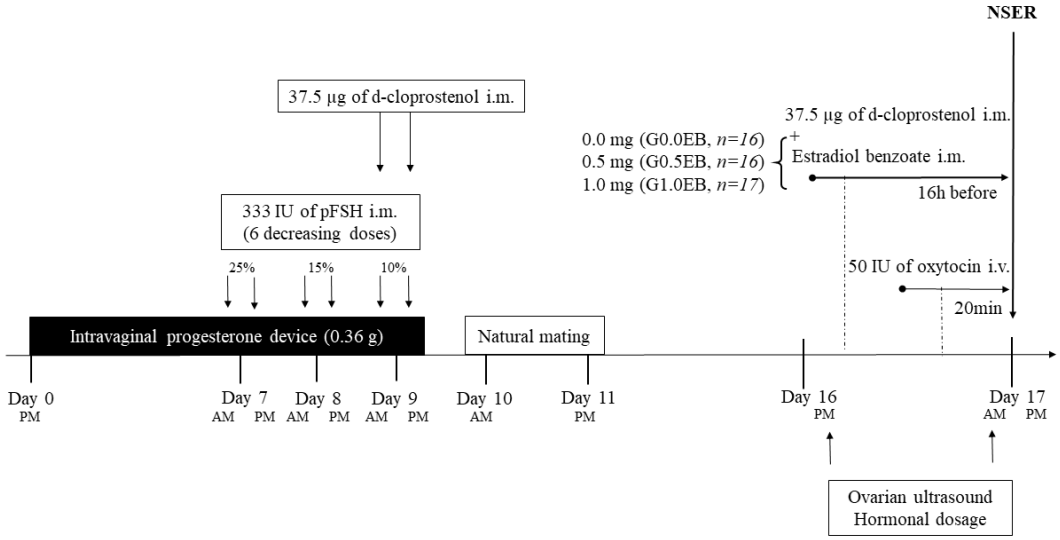
2.1. Ethics, location, and experimental animals

This study was conducted after the approval by the Ethics in Animal Care Committee of Embrapa Dairy Cattle (protocol 6365271119). The experiment was carried out during the non-breeding season, at the experimental farm located in Coronel Pacheco, Minas Gerais, Brazil (latitude 21° 38' 9" S, longitude 43° 19' 9" W). A total of 36 multiparous (MULT) and 13 nulliparous (NULL) crossbred ewes (Lacaune X Santa Inês; n=49), with 4.5 ± 0.2 and 2.5 ± 0.1 years of age (MULT and NULL, respectively) and 3.2 ± 0.1 and 3.1 ± 0.1 body condition score (scale 1 to 5; 1 = emaciated and 5 = obese) were enrolled in the study. All animals were kept in a confinement system, fed corn silage supplemented with a balanced ration and minerals with *ad libitum* access to water.

2.2. In vivo embryo production and NSER procedures

All ewes, at random stages of the estrous cycle, received an intravaginal device (D0) containing 0.36 g of P4 (PRIMER PR® Caprinos e Ovinos, Tecnopec – Agener União, Taboão da Serra, Brazil) kept for nine days. Superovulation was induced by i.m. treatments with 333 IU of pFSH (PLUSET®, Biogénesis Bagó, Curitiba, Brazil) beginning 60 h (D6) prior to P4 device withdrawal (D9). Ewes received pFSH twice daily (12 h intervals), in six decreasing doses (25, 25, 15, 15, 10, 10% of the total dose), as depicted in Figure 1. Additionally, 37.5 µg of a PGF2α analogue (d-cloprostenol; Prolise®, Agener União, Taboão da Serra, Brazil) was injected twice i.m., concurrently with the fifth and sixth doses of pFSH. After P4 device withdrawal, ewes were checked for estrous behavior and naturally mated to fertile rams (ram to ewe ratio 1:7) every 12 h, while in standing estrus.

Figure 1: Schematic representation of the experimental procedures performed to evaluate luteal characteristics, circulating E2 and P4 concentration profiles, and embryo yield in superovulated ewes submitted to a cervical relaxation protocol using different doses of EB (0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin. i.m. intramuscular; i.v. intravenous prior to non-surgical embryo recovery (NSER)



The cervical relaxation protocol started 16 h before NSER. At this time, females were blocked by parity (MULT and NULL) and randomly assigned into three treatment groups to receive 37.5 µg of d-cloprostenol i.m. associated or not with different doses of EB (RIC-BE®, Agener União, São Paulo, Brazil) as follows: 0.0 mg (Control group; n=16, NULL = 3 and MULT = 13); 0.5 mg (0.5EB group; n=16, NULL = 4 and MULT = 12) or 1.0 mg (1.0EB group, n=17, NULL = 6 and MULT = 11). All ewes were treated with 50 IU of oxytocin i.v. (Ocitocina Forte UCB®, Jaboticabal, Brazil) 20 min before NSER.

The procedures for NSER were performed as previously described elsewhere (DIAS *et al.*, 2020). Ewes that were not detected in estrus and/or did not present with a CL 24 h before NSER (n= 13) were indeed submitted to NSER to provide data on feasibility of the NSER technique, but other data from those females were not considered to evaluate superovulatory response nor the NSER procedure efficiency relative to structures recovered. Ova/embryos recovered were classified according

IETS manual (MAPLETOFT; REMILLARD; LINDSEY, 2020) and recorded for all females.

2.3. Ultrasonography and image analysis

Ovarian ultrasound evaluations (B-mode and color Doppler) were performed immediately before the beginning of the cervical relaxation protocol (D16) and prior to the NSER procedure (D17) in all ewes using an ultrasound device (Mindray M5Vet®, Shenzen, China) equipped with a transrectal linear probe, fitted with a rigid protractor (transducer frequency: 8.0 MHz; overall gain: 80%; depth 6.2 cm and dynamic range, DR: 70dB). The color Doppler settings were also the same during all evaluations (pulse repetition frequency, PRF: 0.9 KHz; overall gain 72%; depth 6.2 cm; transducer frequency: 4.6 MHz; and wall filter, WF: 1).

All antral follicles ≥ 5 mm in diameter, eventual luteinized anovulatory follicles (LUF), and CL present on the ovaries were counted, recorded, and measured using the Image J® software (Wayne Rasband, National Institutes of Health, USA). The diameter (average length of the vertical and horizontal axes) and area of the CL were measured using the frame image at the largest CL diameter. Volume of luteal tissue was calculated using a formula for the volume of a sphere ($V = 4/3\pi r^3$, where r = radius and π = 3.1416). For CL with a fluid-filled cavity, the dimensions of the cavity were subtracted from total volume. Diameter, area, and volume of luteal tissue were calculated as the sum of all CLs on the ovaries of a given animal.

The area of ovarian blood perfusion was measured on a frame image containing the largest cross section of the ovary with the strongest color Doppler signals in addition to one frame immediately before and one frame immediately after, using the Image J® software. The ovarian perfusion area (excluding the ovarian pedicle region) was calculated within each frame using the formula: *number of colored pixels /number of total ovarian pixels X 100*. Then, we calculated the average of the three evaluated frames. As for luteal echogenicity, we analyzed pixel brightness (mean pixel value; MPV) and pixel heterogeneity (standard deviation of the MPV) within a B-mode frame at the largest cross section of the ovary with the greatest number of visible luteal structures. Echogenicity was assessed using the Image Pro Plus® 7.0TM software (Media Cybernetics Inc., San Diego, CA, USA) with a scale of 256 shades of gray (0 = black and 255 = white). We used a selection tool to analyze circular areas of interest on the luteal tissue, avoiding eventual luteal cavities, if present.

2.4. Blood sampling and hormonal analysis

Blood samples were collected prior to ultrasound examinations (D16 and D17) by venipuncture of the jugular vein, using 4 mL vacuum tubes (BD Vacutainer® Lithium Heparine Tubes, São Paulo, SP, Brazil). Samples were centrifuged at 1500 × *g* for 10 min, plasma was harvested and stored at -20 °C until further analysis. Plasma progesterone and estradiol concentrations were determined by solid-phase radioimmunoassay (RIA) using I^{125} kits (MP Biomedicals LLC, Orangeburg, NY) according to the manufacturer's instructions. Sensitivity and intra-assay coefficients of variation were 5.0 pg/mL and 2.2% for estradiol and 0.075 ng/mL and 2.7% for progesterone, respectively.

2.5. Statistical analyses

The following data were recorded for all ewes enrolled in the study: number and proportion of ewes in standing estrus; interval from P4 removal to estrous behavior; number and proportion of SOV response (i.e. ewe in estrus with at least one CL on the day before NSER); overall CL count; overall LUF count; number and proportion of ewes presenting LUF on D16; time to pass the Hegar dilator through the cervix; number and proportion of ewes that the Hegar dilator was successfully passed through cervix; mean duration of mandrel-catheter passing through; duration of uterine flushing; duration of the NSER procedure; number and proportion of ewes successfully submitted to the NSER procedure (ewes successfully transposed with mandrel-catheter and flushed); flushing efficiency (percentage of fluid recovered post-infusion relative to the 400 mL of initial volume, calculated using the formula: volume retrieved / 400 mL x 100); CL count in ewes successfully flushed; ova/embryo recovery rate (considering ewes with at least one CL); number of ova/embryos recovered from ewes successfully flushed; number of transferable embryos (Code 1 and 2); number of unfertilized ova; number of non-viable embryos (Code 4); CL count on D16 and D17; luteal tissue diameter (mm), area (mm^2) and volume (mm^3); ovarian blood perfusion (mm^2); luteal tissue mean pixel value and heterogeneity; plasma P4 concentration (ng/mL); plasma E2 concentration (pg/mL).

Statistical analysis was performed using the SAS® Studio software. The Bartlett test was used to determine homogeneity of variances and Shapiro-Wilk test used to verify normality. Parametric data (interval to estrus, CL count, time to pass Hegar,

duration of mandrel passing, duration of flushing, duration of entire procedure, CL count in ewes successfully flushed) were analyzed by one-way ANOVA using the PROC GLM followed by a post-hoc Tukey test to determine differences among means. Non-parametric data (LUF count, number of ova/embryos recovered, transferable and degenerated embryos, unfertilized ova and zona pelucidae) were analyzed using PROC NPAR1WAY with the Wilcoxon test. For binomial data, contingency tables were analyzed using the Chi-Square test to determine statistical differences. Variables with repeated measures over time (plasma P4 and E2 concentrations, CL diameter, luteal tissue area and volume, ovarian blood perfusion and luteal tissue echogenicity and heterogeneity) were evaluated using the PROC MIXED procedure with a repeated statement to assess the interactions treatment and time. The statistical model included main effects of treatment, days of evaluation, and their interaction. Wilcoxon test was used to determine differences between means of ordinal-paired data. Statistical significance was determined based on a P-value of 0.05.

3. Results

Data relative to superovulatory responses and NSER outcomes are shown in Table 1. Table 2 presents superovulatory responses in NULL and MULT ewes.

Table 1: Superovulatory and non-surgical embryo recovery (NSER) outcomes (% or mean ± SEM) in ewes submitted to a superovulation protocol[1] and different doses (0.0, 0.5 and 1.0 mg) of estradiol benzoate plus 37.5 µg of d-cloprostenol 16 h before and 50 IU of oxytocin 20 min prior to NSER, used as a cervical relaxation protocol for NSER

	Estradiol benzoate			
Endpoints	0.0 mg	0.5 mg	1.0 mg	P value
Estrus response (%)	93.7 (15/16)	93.7 (15/16)	88.2 (15/17)	0.80
Interval to estrus (h)	21.7 ± 3.0	24.0 ± 2.8	23. 3 ± 3.2	0.98
Ewes not responding to SOV (%)	33.3 (5/15)	20.0 (3/15)	6.7 (1/15)	0.24
Overall CL count[2]	7.0 ± 1.8 [105]	6.9 ± 1.2 [103]	8.6 ± 1.6 [129]	0.70
Overall LUF count[2]	2.0 ± 0.3 [18]	2.1 ± 0.3 [23]	2.5 ± 0.6 [28]	0.95
Ewes with LUF (%)	90.0 (9/10)	91.7 (11/12)	78.6 (11/14)	0.57
Successful Hegar dilator transposing rate (%)	100.0 (10/10)	83.3 (10/12)	71.4 (9/14)	0.09
Duration of Hegar dilator transposing (min)	2.4 ± 0.9	4.1 ± 1.2	4.5 ± 1.1	0.31
Duration of Mandrel-Catheter transposing (min)	1.6 ± 0.9	2.3 ± 1.0	1.2 ± 0.4	0.82

Duration of uterine flushing (min)	15.0 ± 0.7	20.2 ± 3.9	16.1 ± 0.9	0.20
Duration of NSER procedure (min)	20.4 ± 1.0	25.9 ± 3.3	22.2 ± 1.5	0.33
Successful NSER procedure rate (%)	90.0 (9/10)	83.3 (10/12)	64.3 (9/14)	0.28
Flushing efficiency (%)	100.0	100.0	100.0	-
CL count in ewes successfully flushed	10.1 ± 2.2 [91]	8.3 ± 1.1 [83]	10.8 ± 2.1 [97]	0.60
Recovery rate (%)	60.4^{c} (55/91)	107.2^{a} (89/83)	82.5^{b} (80/97)	0.001
Ova/embryos recovered	6.1 ± 2.2 [55]	8.9 ± 1.7 [89]	8.9 ± 2.7 [80]	0.62
Transferable embryos[3]	3.7 ± 1.5 [33]	6.2 ± 1.7 [62]	4.8 ± 2.1 [43]	0.63
Unfertilized ova	1.9 ± 1.0 [17]	1.8 ± 1.2 [18]	3.7 ± 1.8 [33]	0.49
Degenerated embryos[3]	0.6 ± 0.3 [5]	0.7 ± 0.5 [7]	0.1 ± 0.1 [1]	0.72
Zona pelucidae	0.0 ± 0.0 [0]	0.2 ± 0.1 [2]	0.3 ± 0.2 [3]	0.77
Viability rate (%)	60.0 (33/55)	69.7 (62/89)	53.7 (43/80)	0.10

CL = corpora lutea; LUF = luteinized unovulated follicle.

(n/n) Number of animals, ovarian structures or recovered ova/embryos.

[n] Total sum of animals, ovarian structures or recovered ova/embryos.

[1] Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing doses of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5µg d-cloprostenol at the time of last two pFSH doses.

[2]Considering ewes that manifested estrous behavior with at least one CL on D16

[3]Transferable embryos = embryos Code 1 and Code 2; Degenerated embryos = embryos Code 4

[a,b] Within a row, means without a common superscript differed ($P<0.05$)

Table 2: Superovulatory and non-surgical embryo recovery (NSER) outcomes (% or mean ± SEM) in superovulated[1] nulliparous and multiparous ewes submitted to a cervical relaxation protocol before NSER.

	Category			
Endpoints	Nulliparous	Multiparous	Total	P value
Estrus response (%)	92.3 (12/13)	91.7 (33/36)	91.8 (45/49)	0.60
Interval to estrus (h)	23.0 ± 2.7	26.8 ± 1.9	25.8 ± 1.6	0.65
Ewes not responding to SOV	0.0 (0/12)	27.3 (9/33)	20.0 (9/45)	0.13
Overall CL count[2]	8.2 ± 1.1 [98]	7.4 ± 1.2 [239]	7.6 ± 0.9 [337]	0.59
Overall LUF count[2]	1.9 ± 0.4 [23]	1.9 ± 0.3 [46]	1.9 ± 0.2 [69]	0.78
Ewes with LUF (%)	83.3 (10/12)	63.6 (21/33)	68.9 (31/45)	0.28
Successful Hegar dilator transposing rate	66.7 (8/12)	87.5 (21/24)	80.5 (29/36)	0.33
Duration of Hegar dilator transposing (min)	5.3 ± 1.2	3.1 ± 0.8	3.8 ± 0.7	0.31
Duration of Mandrel-Catheter transposing (min)	2.1 ± 1.2	1.6 ± 0.6	1.7 ± 0.5	0.79
Duration of uterine flushing (min)	21.1 ± 4.9	15.7 ± 0.5	17.2 ± 1.4	0.13
Duration of procedure	25.1 ± 4.2	21.1 ± 0.9	22.3 ± 1.4	0.60

Successful NSER procedure rate (%)	100.0 (8/8)	95.2 (20/21)	96.5 (28/29)	0.82
Flushing efficiency (%)	100.0	100.0	100.0	-
CL count in ewes washed	9.5 ± 1.0 [76]	9.7 ± 1.4 [195]	9.7 ± 1.0 [271]	0.91
Recovery rate (%)	107.9 [a] (84/76)	71.7 [b] (140/195)	82.6 (224/271)	0.001
Ova / embryos number	10.5 ± 2.7 [84]	7.0 ± 1.4 [140]	8.0 ± 1.2 [224]	0.27
Transferable embryos[3]	7.5 ± 1.3[a] [60]	3.9 ± 1.2[b] [78]	4.9 ± 1.1 [138]	0.05
Unfertilized ova	2.0 ± 1.9 [16]	2.6 ± 0.8 [52]	2.4 ± 0.8 [68]	0.55
Degenerated embryos[3]	0.4 ± 0.4 [6]	0.3 ± 0.2 [7]	0.5 ± 0.2 [13]	0.92
Zona pelucidae	0.2 ± 0.3 [2]	0.1 ± 0.1 [3]	0.2 ± 0.1 [5]	0.90
Viability rate (%)	71.5[a] (60/84)	55.7[b] (78/140)	61.6 (138/224)	0.03

CL = corpora lutea; LUF = luteinized unovulated follicle.

( ) Number of animals, ovarian structures or recovered ova/embryos.

[ ] Total sum of animals, ovarian structures or recovered ova/embryos.

[1] Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing doses of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5µg d-cloprostenol at the time of last two pFSH doses.

[2]Considering ewes that manifested estrous behavior

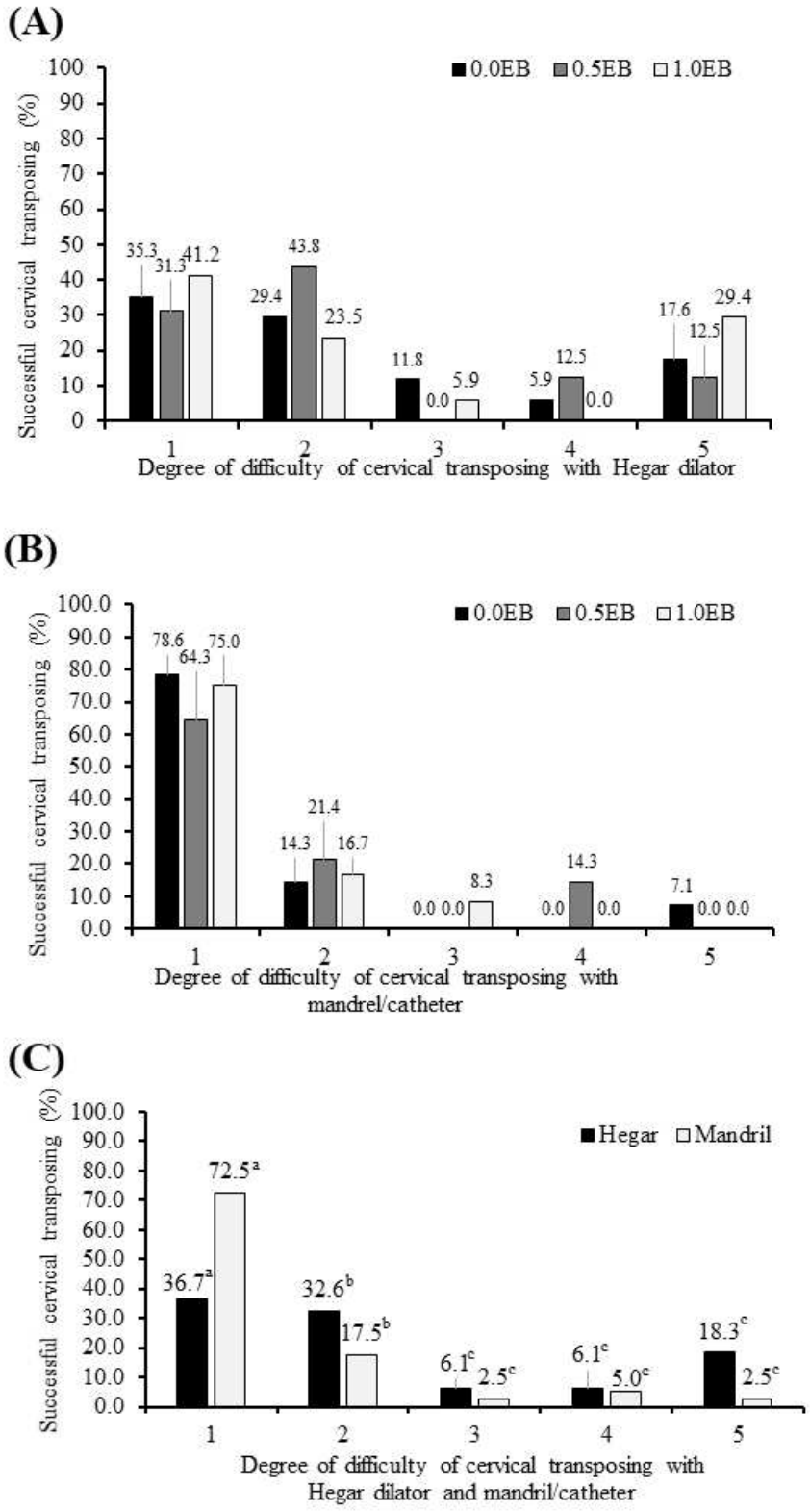
[3]Considering ewes with at least one CL on D16

[3]Transferable embryos = embryos Code 1 and Code 2; Degenerated embryos = embryos Code 4

[a,b] Different letters indicate differences among groups (P<0.05)

Overall, the 0.5EB group had a greater recovery rate compared with groups 0.0 and 1.0EB ($P<0.05$), whereas NULL ewes presented higher recovery and embryo viability rates compared with MULT ewes ($P<0.05$). Three animals in 0.5EB group and one animal in 1.0EB group had an individual embryo recovery rate greater than 100% because the number of recovered structures was greater than CL count. Interestingly, these animals were all nulliparous. As a result, total recovery rate was increased in 0.5EB group and in the nulliparous animals. Time to complete the NSER procedure did not differ among groups nor parity ($P>0.05$). The rate of passing through cervix success and the degree of difficulty using the Hegar dilator versus mandrel-catheter device did not differ among groups (Figure 2; $P>0.05$). In addition, we observed a greater number of animals classified into scores 1 and 2 for passing through cervix with Hegar or mandrel-catheter ($P<0.05$).

Figure 2: Successfully cervical transposing rates (y-axis) in ewes submitted to a 9-day progesterone-based estrus synchronization protocol associated with a superovulatory treatment using 333 IU of pFSH (six decreasing doses starting 60 h before P4 device removal), and a cervical relaxation protocol using different doses of estradiol benzoate (EB; 0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin before non-surgical embryo recovery (NSER). The x-axis represents the degree of difficulty in passing through transposing the cervix with the Hegar dilator (A), mandrel and catheter equipment (B) or both (C). Scale 1 to 5, where (1) very easy, time to pass cervix <1 min; (2) easy, time to pass cervix 1 to 3 min; (3) moderate difficulty, time to pass cervix 3 to 7 min; (4) difficult; time to pass cervix 7 and 10 min; and (5) impossible to penetrate the cervix with dilator or unable to complete cervical passage. ^{a,b}Letters differ between groups and procedure in the same degree of difficulty (Wilcoxon test; $P<0.05$).



Luteal morphology and functional data are shown in Table 3.

Table 3: Luteal characteristics on D16 and D17 (immediately before and after cervical relaxation protocol, respectively) in superovulated ewes submitted to a protocol with different doses (0.0, 0.5 and 1.0 mg) of estradiol benzoate (EB) plus 37.5 µg of d-cloprostenol 16 h before and 50 IU of oxytocin 20 min prior to non-surgical embryo recovery (NSER)

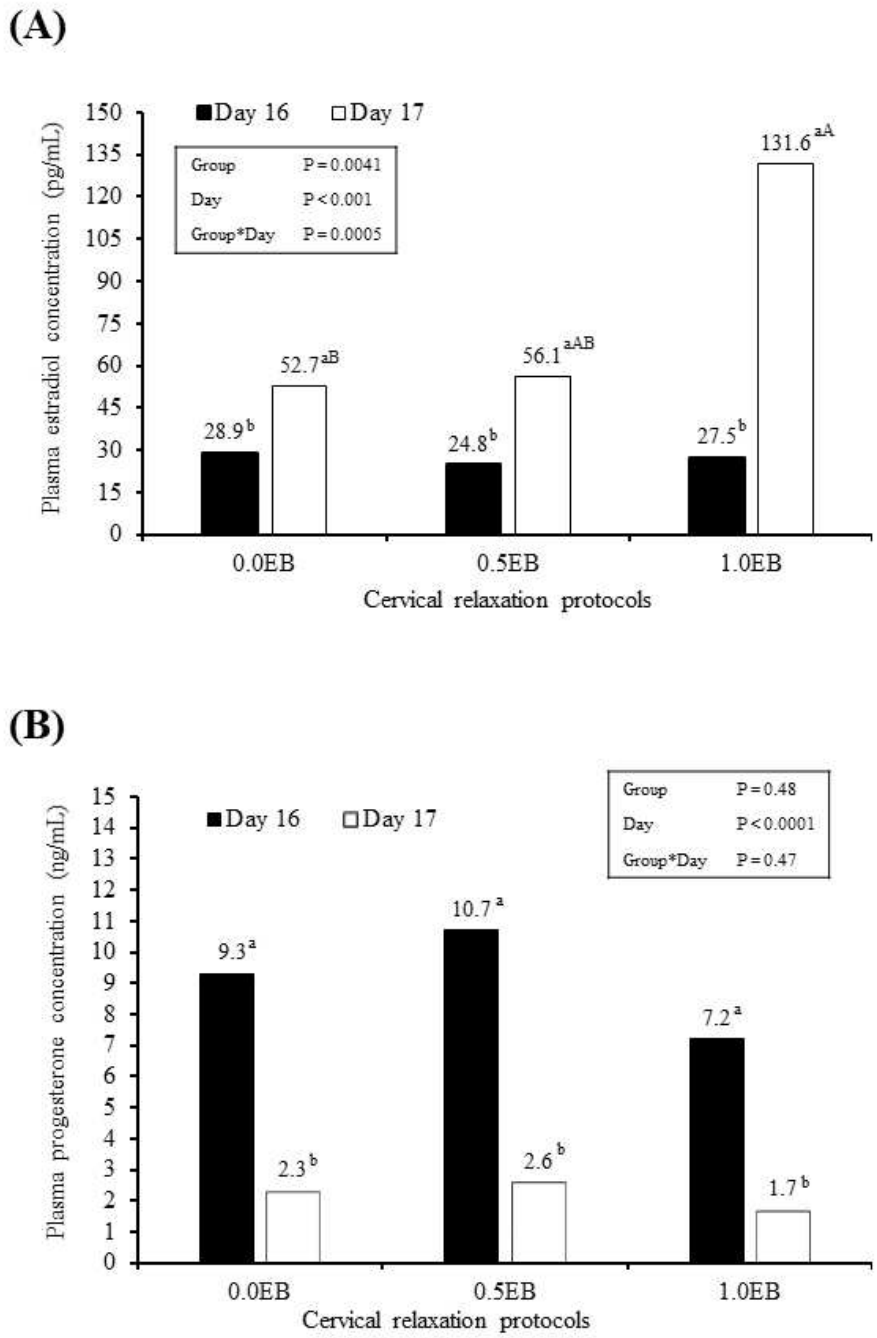
	D16			D17		
Endpoints	0.0EB	0.5EB	1.0EB	0.0EB	0.5EB	1.0EB
Overall CL count	10.1 ± 2.2	8.3 ± 1.1	10.8 ± 2.1	10.1 ± 2.2	8.3 ± 1.1	10.8 ± 2.1
CL diameter (mm)	81.1 ± 14.9^{a}	59.5 ± 7.5^{a}	62.3 ± 11.2^{a}	65.8 ± 12.1^{b}	54.5 ± 5.9^{b}	53.9 ± 9.2^{b}
CL volume (mm^3)	2635.3 ± 476.1^{a}	1710.7 ± 287.4^{a}	1802.4 ± 320.1^{a}	1492.6 ± 303.9^{b}	1232.8 ± 153.4^{b}	859.1 ± 184.7^{b}
CL area (mm^2)	436.7 ± 78.7^{a}	309.7 ± 43.8^{a}	313.0 ± 58.4^{a}	301.2 ± 56.5^{b}	255.5 ± 29.4^{b}	243.6 ± 38.6^{b}
Ovarian blood perfusion (%)	27.0 ± 2.1	30.1 ± 2.8^{a}	24.1 ± 2.1	20.6 ± 2.9	21.0 ± 3.1^{b}	24.5 ± 1.5
CL echogenicity	60.9 ± 3.2	62.0 ± 3.0	59.0 ± 3.3	57.6 ± 2.2	54.1 ± 1.5	60.0 ± 1.4
CL heterogeneity	9.7 ± 0.7	8.9 ± 0.5	9.9 ± 0.4	9.5 ± 0.5	9.1 ± 0.5	9.3 ± 0.4

^{ab} Different letters indicate differences between the moments evaluated within the same group ($P<0.05$). * $P>0.05$ for comparison between group on the same day.

CL – corpus luteum

We did not observe any differences in luteal morphology among groups on D16 and D17($P>0.05$). Luteal diameter, volume, and area decreased from D16 to D17 in all groups ($P<0.05$). The percentage of ovarian blood perfusion decreased from D16 to D17 ($P<0.05$) only in the 0.5EB group. Plasma estradiol concentrations increased from D16 to D17 in all groups (Figure 3A; ($P<0.05$). Within a day, E2 concentrations did not differ among groups on Day 16, whereas it was greater in 1.0EB group compared with 0.0EB group on D17 ($P<0.05$). Plasma progesterone concentrations (Figure 3B) decreased from D16 to D17 in all groups ($P<0.05$).

Figure 3: Plasma concentrations of estradiol (A) and progesterone (B) on D16 and D17 (immediately before and after cervical relaxation protocol, respectively) in ewes submitted to a 9-day progesterone-based estrus synchronization protocol associated with a superovulatory treatment using 333 IU of pFSH (six decreasing doses starting 60 h before P4 device removal), and a cervical relaxation protocol using different doses of estradiol benzoate (EB; 0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin before non-surgical embryo recovery (NSER). ^{a,b}Different lowercase letters indicate differences between days within the same group; ^{A,B}Uppercase letters indicate differences between groups within the same day (Tukey; $P<0.05$).



4. Discussion

This study confirmed that NSER can be successfully performed in superovulated Lacaune x Santa Inês crossbred ewes using a reduced EB dose or even abolishing the use of EB in the cervical relaxation protocol. In addition, this study demonstrates for the first time the feasibility of NSER in nulliparous animals, without affecting procedure duration nor its success rates. We provide evidence that not only EB but also PGF (d-cloprostenol) used to relax the cervix may affect luteal function of

superovulated females submitted to NSER, mainly by triggering the process of luteolysis. Another important finding was that treatment with 1.0 mg EB led to a 2.5-fold increase in plasma E2 concentration compared with controls (0.0EB) together with nearly basal levels of P4 in a phase of the estrous cycle (diestrus) where plasma P4 levels should be at/or close to their zenith. Therefore, this study brings novel information to help elucidate the impact of a hormonal cervical relaxation protocol upon luteal and endocrine function in superovulated sheep submitted to NSER.

Our findings using a PGF2α-OT cervical relaxation protocol in superovulated sheep is reminiscent of previous studies on ewes with synchronized estrus (DIAS *et al.*, 2020) and superovulated goats (PEREIRA; SOHNREY; HOLTZ, 1998a), demonstrating that a single d-cloprostenol treatment 16 h before NSER associated with OT is efficient to promote cervical relaxation. Here we observed similar efficiency in cervix-transposing rates and time intervals for all the steps of the procedure among the three experimental groups, indicating the success and feasibility of this new cervical relaxation protocol for NSER in crossbred sheep. This lack of differences in time to execute the procedures among groups contradicts a previous study by our group, which reported that it took longer to pass the Hegar dilator and mandrel-catheter through the cervix in the control group (no EB) compared with females receiving either 0.5 or 1.0 mg EB (DIAS *et al.*, 2020). This apparent contradiction may be explained by the greater number of oxytocin receptors after superovulation treatments (MCCANN; FLINT, 1990). This study also confirm the efficiency of the NSER technique in nulliparous females, in disagreement with previous studies reporting that NSER was not feasible for this animal category (KAABI *et al.*, 2006; NAQVI *et al.*, 2005). Perhaps the success of NSER is more related to the individual cervical anatomy than their reproductive status or parity. Degrees of difficulty in cervical transposition regardless of the treatment used for relaxation were still observed in this study, as well as in Dias et al. (2020). This corroborates once again that 1) the cervical anatomy of the species is a major obstacle to a successful performance of the technique and 2) it is necessary to previously select the animals for the existence of visible abnormalities on visual inspection that could compromise NSER procedure efficiency (CANDAPPA; BARTLEWSKI, 2012; FONSECA *et al.*, 2018b).

The higher recovery rate observed in the 0.5EB group and nulliparous females may be due to a reduction in the accuracy of the ultrasound evaluation. According to Pinto *et al.* (2017), animals with a high superovulatory response (>8 CL) have a

decreased accuracy and sensitivity of luteal count by ultrasound compared with poor responders (≤4 CL). Still, ultrasound luteal counting remains an effective, less invasive, real-time alternative to assess the number of CL on the ovaries. The occurrence of multioocyte follicles has already been described in sheep and goat primordial follicles (GURAYA *et al.*, 1994; OLIVEIRA *et al.*, 2017), but the literature has no reports of that particular phenomenon in antral follicles. Nonetheless, this may explain the lack of agreement luteal counting and recovered structures. Finally, as expected, females with a higher recovery rate has also a higher mean number of viable embryos recovered.

Previous information suggests that the mechanism by which PGF2α promotes cervical relaxation involves triggering luteolysis and the growth of preovulatory follicles (IVELL, 2000). During the early luteal phase, the pattern of prostaglandin secretion is controlled primarily by the higher concentrations of P4, which inhibits E2 and OT endometrial receptors until the end of the luteal phase. After an exogenous treatment with PGF2α, however, P4 levels decrease and E2 receptors are bound by circulating estrogen, stimulating OT receptors, which eventually triggers the release of more prostaglandins, culminating in luteolysis (SILVIA *et al.*, 1991). This explains why not only EB-treated groups had an increase in E2 concentration on D17, but also controls (0.0EB group) had an almost two-fold increase in E2 concentration 16 h after treatment. In addition, we observed a decrease in P4 concentration associated with the changes in plasma E2 levels on D17, corroborating the proposed mechanism of cervical dilatation as a result of luteolysis, similar to what has demonstrated in superovulated goats (PEREIRA; SOHNREY; HOLTZ, 1998a).

Association of the P4 dosage and the evaluation of CL morphology (i.e., diameter, area, and volume) (GONZALEZ DE BULNES *et al.*, 2000) and echotexture (ARASHIRO *et al.*, 2010; SIQUEIRA *et al.*, 2009) have been used as tools to assess luteal functionality during estrous cycle. It can be noted that the cervical relaxation protocol directly interfered on the luteal function of superovulated ewes, regardless of the use of EB treatment, not only because of the changes in P4 and E2 levels, but also for a noticeable effect on CL morphology, with a reduction in luteal diameter, volume, and area on D17. The exogenous administration of PGF2α induced luteolysis, leading to a functional and physical regression of the CL. This was a result of the loss of communication between endothelial and steroidogenic cells, reducing the blood supply to the luteal tissue and, consequently, dropping P4 concentration (ACOSTA; MIYAMOTO, 2004). However, neither effect on echogenicity nor heterogeneity was

observed. According to Davies et al. (DAVIES *et al.*, 2006) and Arashiro et al. (ARASHIRO *et al.*, 2010), the correlation between steroidogenic function and echogenicity is low and poor, limiting the use of this tool as a predictor of luteal function, while heterogeneity seems to be cycle-dependent (SIQUEIRA *et al.*, 2009), which would justify the lack of any significant difference between groups and moments of observation.

It is important to verify how the exposure to an environment of unexpected hormonal changes can directly or indirectly affect the viability and post-transfer survival of embryos collected by NSER. Previous studies have shown that goat embryos collected non-surgically from donors treated only with a PGF-analogue (FONSECA *et al.*, 2014, 2018a; GARZA *et al.*, 2018; MORAIS *et al.*, 2020) or sheep donors (FIGUEIRA *et al.*, 2019) receiving d-cloprostenol and EB resulted in acceptable pregnancy rates after the transfer of fresh or frozen-thawed embryos. Likewise, treatment with PGF and OT prior to NSER in superovulated goats did not affect the viability of collected embryos (PEREIRA; SOHNREY; HOLTZ, 1998a). Although progesterone levels did not differ between groups, it is noteworthy that P4 was near to basal levels in the 1.0EB group, which may affect embryonic viability to some extent, considering that adequate circulating P4 is essential for embryonic survival (MORRIS; DISKIN, 2008).

5. Conclusion

This study provides evidence that NSER can be efficiently performed after a cervical relaxation protocol without the use of EB in superovulated Lacaune x Santa Inês crossbred ewes, regardless of their category (nulliparous and multiparous). One must consider the isolated contribution of d-cloprostenol on the cervical relaxation protocol to change circulating P4 and E2 profiles. The feasibility of NSER in nulliparous females opens a new frontier for the use of assisted reproduction in young females, further expanding the spectrum of use of NSER in sheep.

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CONFLICTS OF INTERESTS

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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3. Chapter 4 – Single dose of 300 IU hCG in the early luteal phase in superovulated ewes: effects on corpora lutea, progesterone profile, and embryo recovery

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Single dose of 300 IU hCG in the early luteal phase in superovulated ewes: effects on corpora lutea, progesterone profile, and embryo recovery

Dias, JH[1]; Gonçalves, JD[2]; Arrais, AM[3]; Batista, RITP[4]; Souza-Fabjan, JMG[4]; Bastos, R[5]; Siqueira, LGB[6]; Oliveira, MEF[2]; Fonseca, JF[7]

[1]Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, 36570-000, Viçosa, MG, Brazil

[2]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de acesso Prof. Paulo Donato Castellane, s/n, 14884-900, Jaboticabal, SP, Brazil

[3]Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465, Km7, s/n, 23890-000, Seropédica, RJ, Brazil

[4]Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, 24230-340, Niterói, RJ, Brazil

[5]Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego, n° 2000, 28013-642, Campos dos Goytacazes, RJ, Brazil

[6]Embrapa Gado de Leite, Av. Eugênio do Nascimento, 610, 36038-330, Juiz de Fora, MG, Brazil

[7]Embrapa Caprinos e Ovinos, Três Lagoas Farm, Road Sobral –Groaíras, km 04, CP 145, 62010-970, Sobral, CE, Brazil

*Corresponding author: J.F. Fonseca, Tel.: +55 32 3311-7662, Três Lagoas Farm, Road Sobral –Groaíras, km 04, CP 145, 62010-970, Sobral, CE, Brazil

E-mail address: jeferson.fonseca@embrapa.br

ABSTRACT

This study aimed to evaluate the effects of hCG treatment during the early luteal phase on ovarian function, progesterone profile, and embryo yield in superovulated ewes. Superovulated sheep were randomly assigned to receive 300 IU hCG i.m. (GhCG, n=24) or not (GControl, n=25) at 96 h after the removal of the progesterone (P4) device (D13). Non-surgical embryo recovery (NSER) was performed eight days after P4 withdrawal. Ultrasound evaluations were performed on D13, D14, D16, and D17. Blood samples were collected on D14, D16, and D17. Superovulation scores were recorded based on the number of corpora lutea (CL) as follows: 1 (≤ 2), 2 (3 to 5), 3 (6 to 8), and 4 (≥ 9). NSER efficiency, superovulation response, and luteal tissue area were similar

in both groups (P > 0.05). Structural luteolysis tended to be higher in GControl (P = 0.07; 47.0%) while functional luteolysis was similar in both groups (P > 0.05; 0.0 and 5.9 %). The recovery rate was greater (P < 0.05) in GhCG (89.8 %) compared with GControl (71.0 %), with similar overall ova/embryo numbers observed for both groups (P > 0.05). GhCG showed a higher concentration of animals with a superovulatory response score of score 4 (54.5%; P < 0.05) compared with the lowest scores. Plasma progesterone on D16 was higher (P < 0.05) in GhCG ewes (11.1 ± 1.5 vs 6.9 ± 1.5 ng/mL). In conclusion, the hCG treatment improved circulating P4 and embryo recovery rate, tended to maintain luteal functionality, and thus constitutes an additional tool for improving embryo yield in superovulated ewes.

Keywords: embryo, corpus luteum, NSER, superovulation, ovine.

1. Introduction

Multiple ovulation and embryo transfer (MOET) is one of the most effective techniques to increase the spread of genetically superior females, but the variability in superovulatory responses remains a limiting factor for its wider adoption and success in small ruminants (Cognie, 1999). Particularly for these species, premature luteal regression (PRCL) is a major cause for concern as it can reduce the mean number of transferable embryos (Gonźalez-Bulnes et al., 2004; Oliveira et al., 2014), impeding the success of MOET. Moreover, the incidence of PRCL in small ruminants is relatively high, reaching an average of 25% in superovulated sheep regardless of season, body condition score, and age (Rocha et al., 2021).

PRCL is related to an impairment of the corpora lutea (CL) function caused by the premature secretion of uterine prostaglandin F2α (PGF2α) during the early luteal phase of the estrous cycle, leading to the onset of CL regression three to six days after estrus and, ultimately, to low P4 concentrations (< 1 ng/mL) (Saharrea et al., 1998; Cervantes et al., 2007). Although the mechanisms of this process are not fully elucidated, it has been suggested that this phenomenon is associated with an alteration in the ovarian E2:P4 ratio after superovulation, probably due to inadequate follicle luteinization leading to consistently high estradiol (E2) production (Okada et al., 2000; Gonźalez-Bulnes et al., 2004). It is believed that this occurs due to increased E2 production by the greater number of developing follicles after superovulation treatment

with exogenous gonadotrophins (Kafi and McGowan, 1997; Saharrea et al., 1998). CL regression is accompanied by functional luteolysis, where there is an end to or decline in P4 production, and structural luteolysis, where apoptosis of luteal cells occurs resulting in structural changes in the CL and blood flow (Niswender et al., 2000). Consequently, ova transport becomes abnormal, reducing the embryo recovery rate (Ishwar and Memon, 1996).

Several studies argue that the use of luteotropic agents (GnRH or human chorionic gonadotropin (hCG) close to the time of ovulation may prevent or reduce the effects of PRCL (Brasil et al., 2016; Saharrea et al., 1998; Shabankareh et al., 1998; Shabankareh et al., 2012). Treatment with hCG stands out due to its potent luteotropic activity, which can promote an increase in luteal steroidogenic capacity resulting in greater P4 secretion (Farin et al., 1988; Rodrigues et al., 2022; Vergani et al., 2020) in addition to the commonly observed formation of accessory CL due to hCG's LH-like actions (Coleson et al., 2015). A 300 IU dose of hCG treatment on Day7 of the estrous cycle has been shown to significantly increase CL number and features (morphology, perfusion, and echogenicity) as well as circulating P4 concentrations in ewes (Fonseca et al., 2018, Vergani et al., 2020). In superovulated goats (Saharrea et al., 1998) and ewes (Shabankareh et al., 2012), treatment with 500 IU of hCG immediately after estrus prevented PRCL, but it was not able to reduce the number of anovulatory follicles (Armstrong et al., 1982; Kelidari et al., 2010). Nevertheless, one must consider that these previous studies used equine chorionic gonadotropin (eCG) as the superovulatory treatment, which may have contributed to the persistence of anovulatory follicles.

Since the presence of functional CL is the key to reproductive success in females, the pursuit of alternative treatments to prevent early luteolysis/CL regression may be the main strategy to improve MOET results in sheep. Here, we hypothesized that treatment with 300 IU of hCG early in the estrous cycle (four days after estrus) of superovulated ewes would positively affect CL development and, consequently, their P4 secretion, resulting in a greater embryo yield. Thus, the objectives of the present study were to evaluate the effects of a single treatment of 300 IU of hCG in ewes superovulated with commercial pFSH on ovarian function, superovulation response, and embryo yield.

2. Materials and methods

2.1. Ethics, location, and experimental animals

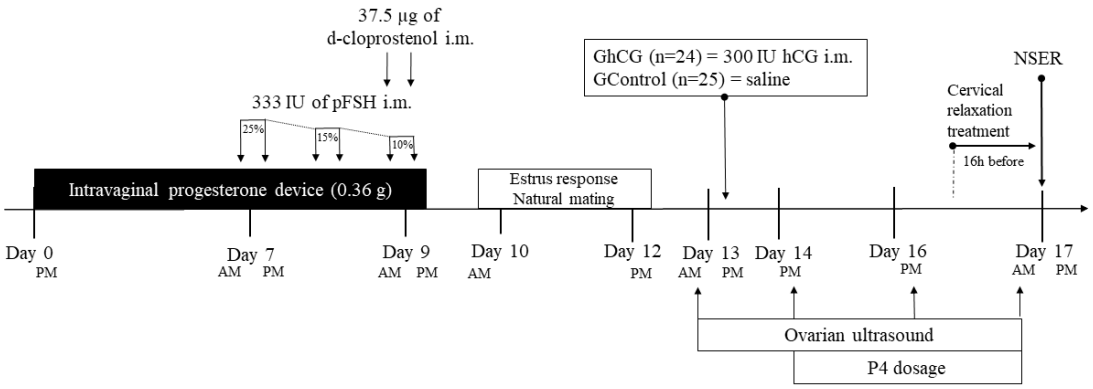
This study has been approved by the Embrapa Dairy Cattle Ethics in the Use of Animals Committee (Juiz de Fora, Brazil, 2019; protocol number 6365271119). The experiment was carried out during the non-breeding season at Embrapa's Experimental Farm "José Henrique Bruschi" located in Coronel Pacheco, MG, Brazil (latitude 45′51″S, longitude 43°20′59″W). A total of 36 multiparous and 13 nulliparous crossbred ewes (Lacaune X Santa Inês) aged 4.6 ± 0.2 and 2.5 ± 0.1 years old, respectively, and with body condition scores of 3.2 ± 0.1 and 3.0 ± 0.1, respectively (scale 1 to 5; 1 = emaciated and 5 = obese), were enrolled. All animals were kept in a confinement system and provided with feed supplemented with corn silage, concentrate, and a mineral salt mixture (Caprinofós® Tortuga, São Paulo, Brazil). Water was available ad libitum.

2.2. Experimental design

Anestrus ewes were submitted to an ovulation-induced protocol associated with a superovulation protocol, receiving intravaginally a 0.36 g of progesterone device (D0; PRIMER PR® Caprinos e Ovinos, Tecnopec – Agener União, Taboão da Serra, Brazil) for nine days and six i.m. injections of 333 IU of pFSH (PLUSET®, Biogénesis Bagó, Curitiba, Brazil) in decreasing doses (25, 25, 15, 15, 10, 10%) beginning 60 h before P4 device withdrawal. Two doses of 37.5 µg of d-cloprostenol (Prolise®, Agener União, Taboão da Serra, Brazil) were injected i.m. concurrently with the fifth and sixth pFSH doses. After P4 device withdrawal, the ewes were observed for estrous behavior and naturally mated with fertile rams (1:7 ram to ewe ratio) every 12 h while in standing estrus. On D13 (96 h after P4 device withdrawal), animals were randomly assigned to groups that would receive either a single treatment of 300 IU of hCG (Vetecor®, Ceva Saúde Animal, Paulínia, Brazil) (GhCG; n=24) or no treatment (GControl; n=25). The experimental procedures are demonstrated in Figure 1.

Figure 1: Schematic representation of the experimental design performed to evaluate the effect of a single treatment of 300 IU of hCG 96 h after progesterone device

withdrawal on corpora lutea features, progesterone profile, and embryo yield. *NSER=non-surgical embryo recovery.



Animals were submitted to the same cervical relaxation protocol described by Dias et al. (2020) beginning 16 h before non-surgical embryo recovery (NSER). The NSER procedure as described by Dias et al. (2020) was performed on all females to verify the efficiency of the technique; however, only animals displaying an estrus response and with at least one CL on D16 were considered for the NSER procedure (duration and success of cervical transpositions and flushing; ova/embryo recovered). All the structures recovered (non-fertilized ova, degenerated or atretic embryos, embryos of different developmental stages, and zona pellucidae) were recorded and the embryos were classified according to the International Embryo Technology Society guidelines (IETS Manual; Mapletoft et al., 2020).

2.3. Ultrasound assessment and image analysis

Ovarian ultrasound evaluations (B-mode and Color Doppler) were performed at four timepoints: 12 h before hCG treatment (D13), 24 h after hCG treatment (D14), 24 h before NSER (D16), and immediately before NSER (D17). The ovaries of all ewes were scanned using a portable ultrasound device (Mindray M5Vet®, Shenzen, China) equipped with a transrectal linear transducer attached to a rigid handle protractor (frequency 8.0 MHz; overall gain 80%; depth 6.2 cm, and dynamic range, DR, 70 dB). The Color Doppler examinations were performed using the same ultrasound machine and transducer at different settings (frequency 4.6 MHz; pulse repetition frequency (PRF) 0.9 KHz; overall gain 72%; depth 6.2 cm, and Wall Filter, WF, 1).

All CL and luteinized anovulatory follicles (LUF; follicles ≥ 5 mm in diameter), if present, were counted, recorded, and measured using Image J® software (Wayne Rasband, National Institutes of Health, USA). The diameter (mean length of the vertical and horizontal axes) and area of the CL were measured using the frame image frozen at the largest CL area. For CL with a fluid-filled cavity, the area of the cavity was subtracted from the total CL area to determine only the luteal tissue area. Animals presenting CL with a diameter of ≤ 5 mm on D16 but not on D14 were considered a regressive CL. Ewes were classified according to a superovulatory response score as follows: score 1: no superovulatory response (≤ 2 CL); score 2: low superovulatory response (3 to 5 CL); score 3: medium superovulatory response (6 to 8 CL); and score 4: high superovulatory response (≥ 9 CL).

The area of ovarian blood perfusion was assessed using a cross-sectional image of the ovary at the strongest Color Doppler signal and the frames taken immediately before and after the cross-sectional image with the help of Image J® software. The ovarian perfusion area (excluding the ovarian pedicle region) was calculated for each image using the formula: number of colored pixels / number of total ovarian pixels x 100. Then, the average of the areas evaluated was calculated. Luteal echogenicity was analyzed by measuring pixel brightness (mean pixel value, MPV) and pixel heterogeneity (standard deviation of MPV) within a B-mode frame at the largest cross-sectional area of the ovary with the greatest number of visible luteal structures. Echogenicity was analyzed using Image Pro Plus® 7.0TM software (Media Cybernetics Inc., San Diego, CA, USA) using a scale of 256 shades of gray (0=black; 255=white). A selection tool was used to analyze circular areas of interest on the luteal tissue, avoiding eventual luteal cavities, if present.

2.4. Blood sampling and hormonal analysis

Along with the ultrasound evaluations, blood samples were collected on D14, D16, and D17 by venipuncture of the jugular vein using vacuum tubes (BD Vacutainer®, São Paulo, SP, Brazil). Samples were centrifuged at 1500 × *g* for 10 minutes and plasma was stored at -20 °C for further analysis. Plasma P4 concentrations were determined by solid-phase radioimmunoassay (RIA) according to the manufacturer's instructions (MP Biomedicals LLC, Orangeburg, NY). The sensitivity and intra-assay coefficient of variation were 0.075 ng/mL and 2.7%, respectively.

2.5. Endpoints

The following variables were recorded for all ewes enrolled in the study: estrous response (percentage and proportion); overall CL count (mean number ± SEM in ewes with estrus behavior); ewes with at least one CL on D14 and D16 (percentage and proportion); functional luteolysis (percentage and proportion; plasma P4 ≤1 ng/mL); structural luteolysis (percentage and proportion; CL diameter ≤ 5 mm); overall LUF count (mean number ± SEM in ewes with estrus behavior); ewes with LUF (percentage and proportion); duration of cervical transposing by Hegar dilator; ewes successfully transposed by Hegar dilator; duration of mandrel/catheter transposing; duration of uterine flushing; duration of NSER procedure; successful NSER procedures (percentage and proportion; ewes successfully transposed with mandrel-catheter and flushed); flushing efficiency (percentage of fluid recovered post-infusion calculated using the formula: volume retrieved / 400 mL x 100); CL count in successfully flushed ewes (mean number ± SEM); recovery rate (percentage and proportion; ewes >1CL); ova/embryos recovered (mean number ± SEM in successfully flushed ewes); unfertilized ova (mean number ± SEM); degenerated embryos (mean number ± SEM; IETS Code 4); transferable embryos (mean number ± SEM; IETS Code 1 and 2); freezable embryos (mean number ± SEM; IETS Code 1); morulae (mean number ± SEM); blastocysts (mean number ± SEM); zona pellucidae (mean number ± SEM); viability rate (percentage and proportion of viable embryos); CL diameter (mm^2); luteal tissue diameter (mm) and area (mm^2); area of ovarian blood perfusion (mm^2); CL echotexture (mean pixel value and pixel heterogeneity); plasma P4 concentration (ng/mL).

2.6. Statistical analysis

Statistical analysis was performed using SAS® University Edition software. The Bartlet test was used to assess the homogeneity of variances and the Shapiro-Wilk test was used to examine the normality of the residues. Parametric data (CL and LUF count, duration of Hegar dilator, mandrel-catheter, and flushing procedures, and number of structures recovered) were analyzed using a one-way ANOVA (PROC GLM) and compared using the Tukey test. Non-parametric data that it was not possible to normalize following logarithmic transformation (transferable, freezable and degenerated embryos, unfertilized ova, and morulae, blastocyst, and zona pellucidae numbers) were analyzed using PROC NPAR1WAY with the Wilcoxon test. Repeated measures variables (CL diameter, CL area, ovarian blood perfusion, ovarian

echotexture, and P4 plasma) were analyzed using the PROC MIXED procedure with the groups (GhCG and GControl), animal, evaluation timepoints (D13, D14, D16 or D17), and their interaction included as the main effects and repetition applied as a random factor. For binomial data (estrus response, ewes with no CL on D14 and D16, ewes with LUF, successful Hegar transposing. successful mandril-catheter transposing, flushing efficiency, recovery rate, and proportion of viable embryos), contingency tables were analyzed using the Fisher exact test to determine statistical differences. A P-value of less than 0.05 was understood to indicate statistical significance.

3. Results

The data on superovulatory responses and NSER efficiency are shown in Table 1. There was no difference (*P > 0.05*) between the groups for any of the recorded NSER procedure parameters. The recovery rate was greater for GhCG (*P < 0.05*), but there was no difference (*P > 0.05*) in the mean number of ova/embryos or unfertilized, viable or degenerated embryos recovered from both groups.

Table 1: Superovulation and non-surgical embryo recovery (NSER) outcomes (% or mean ± SEM) of ewes submitted to an estrus synchronization protocol associated with a superovulatory treatment[1] and receiving or not a single dose of 300 IU hCG at 96h after P4 device withdrawal.

Endpoints	GhCG	GControl	P value
Estrous response (%; n/n)	91.7 (22/24)	92.0 (23/25)	0.69
Overall CL count[2] (n; total)	8.4 ± 1.3 [186]	6.6 ± 1.2 [151]	0.30
Ewes with at least one CL on D14[2] (%; n/n)	77.3 (17/22)	65.2 (15/23)	0.51

Ewes with at least one CL on D16[2] (%; n/n)	86.4 (19/22)	73.9 (17/23)	0.46
Functional luteal regression (%)[3] (%; n/n)	0.0 (0/19)	5.9 (1/17)	0.47
Structural luteal regression (%)[3] (%; n/n)	15.8 (3/19)	47.0 (8/17)	0.07
Overall LUF count[2] (n; total)	1.8 ± 0.3 [39]	1.9 ± 0.3 [43]	0.84
Ewes with LUF (%; n/n)	84.2 (16/19)	88.2 (15/17)	0.89
Duration of Hegar dilator transposing[3] (min)	2.6 ± 0.7	3.6 ± 0.9	0.38
Successful Hegar dilator transp. rate[3] (%; n/n)	89.5 (17/19)	70.6 (12/17)	0.21
Duration of mandrel-catheter transp.[3] (min)	2.0 ± 0.6	1.4 ± 0.8	0.57
Duration of uterine flushing[3] (min)	16.5 ± 0.7	18.2 ± 3.3	0.93
Duration of procedure[3] (min)	23.3 ± 1.2	22.5 ± 2.7	0.39
Successful NSER procedure[3] (%; n/n)	84.2 (16/19)	70.6 (12/17)	0.43
Flushing efficiency[3] (%)	100.0	100.0	-
CL count in ewes washed (n; total)	9.8 ± 1.4 [157]	9.5 ± 1.4 [114]	0.88
Recovery rate (%; n/n)	89.8 (141/157)[a]	71.0 (81/114)[b]	0.001

Ova / embryos number (n; total)	8.8 ± 1.8 [141]	6.7 ± 1.7 [81]	0.57
Unfertilized ova number (n; total)	3.2 ± 1.2 [51]	1.4 ± 0.9 [17]	0.35
Degenerated embryos (n; total)	0.3 ± 0.2 [5]	0.7 ± 0.3 [8]	0.48
Transferable embryos (n; total)	5.2 ± 1.4 [84]	4.5 ± 1.5 [54]	0.73
Freezable embryos (n; total)	5.0 ± 1.3 [80]	4.5 ± 1.5 [54]	0.81
Morulae (n; total)	1.8 ± 0.6 [29]	1.6 ± 0.6 [19]	0.72
Blastocysts (n; total)	3.4 ± 1.2 [55]	2.9 ± 1.1 [35]	0.94
Zona pelucidae (n; total)	0.1 ± 0.1 [1]	0.2 ± 0.2 [2]	0.90
Viability rate (%; n/n)	59.6 (84/141)	66.7 (54/81)	0.32

CL = corpora lutea; LUF = luteinized anovulatory follicle.

[1]Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing injections of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5µg d-cloprostenol along with the fifth and sixth pFSH doses.

[2]Only ewes detected in estrus

[3]Ewes with at least one CL on D16.

[a,b]Within a row, means with different superscripts did differ between groups (Tukey´s test, P<0.05)

There was a significant reduction (P < 0.05) in average CL diameter on D16 and D17 in the two groups (Fig 2). The diameter of luteal tissue, ovarian echotexture (mean pixel value and pixel heterogeneity) also decreased (P < 0.05) from D16 to D17 (Table 2). The ovarian blood perfusion increased (P < 0.05) on D16 as compared with other

timepoints, with no differences between the groups or their interactions observed (P > 0.05). The superovulatory response score and embryo viability rate calculated according to the superovulatory response score are shown in Figure 3.

Figure 2. Mean corpus luteum diameter (A) and corpus luteum in structural regression (B) of superovulated ewes treated (GhCG, n=24) or not (GControl, n=25) with 300 IU of hCG administered i.m. 96 h after P4 device withdrawal. *Evaluation timepoints: D13=12 h before hCG administration; D14=24 h after hCG administration; D16=24 h before NSER procedure; D17=immediately before the NSER procedure. a,b,cDifferent superscripts for means indicate they varied at different evaluation timepoints (Tukey's test; P < 0.01).

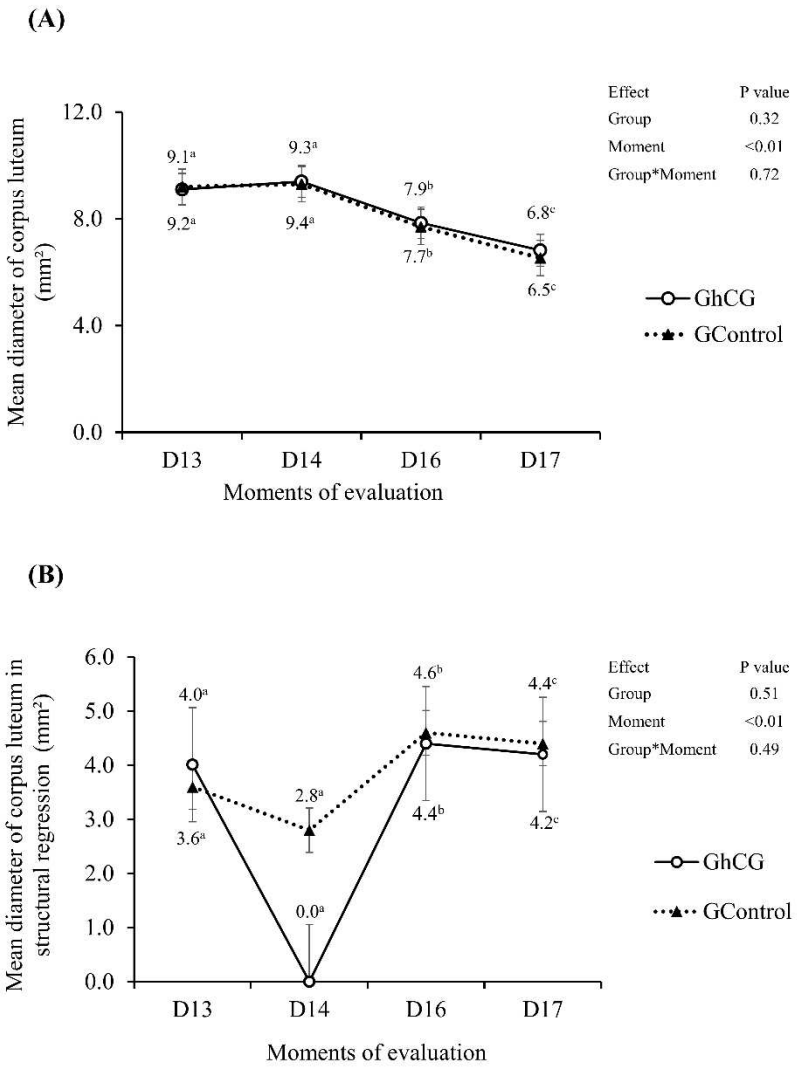


Table 2. Ovarian structures assessed by ultrasonography on D13 (12h prior to hCG treatment), D14 (24h after hCG treatment), D16 (immediately before the beginning of a cervical relaxation protocol) and D17 (16h after the cervical relaxation protocol) in superovulated ewes[1].

	D13		D14		D16		D17	
End points	hCG	Control	hCG	Control	hCG	Control	hCG	Control
Overall CL count	8.6 ± 1.5	6.7 ± 1.1	8.3 ± 1.4	6.5 ± 1.2	7.9 ± 1.3	6.2 ± 1.2	7.8 ± 1.3	6.2 ± 1.2
Overall LUF count	4.8 ± 0.9^{a}	5.1 ± 1.0^{a}	4.1 ± 1.0^{ab}	3.6 ± 0.7^{ab}	1.7 ± 0.3^{bc}	1.8 ± 0.3^{b}	1.4 ± 1.3^{c}	1.6 ± 1.2^{b}
Diameter of luteal tissue* (mm)	77.3 ± 14.3^{a}	61.7 ± 11.2^{a}	77.9 ± 13.8^{a}	59.9 ± 11.2^{a}	60.0 ± 10.1^{b}	46.2 ± 8.7^{b}	49.3 ± 7.7^{b}	37.5 ± 7.3^{b}
Area of luteal tissue* (mm^2)	219.4 ± 43.5	174.6 ± 34.7	246.4 ± 46.1	188.1 ± 37.9	294.4 ± 52.1	231.2 ± 43.7	231.5 ± 35.0	167.0 ± 32.6
Ovarian blood perfusion (mm^2)	12.4 ± 1.7^{a}	12.2 ± 2.5^{a}	16.7 ± 2.5^{ab}	14.7 ± 2.5^{a}	24.1 ± 2.4^{b}	26.3 ± 2.2^{b}	18.4 ± 2.3^{ab}	15.1 ± 2.6^{a}
Luteal mean pixel value (scale 0 to 255)	90.1 ± 2.0^{a}	96.4 ± 2.4^{a}	84.0 ± 5.8^{a}	85.9 ± 4.6^{a}	58.2 ± 4.3^{b}	59.3 ± 2.4^{b}	47.1 ± 5.2^{b}	38.3 ± 5.4^{c}

Luteal pixel heterogeneity	12.6 ± 0.2[a]	13.6 ± 0.4[a]	11.5 ± 0.8[a]	11.8 ± 0.6[ab]	9.3 ± 0.6[ab]	9.7 ± 0.3[b]	7.7 ± 0.8[b]	6.0 ± 1.0[c]

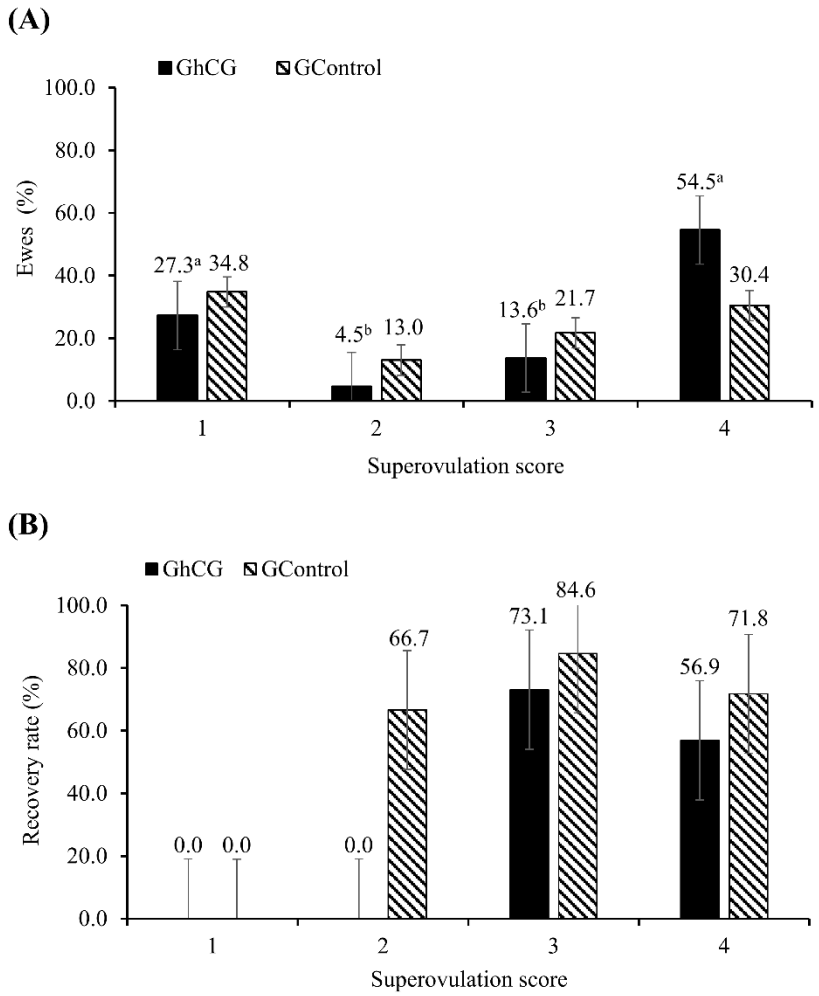
CL = Corpus luteum; LUF = Luteinized anovulatory follicle.

*Sum of luteal tissue if more than 1 CL.

[1]Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing injections of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5µg d-cloprostenol along with the fifth and sixth pFSH doses.

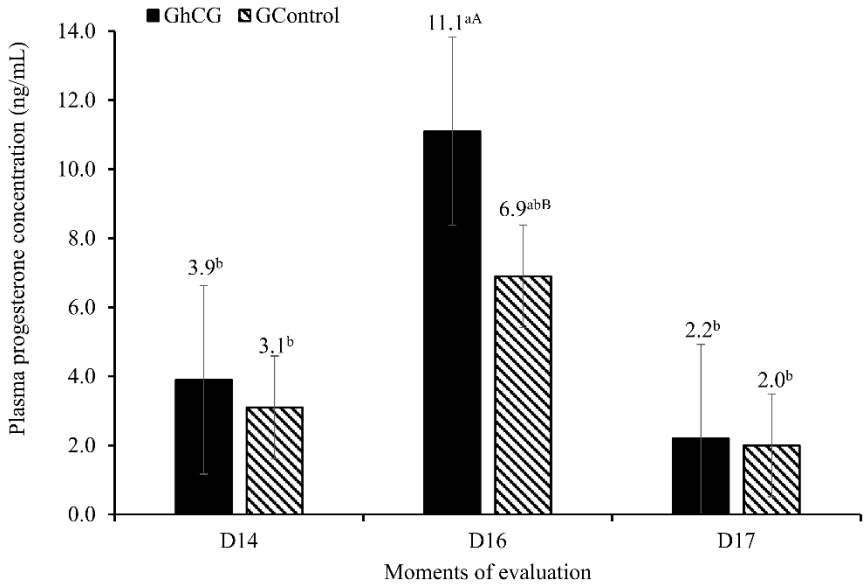
[a,b,c]Within a row, means with different superscripts did differ among evaluation timepoints within the same group (Tukey´s test, $P<0.05$).

Figure 3: Percentage of ewes (A) and viable embryos (B) collected from superovulated ewes treated (GhCG, n=24) with 300 IU of hCG and untreated superovulated ewes (GControl, n=25) according to superovulatory response scores (score 1: ewes with ≤2 CL; score 2: ewes with 3 to 5 CL; score 3: ewes with 6 to 8 CL; score 4: ewes with ≥9 CL). a,bDifferent letters indicate differences between the scores within the same group (Fisher's Exact Test; P < 0.05).



Functional luteolysis was not observed in GhCG while 5.9% of the animals in GControl presented functional luteolysis (0 > 0.05). Structural luteolysis was observed in 15.8% and 47.0% of the animals in GhCG and GControl, respectively (P > 0.05). Plasma P4 concentration on D16 was greater for GhCG (11.1 ± 1.5) compared with GControl (6.9 ± 1.5; P < 0.05) and higher than on D14 (3.9 ± 0.6 and 3.1 ± 0.8) and D17 (2.2 ± 0.4 and 2.0 ± 0.7), with no differences observed between the groups (P > 0.05). Data on plasma P4 concentration are shown in Figure 4.

Figure 4: Plasma progesterone (P4) concentration on D14, 16, and 17 (24 h before hCG treatment, immediately before the cervical dilation protocol, and after the cervical dilation protocol, respectively) in ewes submitted to a nine-day P4-based estrus synchronization protocol associated with a superovulatory treatment of 333 IU of pFSH (in six decreasing doses from 60 h before P4 device removal) and administration of 300 IU of hCG 96 h after P4 device withdrawal. a,bDifferent superscripts for means indicate they varied at different evaluation timepoints. A,B Different superscripts for means indicate they varied between groups at the same evaluation timepoint.



4. Discussion

The findings of this study contribute to a better understanding of the mechanisms involved in the maintenance of ovarian luteal function in superovulated ewes treated with a luteotropic agent and the treatment's effects on in vivo embryo production. Firstly, we demonstrate that plasma progesterone (P4) concentration is increased by a single hCG treatment in superovulated ewes, corroborating the findings of previous studies (Kelidari et al., 2010; Shabankareh et al., 2012). Furthermore, hCG appears to influence the maintenance of luteal structure. This confirms the potential of hCG treatment to improve embryo production (McNeill et al., 2006), as validated by the higher recovery rate observed in treated ewes, without affecting either the superovulatory response or the quality of the recovered embryos.

The treatment was not found to be an efficient means of preventing PRCL since the groups presented similar proportions of functional and structural regression. This study was based on the protocol described by Saharrea et al. (1998), whose study found a 10-fold higher number of normal CL and a 6-fold lower number of regressing CL in treated goats, demonstrating the effectiveness of hCG in maintaining luteal functionality. It is important to emphasize that in the present study, however, GControl experienced an almost three-fold increase in the number of animals in structural regression compared with GhCG and no functional regression was observed in GhCG, corroborating the positive effect of hCG treatment observed in Saharrea et al. (1998). The lack of significant treatment effects in this study may be due to the later administration of the treatment protocol. Further studies where the hCG treatment is administered in superovulated ewes at different timepoints should be conducted to identify the optimal administration time for the prevention of PRCL.

The PRCL prevention triggered by hCG may be achieved by hCG's LH-like action through the mechanism of converting small luteal cells into large luteal cells (Farin et al., 1988). This promotes an increase in the steroidogenic capacity of the CL and a greater supply of P4 (Gamboni et al., 1984) and, consequently, improves luteal functionality. The data recorded in this study do reveal an increase in P4 concentration but not in luteal morphology, as observed by Gamboni et al. (1984) and Vergani et al. (2020). Likewise, the treatment did not affect ovarian perfusion, which is contrary to expectations due to the angiogenic effects of hCG (Waseda et al., 2003). Both groups displayed the same luteal characteristics (morphology, perfusion, and echogenicity) even during structural luteolysis. The beneficial effect of hCG on luteal function is dependent on the time of treatment administration and the dose administered and may prevent PRCL and increase luteal morphology and progesterone concentration (Kelidari et al., 2010) or not (Aslan et al., 2011). In the present study, a single dose of hCG was observed to increase P4 concentrations, confirming that changes in the timing of hCG administration can impact the pro-luteal effects of this gonadotropin.

Despite this, it is important to emphasize the positive effects that hCG can exert directly on the embryo. These effects are associated with an increase in P4 secretion, promoting an improvement in uterine function and, consequently, an increase in fertility (Côrtes et al., 2021). The luteal-enhancing action of hCG may allow a healthier P4 supplementation compared with exogenous P4 supplementation. Deleterious effects

on luteinizing hormone concentration (Burke et al., 1994) and CL lifespan (Parr et al., 2017) have been observed in heifers after exogenous P4 supplementation during the early luteal phase, although treated groups presented higher P4 concentrations (Burke et al., 1994).

A noteworthy finding of this study is the relationship between the superovulatory response and embryonic recovery with superovulatory response scores. Both groups had animals of all superovulatory scores, but GhCG had a concentration of animals at score 4 as compared with scores 2 and 3. This pattern was not observed in GControl, where the animals were distributed more evenly across scores. A similar result was observed after LH administration 24 h after P4 device withdrawal (Oliveira et al., 2012) since changes in the FSH/LH ratio are associated with higher ovulation rates (D'Alessandro et al., 2005). It is also possible that hCG treatment improved the ovulatory rates of "low-response sheep," as previously reported after treatment to induce ovulation in dairy cows (De Rensis et al., 2008).

A higher proportion of viable embryos in females with a higher number of CL is expected due to the embryotrophic effects of P4 (Diskin and Morris, 2008), but high-response animals are likely to experience a reduction in fertilization rates in response to the estrogenic overstimulation that affects sperm transport and causes failures in the sperm/oocyte fertilization process (Armstrong and Evans, 1983). Decreased fertility in superovulated cows (Hawk, 1988) and sheep (Evans and Armstrong, 1984) has been observed and is related to asynchrony in oocyte maturation and, consequently, premature ovulation and/or failures in sperm transport (Armstrong, 1993). Surprisingly, high-response sheep in both groups achieved a high viability rate (above 50%) in contrast to the existing literature. Although a considerable number of unfertilized structures were recovered, as in Armstrong and Evans (1983), the average number was close to that found in superovulated Santa Inês ewes (2.0-2.8 unfertilized eggs per ewe; Oliveira et al., 2014).

5. Conclusion

We conclude that the administration of 300 IU of hCG in superovulated ewes during the early luteal phase has a positive effect on the maintenance of luteal function, can induce an increase in P4 levels, improves embryo recovery rates, and tends to maintain the luteal structure. Treatment with hCG did not affect the effectiveness of NSER nor embryonic viability, but it did enhance superovulatory scores. Further studies are still required to establish the optimal time for hCG treatment, but this treatment has the potential to be an additional tool for improving embryo yield in donor ewes.

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Conflict of interest

Authors declare that they do not have any actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations.

AUTHORS CONTRIBUTIONS

JFF elaborated the hypothesis, discussed the experimental design. JHD collected and analyzed the data from the animals and wrote the first version of the manuscript. JGD, AMA and RITPB assisted with the animals and helped collecting and analyzing the data. RB analyzed hormonal dosage. JFF, RITPB, LGBS, MEFO and JMGS-F discussed the design of the experiment and analyzed the data. JHD elaborated and worked on the statistics. JFF, MEFO and JMGS- F approved the final version of the manuscript.

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4. Chapter 5 - 333IU of pFSH is effective to promote follicular growth, superovulatory response, and embryo yield in White Dorper ewes

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333IU of pFSH is effective to promote follicular growth, superovulatory response, and embryo yield in White Dorper ewes

Dias, JH[1]; Vergani, GB[2]; Gonçalves, JD[2]; Oliveira, TA[3]; Pereira, VSA[4]; Esteves, SN[4]; Garcia, AR[4]; Batista, RITP[3]; Oliveira, MEF[2]; Souza-Fabjan, JMG[3]; Fonseca, JF[5]

Afiliação

[1]Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, CEP 36570-000, Viçosa, MG, Brazil.

[2]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de acesso Prof. Paulo Donato Castellane, s/n, CEP 14884-900, Jaboticabal, SP, Brazil

[3]Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil

[4]Embrapa Pecuária Sudeste, Rod. Washington Luiz, km 234, CEP 13560-970, São Carlos, SP, Brazil.

[5]Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, km 04, CP 145, CEP 62010-970, Sobral, CE, Brazil.

Electronic address: jeferson.fonseca@embrapa.br.

Abstract

This study evaluated the effect of 250, 333, or 400 IU of a commercial pFSH on follicular growth, superovulatory (SOV) response, and embryo yield in White Dorper sheep. Ewes received an induced-ovulation protocol and a superovulation starting 60 h before progesterone (P4) withdrawal, which consisted of six decreasing doses of 250 IU (G250; n=15), 333 IU (G333; n=15), or 400 IU (G400; n=14) of pFSH. Ewes also received 50 µg of GnRH analog on D10 and 300 IU of hCG on D13. Ewes were matted and submitted to non-surgical embryo recovery (NSER). Ultrasound evaluations were performed on D6, D7, D8, D9, and D16. The overall responding donors' rate (≥3 CL) was 86.4% (38/44). The mean number of large follicles was superior ($P<0.05$) in all groups on D8 (6.2±0.9, 7.7±0,.9 and 8.3±1.1) and D9 (11.1±0.6, 10.4±1.0 and 11.5±0.9). NSER procedures were successfully performed in 60.5% (23/38) of females, with a shorter ($P<0.05$) duration of uterine flushing in G333. The mean number of recovered ova/embryo tended ($P=0.06$) to be superior in the G333 (7.3±2.8) and G400 (6.0±1.3) than in G250 (2.7±1.2), with no difference ($P>0.05$) in the recovery

rate (44.4; 57.3 and 50.0%) and numbers of transferable embryos (2.3±1.1; 6.1±2.6 and 4.5±1.3) among groups. All protocols were efficient in promoting follicular growth and adequate superovulatory responses, but there was a tendency for a greater number of ova/embryos recovered in G333 and G400.
Keywords: follicular dynamics; FSH; MOET; ovarian superestimulation; ovine.

1. Introduction

Biotechnologies such as multiple ovulation and embryo transfer (MOET) were introduced in animal reproduction allowing an acceleration of genetic gain in several species, including ewes (Cognié et al., 2003). For MOET success, an effective superovulatory (SOV) treatment is essential and considered the most important aspect of *in vivo* embryo production. It is responsible for promoting a greater stimulus of follicular development than would occur physiologically and, consequently, increasing the number of ovulation and viable embryos per reproductive cycle (Bartlewski et al., 2016). The individual variability, however, can directly or indirectly affect SOV response, fertilization, and the number of viable embryos recovered (Bartlewski et al., 2016).

Recent studies using SOV treatment with porcine stimulating-follicle hormone (pFSH) associated or not with exogenous gonadotropins (i.e., eCG and/or GnRH) supplementation at the end of treatment (Maciel et al., 2019; Figueira et al., 2020) have reached good SOV responses. It is well known that SOV treatment only with eCG is not recommended due to ovarian overstimulation, which induces a greater incidence of luteinized anovulatory follicles (LUFs) and high estradiol concentrations (Bartlewski et al., 2016). In addition, it has been demonstrated that deleterious effects on embryo number and quality (Menchaca et al., 2009) and a tendency to increase the number of degenerated embryos (Luna-Palomera et al., 2019) may occur after FSH-eCG association, compared to FSH treatment alone.

Commercially available pFSH preparations contain a lower (400 mg pFSH presentation) or equivalent (500 IU pFSH) amount of LH in their composition, which may affect ovulatory response and embryonic quality (D'Alessandro et al., 2005). Equivalent FSH:LH ratio has already been applied at various concentrations in cows (Tegegne et al., 1994) and sheep (D'Alessandro et al., 1996; Wierzchos et al., 1992; Lagares et al., 2021). Indeed, comparisons between both commercial pFSH preentations in heifers showed no difference in the efficiency to promote follicular

development and superovulatory stimulation (Kelly et al., 1997; Mikkola and Taponen, 2017).

In sheep, a decreasing dose of 200-250 IU (Cordeiro et al., 2003) appears to be sufficient to induce a desirable ovulatory response with a superior number of viable embryos compared to using higher doses (500-1000 IU; Wierzchos et al., 1992; D'Alessandro et al., 1996). The mean number of CL count and recovered structures using 250 IU of pFSH (7.6 ± 3.1 and 6.3 ± 2.4; Wierzchos et al., 1992) were similar to those achieved using 133 mg (6.6 ± 1.4 and 5.5 ± 1.6; Figueira et al., 2020) or 200 mg (14.9 ± 6.9 and 6.2 ± 4.3; Maciel et al., 2019). Our group found comparable results using an intermediate dose (333 IU; Dias et al., 2022) in dual-purpose crossbred ewes (Lacaune x Santa Inês). Dias et al. (2022) used a shorter superovulatory treatment (9d) compared to others that have a longer progestin duration (12-14d), which may have effects on the embryonic population (Oliveira et al., 2020).

The repeatability of superovulatory treatment depends directly on the ovarian status and response to treatment, which is influenced the by individual response, such as the breed (González-Bulnes et al., 2004). Therefore, it is vital to verify the SOV response in different sheep breeds aiming to increase the MOET success. Dorper sheep are known for their reproductive performance, with a high ovulatory rate and SOV response (Cloete et al., 2000), and the current study may serve as a model for other prolific breeds. Considering the differences between the existing protocols and breeds tested in these studies and the possibility of introducing a new SOV protocol in White Dorper sheep breed, this study aimed was to evaluate the effect of different doses of commercial pFSH (250, 333, and 400 IU) on the superovulatory response and *in vivo* embryo production in White Dorper ewes.

2. Material and methods

Ethics, location, and experimental animals

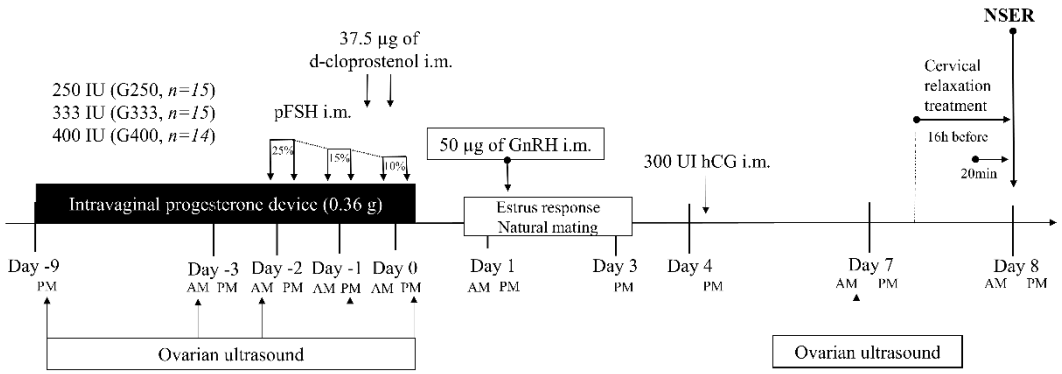
This study was conducted after the approval of the Ethics in Animal Care Committee of Embrapa Dairy Cattle, Brazil (protocol 6365271119). The experiment was carried out during the non-breeding season, in São Carlos, São Paulo, Brazil (latitude 22° 0' 55" S, longitude 47° 53' 28" W). A total of 44 multiparous White Dorper ewes, with an average of 5.1 ± 0.2 years of age, 61.1 ± 1.2 kg of body weight, and 3.4 ± 0.5 of body condition score (scale 1 to 5; 1 = emaciated and 5 = obese) were enrolled in the study. All animals were placed in pasture containing *Panicum maximum cv* and

Aruana grass and supplemented with a balanced concentrate ratio daily, according to nutritional demand. Drinking water was available *ad libitum*.

Superovulation design

All anestrus ewes received an ovulation-induced protocol based on the insertion of an intravaginal device (Day 0; D0) containing 0.36 g of progesterone (P4; PRIMER PR® Caprinos e Ovinos, Tecnopec – Agener União, Taboão da Serra, Brazil) for nine days plus two injections of 37.5 µg of prostaglandin F2α (PGF2α) analog (d-cloprostenol; Prolise®, Agener União, Taboão da Serra, Brazil) administered i.m., concurrently with the fifth and sixth doses of pFSH. Superovulation treatment started 60 h before P4 withdrawal, with twice daily (12 h intervals) injections of a decreasing (25, 25, 15, 15, 10, 10% of the total dose) dose of 250 IU (G250; n=15), 333 IU (G333; n=15) or 400 IU (G400; n=14) of pFSH (PLUSET®, Biogénesis Bagó, Curitiba, Brazil), as showed in Figure 1. All ewes also received 50 µg of GnRH analog (Gestran Plus®, Agener União, Taboão da Serra, Brazil) 24 h after P4 device withdrawal and 300 IU of hCG (Vetecor®, Ceva Saúde Animal, Paulínia, Brazil) 96 h after P4 device withdrawal. Ewes were checked for estrus and naturally mated starting 12 h after P4 withdrawal by fertile rams (ram to ewe ratio 1:7) every 12 h, while in standing estrus.

Figure 1: Schematic representation of the experimental procedures performed to evaluate the follicular development, superovulatory response and embryo yield of ewes submitted to a superovulatory treatment with 250 IU (G250), 333 IU (G333) and 400 IU (G400) of pFSH associated to an estrus-induction protocol (9-days progesterone (P4) plus 37.5µg of d-cloprostenol).



Embryo recovery procedures

Embryo recovery was performed by the non-surgical embryo recovery (NSER) technique, as previously described (Dias et al., 2020). Ewes received a relaxation protocol containing an injection of 37.5 µg of d-cloprostenol i.m. 16 h before the beginning of procedures plus 50 IU of oxytocin i.v. (Ocitocina Forte UCB®, Jaboticabal, Brazil) 20 min before NSER. Ova/embryos recovered were recorded and classified according to the International Embryo Technology Society guidelines (IETS Manual; Mapletoft et al., 2020).

Ultrasonography and image analysis

Ovarian ultrasound evaluations (B-mode) were performed 24 h before the first pFSH injection (D6) and concomitantly with the first (D7), fourth (D8), and sixth (D9) doses of pFSH and 24 h before the NSER procedures (D16) in all ewes. It was used an ultrasound device (Mindray M5Vet®, Shenzen, China) equipped with a transrectal linear probe, fitted with a rigid protractor (transducer frequency: 8.0 MHz; overall gain: 80%; depth 6.2 cm and dynamic range, DR: 70dB).

All antral follicles (≥ 2 mm) and CL present on the ovaries were counted and measured using the Image J® software (Wayne Rasband, National Institutes of Health, USA). Follicles ≥ 5 mm in diameter counted at 24 h before the NSER procedures (D16) were defined as luteinized anovulatory follicles (LUF). The diameter (average of the vertical and horizontal axes) of the follicles was measured using the frame image where the follicle had its largest diameter. Follicles were classified according to the diameter, being small < 3 mm, medium between 3.1 to 5.0 mm, and large > 5 mm. The mean number of follicles according to the classes was evaluated.

Statistical analyses

The following data were recorded for all ewes enrolled in the study: estrus response; interval to estrus; overall CL count; overall LUF count; ewes with LUF (%; number of ewes with LUF/number of ewes); mean antral follicle diameter (mm; D6, D7, D8, D9); responding donors' rate (considering ≥ 3 CL; proportion of ewes responding to SOV); duration of Hegar dilator transposing (mean); successful Hegar dilator transposing rate (number/proportion of ewes successfully transposed by Hegar dilator); duration of mandrel-catheter transposing (mean); duration of uterine flushing

(mean); NSER success (proportion of ewes successfully submitted to NSER procedure); flushing efficiency rate (percentage of fluid recovered post-infusion calculated with the formula: volume retrieved / 400 mL x 100); CL count in ewes successfully flushed; recovery rate (total recovered structures x 100 / total CL number; considering responding donors); average recovery rate (mean number of individual recovery rates); ova/embryos recovered (mean); transferable embryos (mean; IETS Code 1 and 2); freezable embryos (mean; IETS Code 1); degenerated embryos (mean; IETS Code 3); unfertilized ova (mean); morulae (mean); blastocyst (mean); zona pelucidae (mean).

Statistical analyses were performed using the SAS® Studio software. The Bartlett test was used to assess the homogeneity of variances followed by the Shapiro-Wilk test to verify the normality of the data. Data (duration of Hegar transposing, ova/embryos recovered) were transformed to log to fit a normal distribution, and results are presented as non-transformed data. Parametric data (CL count, LUF count, duration of Hegar transposing, ova/embryos recovered, transferable embryos, freezable embryos) was analyzed by one-way ANOVA (PROC GLM) followed by a post-hoc Tukey test to determine differences among means. Non-parametric data (interval to estrus, duration of mandrel transposing, duration of flushing, average recovery rate; mean number of follicles in the same day, non-viable embryos recovered, unfertilized ova; morulae, and blastocyst) were analyzed using PROC NPAR1WAY with the Wilcoxon test. For binomial data (estrus response, ewes with LUF, responding donors' rate, successful Hegar dilator transposing, NSER success, recovery rate) contingency tables were analyzed using the Chi-Square test to determine statistical differences. Repeated measures variables (follicle diameter and mean number of follicles) were evaluated using the PROC MIXED procedure with a repeated statement to assess the interaction between treatment and time. The statistical model included the main effects of treatment, days of evaluation, and their interaction. Statistical significance was determined based on a P-value of 0.05. P-values of 0.06 was considered as a tendency for significant differences.

3. Results

Overall, the estrus response and the responding donors' rate (animals presenting ≥3 CL) were 97.7% (43/44) and 86.4% (38/44), respectively. Ovarian responses to SOV treatments are shown in Table 1.

Table 1: Ovarian response outcomes (% or mean ± SEM) in White Dorper ewes submitted to estrus induction protocol (9-days P4 plus two injections of 37.5 µg of d-cloprostenol at day of device withdrawal) associated to a superovulation protocol consisting in different doses (250, 333 and 400 IU) of pFSH administered in six decreasing doses 12h-apart plus one injection of 50 µg of GnRH analogue and 300 IU of hCG 24 and 96h after P4 withdrawal, respectively.

	pFSH treatments			
Endpoints	250 IU	333 IU	400 IU	P value
Number of animals	15	15	14	
Estrus response (%)	93.3 (14/15)	100.0 (15/15)	100.0 (14/14)	0.36
Interval to estrus (h)	24.0 ± 2.9	23.2 ± 1.8	24.0 ± 2.2	0.89
Overall CL count[1]	8.1 ± 2.1 [113]	9.9 ± 1.6 [148]	13.0 ± 1.4 [182]	0.13
Overall LUF count	2.6 ± 0.8 [18]	2.7 ± 0.6 [27]	1.6 ± 0.4 [21]	0.29
Ewes with LUF (%)	60.0 (9/15)	92.4 (12/13)	71.4 (10/14)	0.15
Antral follicle diameter (mm)[2]				0.004
Day 6	4.4 ± 0.2c (1.4 – 12.8)	4.7 ± 0.2c (1.3 – 13.9)	4.0 ± 0.2b (1.8 – 8.8)	
Day 7	○ ± 0.1c (1.5 – 9.2)	4.1 ± 0.1c (1.4 – 9.6)	4.2 ± 0.1b (1.8 – 7.9)	
Day 8	5.2 ± 0.1b (2.6 – 10.5)	5.2 ± 0.1b (1.7 – 10.5)	5.7 ± 0.1a (1.7 – 14.5)	
Day 9	6.0 ± 0.1a (1.7 – 14.0)	6.1 ± 0.1a (1.3 – 13.9)	6.2 ± 0.1a (1.2 – 12.2)	

CL = corpora lutea; LUF = luteinized unovulated follicle;

( ) Number of animals, ovarian structures or recovered ova/embryos;

[ ] Total sum of ovarian structures.

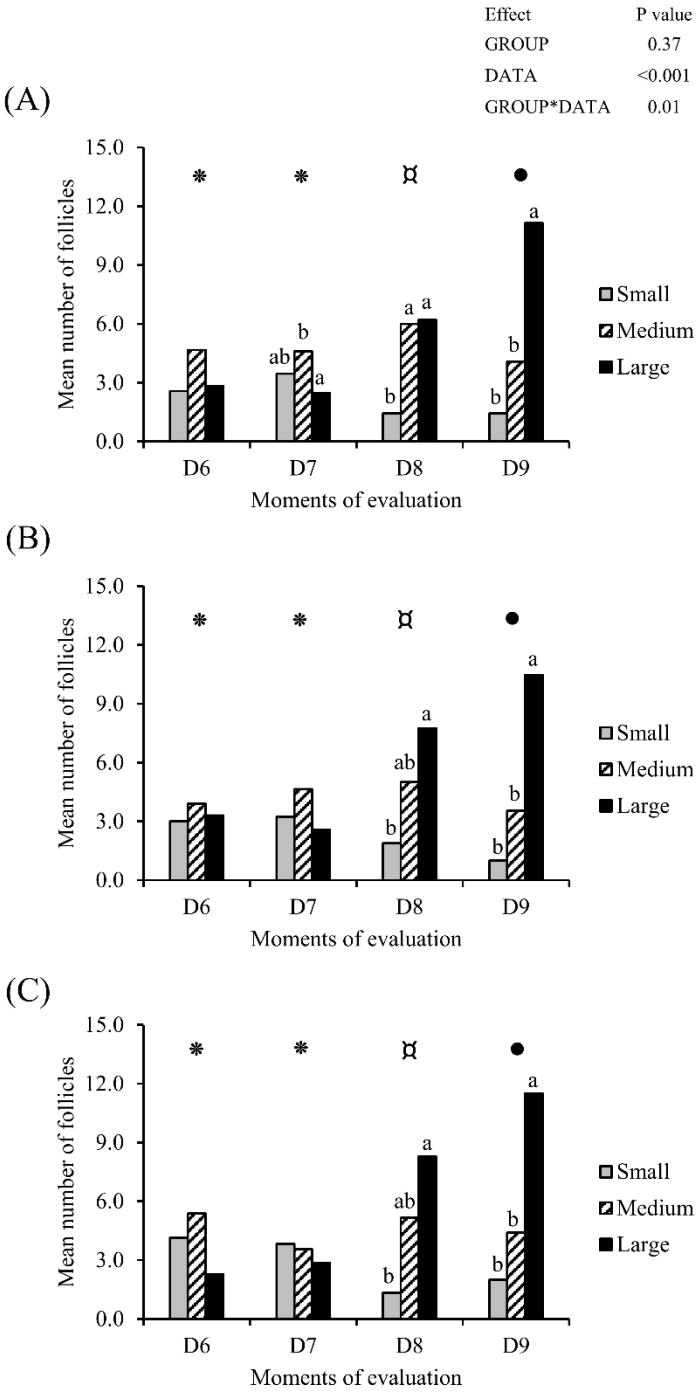
[1] Considering responding ewes that manifested estrus behavior

[2] ( ) Minimum and maximum diameters range

[a,b,c] Different letters indicate differences between days of evaluation (Tukey test; $P<0.001$)

The mean diameter of follicles presented interaction (P<0.05) between groups and moments, with a greater (P<0.001) diameter on D9 compared to D8, D7, and D6 for G250, G333, and G400 respectively, but there was no difference (P>0.05) among groups. The follicular population was different within the same day in the group (P<0.05; Fig. 2), and the mean numbers of antral follicles increased (P<0.001) among days but there was no difference (P>0.05) among groups.

Figure 2: Follicular population according to their diameter classification (Small, < 3.0 mm; Medium, 3.1 to 5.0 mm; and Large, > 5.0 mm) of White Dorper ewes submitted to an estrus inducing-protocol (9-days P4 device plus 37.5µg of d-cloprostenol injections at fifth and sixth doses of pFSH and 50µg of GnRH and 300IU of hCG at 24 and 96h after P4 withdrawal) and superovulatory treatment using 250 IU (A), 333 IU (B) or 400 IU (C) of pFSH, observed 24h before (D6), at the time of the first (D7), fourth (D8) and fifth (D9) pFSH dose injections. X-axis = moments of evaluation; y-axis = mean number of follicles. ^{a,b,c}Letters means difference among classes in the same day and in the same group (Kruskall-Wallis test, P<0.05). *¤•Symbols means difference among days in the same group.


Effect P value
GROUP 0.37
DATA <0.001
GROUP*DATA 0.01
(A)
(B)
(C)
Mean number of follicles
15.0
12.0
9.0
6.0
3.0
0.0
D6
D7
D8
D9
Moments of evaluation
Small
Medium
Large
a
ab
b
*
¤
●

Data regarding SOV response and NSER procedure are shown in Table 2. The CL count in ewes successfully flushed was greater ($P<0.05$) in G333 (12.7 ± 2.3) and G400 (12.0 ± 1.3) groups in comparison to G250 (6.0 ± 1.1) group, although the overall CL count did not differ ($P>0.05$) among groups. The G333 and G400 groups tended ($P=0.06$) to present a greater number of ova/embryos recovered in comparison of G250 (Table 2). Numbers of transferable and freezable embryos, unfertilized ova, non-viable embryos, morulae and blastocyst did not differ ($P>0.05$) among groups.

Table 2: End points of non-surgical embryo recovery (NSER) procedures and embryo yield (mean ± SEM) in White Dorper ewes submitted to superovultatory treatment containing 250, 333 or 400IU of pFSH after a synchronized estrus induction protocol (9-days P4 plus d-cloprostenol) and successfully flushed by NSER.

	pFSH treatments			
Endpoints	250 IU	333 IU	400 IU	P value
Number of animals	15	15	14	-
Estrus response (%)	93.3 (14/15)	100.0 (15/15)	100.0 (14/14)	0.36
Responding donors' rate (%) ²	78.6 (11/14)	86.7 (13/15)	100.0 (14/14)	0.20
Duration of Hegar dilator transposing (min)	5.6 ± 1.3	5.7 ± 1.2	4.7 ± 1.1	0.70
Successful Hegar dilator transposing rate	63.6 (7/11)	53.8 (7/13)	85.7 (12/14)	0.26
Duration of Mandrel-Catheter transposing (min)	3.2 ± 1.5	0.7 ± 0.1	3.1 ± 1.1	0.11
Duration of uterine flushing (min)	18.4^{a} ± 0.6	14.6^{b} ± 0.7	18.3^{ab} ± 1.9	0.049
NSER success (%)	54.5 (6/11)	53.8 (7/13)	71.4 (10/14)	0.28

Flushing efficiency (%)	100.0	100.0	100.0	-
CL count in ewes successfully flushed[1]	6.0[b] ± 1.1 [36]	12.7[a] ± 2.3 [89]	12.0[a] ± 1.3 [120]	0.03
Recovery rate (%)	44.4 (16/36)	57.3 (51/89)	50.0 (60/120)	0.36
Average recovery rate	34.0 ± 13.8 (0 - 80.0)	51.6 ± 10.7 (0 - 95.8)	47.9 ± 9.5 (0 - 91.7)	0.71
Ova/embryos recovered	2.7 ± 1.2 [16]	7.3 ± 2.8 [51]	6.0 ± 1.3 [60]	0.06
Transferable embryos[3]	2.3 ± 1.1 [14]	6.1 ± 2.6 [43]	4.5 ± 1.3 [45]	0.38
Freezable embryos[3]	2.2 ± 1.1 [13]	5.3 ± 2.2 [37]	4.2 ± 1.3 [42]	0.46
Degenerated embryos[3]	0.0 ± 0.0 [0]	0.9 ± 0.5 [6]	0.8 ± 0.4 [8]	0.33
Unfertilized ova	0.2 ± 0.2 [1]	0.1 ± 0.1 [1]	0.5 ± 0.3 [5]	0.66
Morulae	1.5 ± 0.8 [9]	4.4 ± 2.5 [31]	1.6 ± 0.8 [16]	0.64
Blastocyst	0.8 ± 0.5 [5]	1.7 ± 0.8 [12]	2.9 ± 1.2 [29]	0.55
Zona pelucidae	0.2 ± 0.2 [1]	0.1 ± 0.1 [1]	0.2 ± 0.1 [2]	0.98

CL = corpora lutea; LUF = luteinized unovulated follicle.

( ) Number of animals, ovarian structures or recovered ova/embryos.

[ ] Total sum of animals, ovarian structures or recovered ova/embryos.

[1] Considering responding ewes that manifested estrus behavior

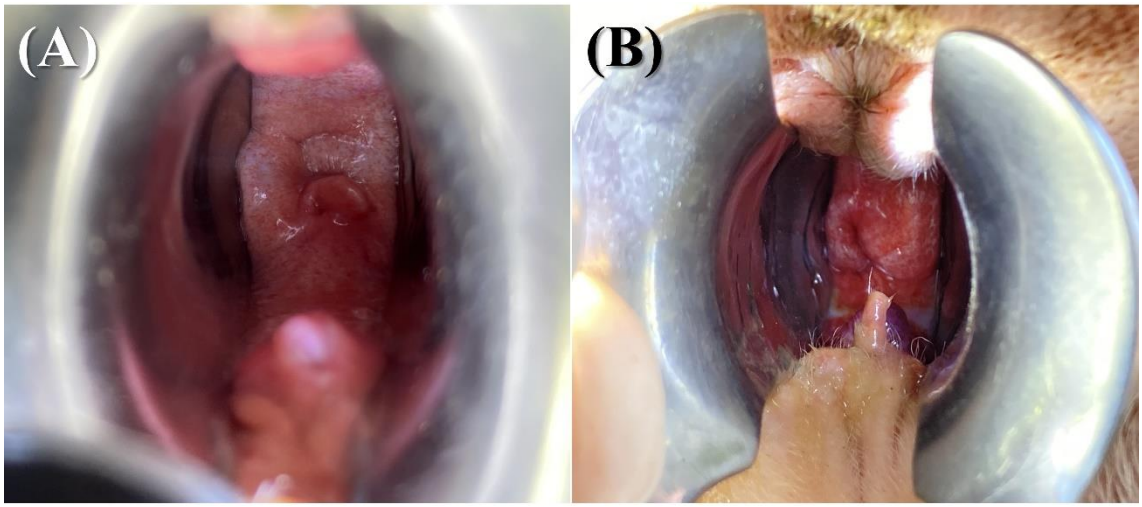
[2] Considering animals with ≥ 3 CL

[3] Transferable embryos = Codes 1 and 2; Freezable embryos = Code 1 and Degenerated embryos = Code 4.

[a,b] Different letters indicate differences between groups (Tukey test; $P<0.05$)

The NSER procedure was successfully performed in 60.5% (23/38) of animals. In 15 animals, it was not possible to perform the NSER, due to anatomical problems such as vestibulo-vaginal stenosis (34.2% or 13/38; Fig. 3 B) and incomplete cervical retraction (5.3% or 2/38). Thus, NSER was 100% for ewes without anatomical problems. Examples of normal cervical os and with vestibulo-vaginal stenosis can be seen in Figure 3. Despite similar adult age in comparison to other counterparts ($P>0.05$), these animals had in common no information about parity. The duration of cervical transposing with Hegar dilator and mandrel-catheter did not differ among groups ($P>0.05$), but there was an increased time in G250 (18.4 ± 0.6 min; $P<0.05$) to present longer duration of uterine flushing compared to G333 (14.6 ± 0.7 min) and G400 (18.3 ± 1.9 min).

Figure 3: Vaginoscopy in ewes showing total vaginal speculum introduction and complete cervical ostium visualization (A). Vaginal speculum positioned into vestibulo before himenal prega (B).



4. Discussion

The results of this study confirms the efficiency of ovarian superstimulation using a commercial pFSH (ratio 1:1 FSH and LH) promoting superovulatory (SOV) response in approximately 90% of animals, as observed in studies of D'Alessandro et al. (1996) and Cordeiro et al. (2003). This study found a similar or even higher mean CL count (10.1–13.5/ewe) with reduced doses of pFSH (250-400 IU), in comparison to

other studies using 500 or 750 IU (8.3-9.1/ewe) (Wierzchos et al., 1992; Ciornei et al., 2022). Furthermore, it confirms that a better SOV response is directly related to a greater number of recovered structures.

Our NSER success rate (60.5 %) in White-Dorper ewes was lower than in Dias et al. (2020) using the same cervical relaxation protocol in Dorper ewes (83.3%). Differentially from Dorper ewes of the reported study, which were tested near after post-partum (120 to 120 days), 15 ewes from the present study had no parturition history. Interesting, the animals presented vestibulo-vaginal stenosis that prevents cervical clipping or when clipped cervix could not be adequately exposed as expected (Fonseca et al., 2019). Thus, the unsuccess could be associated to long time cervical closure and/or nulliparous status. In spite of 67% of NSER success reported in crossbreed nulliparous Lacaune-Santa Inês donors (Dias et al., 2022), it is important to highlight that previous vaginoscopy inspection with total vaginal speculum introduction and complete cervical ostium visualization (Fig. 3 B) must be advocated as a decisive step for reaching success in NSER. This situation highlight the importance of selecting animals before superovulation followed by NSER for avoiding animals with reproductive anatomic problems (Prellwitz et al., 2019) as well as using animals after and near to a known partum (Fonseca et al., 2021). Therefore, the failure of NSER is more linked to anatomical characteristics than to superovulatory treatment since the different doses of pFSH did not affect NSER success. It is possible that higher doses of pFSH tend to facilitate the NSER procedure due to the greater number of available CLs, which increases prostaglandin precursors and, consequently, the degrees of cervical relaxation (McCann and Flint, 1990). In fact, the 250IU dose of pFSH generated an increase in the duration of uterine flushing and presented a lower CL count in ewes successfully flushed, corroborating that the success of NSER may also be associated with CL count.

The groups showed a similar pattern of follicular growth at the times evaluated and the protocol was effective in recruiting follicle growth, since there was a change in the follicular population between the first and fourth application of pFSH and an increasing number of follicles among days. A curious fact found is the number of large follicles observed on D8, that was considerably superior than in other studies using a commercial pFSH with lower amounts of LH (Bartlewski et al., 2008; Gonzalez-Bulnes et al., 2002, 2000). This may be a result of the number of medium follicles observed before the beginning of superovulatory treatment, which developed more rapidly with

exogenous FSH supplementation. Ovarian status at the start of superstimulation directly influences response to treatment (Nasser et al., 1993) and embryo yield (Menchaca et al., 2007).

Apparently, the presence of medium and large follicles on D7 did not affect the superovulatory response in any of the treatments evaluated. The overall mean CL count was similar to studies using P4-FSH-GnRH protocol (Brasil et al., 2016), P4-FSH-eCG (Menchaca et al., 2009), or Day 0 protocol (Menchaca et al., 2009, 2007). However, the number of recovered structures and viable embryos (i.e., transferable and freezable) were lower than expected. Veiga-Lopez et al. (2008) associate earlier estrus onset intervals and consequent earlier LH surge to a greater number of degenerated embryos and reduced embryonic quality. However, the occurrence of degenerated embryos in this study (11.0%) was relatively low, corroborating to a similar protocol (P4-FSH-GnRH; 14%) (Brasil et al., 2016). It also does not seem to be associated with failures in the fertilization process (Armstrong and Evans, 1983), as the incidence of unfertilized ova was also low. However, we cannot discard the hypothesis that this lower number of the recovered structures is associated with the early estrus behavior and LH surge (Gonzalez-Bulnes et al., 2002), although the groups presented estrus incidence within the average range observed in previous studies (Cordeiro et al., 2003).

A cause of concern is the growth pattern of follicles. A considerable number of animals presented large follicles (diameter up to > 5 mm) in the beginning of SOV treatment and a diameter ≥ 10 mm at the end of treatment. In general, the average diameter of preovulatory follicles ranges from 6 to 7 mm in sheep (Barrett et al., 2004; Figueira et al., 2020). This maintenance of follicular growth above the preovulatory mean diameter associated with endogenous discharge of LH can cause an increase in LUF incidence, resulting in early regression of the CL and reductions in the number and quality of the embryonic yield. It is worth remembering that in addition to endogenous LH, this commercial drug has a greater amount of LH compared to others, and we applied a single dose of GnRH in the end of treatment, promoting an exogenous supplementation to the treatment. Interestingly, the average number of LUF in this study was within expectations (Brasil et al., 2016; Figueira et al., 2020). In heifers, increased LH resulted in reduction of fertilization and transferable embryos (Bó and Mapletoft, 2020), although difference in number and quality of transferable embryos were not seen when compared formulations with low or equivalent LH

(Mikkola and Taponen, 2017). Also, the subtle interaction between FSH:LH can affect P4 and E2 and result in injuries in the transport of oocyte/embryos (Bettencourt et al., 2008). Gonzalez-Bulnes et al. (2000) report that an increase in plasma LH support can increase the number of atretic follicles in sheep, due to a deregulation of the mechanism that regulates follicular atresia or by saturation of LH receptors.

In the present study, CL count was higher compared to other studies in sheep that found an average of 7 (Lagares et al., 2021) to 9 (Ciornei et al., 2022) CL / ewe using the same commercial formulation of pFSH and doses between 250 to 500 IU. The recovery and viability rates were also similar to those studies (Cordeiro et al., 2003; Lagares et al., 2021; Ciornei et al., 2022). This demonstrates once again that the protocol is effective and promotes suitable rates of embryonic viability, but that it may need some adjustments, such as quantity and administration times, to increase the number of embryos recovered. It is important to mention that, in this study, the protocols using medium (333 IU) or high (400 IU) dose of pFSH showed a better superovulatory response in animals submitted to NSER and a tendency for a greater number of recovered structures. D'Alessandro et al. (1996) found a greater number of transferable embryos and a reduction in the number of unfertilized ova using 250 IU of pFSH compared to 750 or 1000 IU, although this group had a lower ovulation rate, corroborating our study. Wierzchos et al. (1992) also found a higher number of unfertilized ova using 750 IU, but with no difference in the average of ovulations or structures and viable embryos recovered between the groups.

In fact, 750 and 1000 IU of pFSH not only appear to be more harmful to the superovulatory response, but also make the protocol more expensive by reducing the number of doses / flasks. In the present study, using 400 IU of pFSH we found very similar rates of superovulatory response and embryonic yield than using 500 IU in Suffolk (Ciornei et al., 2022) and Altamurana ewes (D'Alessandro et al., 1996), which also seems to make the use of the commercial formulation more viable as it increases profitability. In addition, although other studies recommend the use of 250 IU of pFSH in Santa Inês ewes (Cordeiro et al., 2003; Lagares et al., 2021), in this study with White-Dorper ewes, 250 IU was the protocol with the lowest numbers of recovered structures (approximately one-third of that found in groups 333 or 400 IU). Bó and Mapletoft (2020) suggest that doses closer to physiological levels are more appropriate to induce superstimulation, since while low doses of pFSH do not reach the physiological threshold, while very high doses can result in saturation of FSH receptors

or excess LH. It is worth remembering that the SOV response is variable and dependent on several factors, including age and breed (Bartlewski et al., 2016). It is possible that this dosage is more advisable in another animal category or a breed with a higher prolificacy, being important to carry out this study in different categories, breeds, and environments.

5. Conclusion

The three pFSH doses (250, 333 or 400 IU) used in the superovulatory treatments were efficient in promoting preovulatory follicular growth, similar overall CL count and embryo yields. None of the doses affected NSER success. A greater CL count in ewes successfully flushed and a tendency of a greater number of ova/embryo recovered was registered to 333 or 400 IU than 250 IU of pFSH. Furthermore, this study confirms the need for prior selection of animals as a determinant for NSER success. The 333 IU may be the most suitable pFSH dose for superovulation in White Dorper ewes considering superovulatory response and embryo yield compared to higher doses, reducing the protocol cost.

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CONFLICTS OF INTERESTS

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

AUTHORS CONTRIBUTIONS

JFF and JMGS-F elaborated the hypothesis, discussed the experimental design. VSAP, SNE and ARG supported the experimental flock and field infrastructure. JHD

collected and analyzed the data from the animals and wrote the first version of the manuscript. GBV, JDG, TAO, RITPB, MEFO, JMGS-F and JFF assisted with the animals and helped collecting and analyzing the data. JFF, RITPB, LGBS, MEFO and JMGS- F discussed the design of the experiment and analyzed the data. JHD elaborated and worked on the
statistics. ARG, RITPB, MEFO, JMGS- F and JFF approved the final version of the manuscript.

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5. CONCLUSIONS

This study allows the understanding of the physiological mechanism of cervical dilatation and the application of drugs that promote cervical relaxation. Thus, it was proved that it is possible to remove estradiol benzoate from the cervical relaxation protocol, promoting an adequate cervical dilation without injuries to the non-surgical embryo recovery (NSER) procedure or animal welfare in synchronized and/or superovulated ewes.

It also confirms that NSER is an effective technique performed successfully in different breeds and categories of animals. Further, estradiol benzoate withdrawal allows the spread of NSER in countries where the use of this hormone is restricted and encourages evaluation of the technique in other breeds. It is essential to select the animals regarding their reproductive history and the presence or not of anatomical abnormalities in the cervix before submitting them to the procedure, to avoid unwanted expenses with hormonal protocols.

The administration of the cervical relaxation protocol caused an increase in estradiol and a decrease in progesterone concentrations post-protocol, and the isolated effect of prostaglandin F2α should also be considered. More studies verifying the impact of changes in the progesterone and estradiol profiles during early luteal phase on embryonic development and viability should be carried out.

The hCG supplementation in superovulated ewes in this study was able to increase P4 concentration, superovulatory scores and tended to maintain luteal structure, although it had no effect on functional luteal regression, being an excellent alternative to increase the embryonic yield, but an adjustment of the time of administration may be necessary for better results.

Superstimulation with doses of 250, 333 or 400 IU of a commercial pFSH (1FSH:1LH) was efficient in promoting ovarian superstimulation. The three groups showed similar follicular growth, overall CL count and embryo production, without impairing the NSER procedure, although there was a tendency to a greater number of ova/embryo recovery in the groups using 333 or 400 IU of pFSH, suggesting that the dose of 333 IU of pFSH is the most recommended for cost-effectiveness.