LUCAS MACHADO FIGUEIRA

IN VIVO EMBRYO PRODUCTION IN LACAUNE EWES COLLECTED BY TRANSCERVICAL TECHNIQUE

LAVRAS – MG
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Prof[a]. Dr[a]. Nadja Gomes Alves

Orientadora

Prof. Dr. Jeferson Ferreira da Fonseca

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APROVADO em 6 de dezembro de 2019.

Prof[a]. Dr[a]. Iraídes Ferreira Furusho Garcia - UFLA

Prof[a]. Dr[a]. Joanna Maria Gonçalves de Souza-Fabjan - UFF

Prof. Dr. Renato Ribeiro de Lima – UFLA

Prof[a]. Dr[a]. Nadja Gomes Alves – UFLA
Orientadora

Prof. Dr. Jeferson Ferreira da Fonseca – EMBRAPA
Coorientador

LAVRAS – MG
2020

Em memória de meu pai, por todos os ensinamentos de vida, mas principalmente pelos exemplos...

Em memória de meu avô paterno, pelo forte exemplo de persistência em seus projetos...

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EPÍGRAFE

“What a man can be, he must be. This need we call self-actualization.”

Abraham Maslow

RESUMO GERAL

Os programas de múltipla ovulação e transferência de embriões (MOTE) têm o potencial de acelerar o melhoramento genético de raças ovinas de interesse produtivo como a Lacaune. O objetivo geral da tese foi avaliar pontos estratégicos para aumento da eficiência da MOTE empregando a técnica de recuperação não cirúrgica dos embriões (RNCE) na raça Lacaune. No primeiro estudo, o interesse zootécnico da raça foi revisado. No segundo estudo, a avaliação ultrassonográfica da cérvix foi incorporada na rotina de procedimentos de recuperação não cirúrgica de embriões, avaliando seu potencial em predizer a penetração transcervical em função do desalinhamento cervical. Dois momentos foram escolhidos para a realização do exame: (1) 12 h após o início do estro no e (2) 30 min antes da RNCE. A avaliação no estro foi ineficiente devido a alta incidência de falsos negativos (52%). Entretanto, na avaliação antes da RNCE, a sensibilidade e especificidade foram 100%, demonstrando o potencial da ferramenta para a seleção de doadoras. O terceiro estudo avaliou os efeitos da duração dos protocolos de indução de estro com progestágenos por seis (G-6) ou (G-9) dias na dinâmica folicular preovulatória, resposta ovulatória e produção de embriões em ovelhas Lacaune submetidas à RNCE. A taxa de transposição cervical cinco a seis dias após ovulação foi de 95,2%. Houve maior ($P < 0,05$) resposta ovulatória ($2,9 \pm 0,3$ vs $1,9 \pm 0,3$) e produção de embriões ($1,3 \pm 0,4$ vs $0,4 \pm 0,2$) em G-9 comparado ao G-6. No quarto estudo, as ovelhas foram submetidas aos protocolos anteriores, mas foi avaliado a superovulação com 133 mg de FSHp. O G-9 superou o protocolo G-6, apresentando maior ($P < 0,05$) produção de embriões após a RNCE ($3,5 \pm 1,1$ vs $1,8 \pm 0,7$), realizada nos dias 6 e 7 após o estro. Como a dose de FSHp utilizada foi inadequada para aumentar a resposta SOV e a produção de embriões, foi realizado um quinto estudo, utilizando o protocolo de nove dias e comparando as doses de 100 (G-100) e 200 mg (G-200) de FSHp. A dose de 100 mg foi insuficiente para o recrutamento folicular e houve maior ($P < 0,05$) resposta SOV em G-200 ($11,6 \pm 1,2$) comparado ao G-100 ($2,6 \pm 0,7$). A taxa de recuperação de estruturas e o número de embriões viáveis foram maiores no G-200 (67,8% e $6,9 \pm 1,1$) comparado ao G-100 (27,6% e $1,0 \pm 0,5$). No sexto estudo os embriões foram criopreservados por técnicas de congelamento lento e vitrificação. Após descongelamento/ reaquecimento, os embriões foram usados aleatoriamente na transferência de embriões em tempo fixo para receptoras sincronizadas. Embora desafiados aos danos celulares inerentes ao processo de criopreservação, os embriões recuperados pela RNCE após tratamento de dilatação cervical foram capazes de estabelecer a prenhez e a parição, mas resultados satisfatórios foram obtidos apenas com a técnica de congelamento lento (39,4 e 20,9%, respectivamente). A presente tese apresenta resultados que atestam a viabilidade da realização de programas de múltipla ovulação e transferência de embriões baseados em recuperação não cirúrgica de embriões em ovelhas da raça Lacaune.

Palavras-chave: Colheita não cirúrgica Dilatação cervical. MOTE. Ovinos leiteiros. Superovulação.

GENERAL ABSTRACT

Multiple ovulation and embryo transfer (MOET) programs has the potential to accelerate the genetic improvement of sheep breeds of productive interest such as Lacaune. The general objective of this thesis was to evaluated strategic points to increase the efficiency of MOTE applying the non-surgical embryo recovery (NSER) technique following cervical dilation treatment in Lacaune ewes. In the first study, the zootechnical interest of the breed was reviewed. In the second, the ultrasonographic cervical evaluation was incorporated in the routine of procedures aiming NSER, to evaluate its potential to predict the transcervical penetration by cervical misalignment. Two different times was chosen to perform the exam: (1) 12 h after estrus onset in the first study and (2) immediately before NSER in the third study. The evaluating at the estrus was inefficient due to the high incidence of false negatives (52%). However, the evaluation before NSER, the sensibility and specificity were 100%, demonstrating the potential of the tool to donors selecting. The third study evaluated the effects of the duration of progestogen-based estrus induction protocols for six (G-6) or nine (G-9) days on preovulatory follicular dynamic, ovulatory response and embryo yields in Lacaune ewes by NSER technique. A rate of 95.2% of transcervical penetration was achieved 5 to 6 days after ovulation. There was higher ($P < 0.05$) ovulatory response (2.9 ± 0.3 vs 1.9 ± 0.3) and embryo yield (1.3 ± 0.4 vs 0.4 ± 0.2) in G-9 compared to the G-6. In the fourth study, the ewes were submitted again to 6 and 9-days protocols, but superovulated with the 133 mg of porcine FSH (pFSH). The G-9 outperformed G-6 days protocol, showing higher ($P < 0.05$) embryo yield after NSER (3.5 ± 1.1 vs 1.8 ± 0.7) performed on day 6 to 7 after estrus onset. As the pFSH dose used was inadequate to increase superovulatory (SOV) and embryo yield responses, it was conducted a fifth study, using the G-9 protocol and comparing the doses of 100 (G-100) and 200 mg (G-200) of pFSH. The 100 mg dose was insufficient to recruitment follicular and there were higher ($P < 0.05$) SOV response in G-200 (11.6 ± 1.2) than in G-100 (2.6 ± 0.7). The structures recovery rate and the number of viable embryos were higher in G-200 (67.8% and 6.9 ± 1.1) than in G-100 (27.6% and 1.0 ± 0.5). In the sixth study, the recovered embryos were cryopreserved by either slow freezing (SF) and vitrification techniques and were randomly used for fixed-time embryo transfer (FTET) to synchronized recipients. Although challenged to cellular damage inherent in the cryopreservation process, embryos recovered by NSER after cervical dilation treatment were able to establish pregnancy and lambing after FTET, but satisfactory results were obtained only with the SF technique (39.4 and 20.9 %, respectively). The present thesis presents results that attest the feasibility of carrying out multiple ovulation and embryo transfer programs based on NSER in Lacaune ewes.

Keywords: Cervical dilation. Non-surgical recovery. Dairy sheep. MOTE. Superovulation.

Produção *in vivo* de embriões em ovelhas Lacaune coletadas pela técnica transcervical
Elaborado por **Lucas Machado Figueira (UFLA)** e orientado por Dra**. Nadja Gomes Alves (UFLA)** e **Dr. Jeferson Ferreira da Fonseca (EMBRAPA)**

Na presente tese foram estudados diversos pontos estratégicos da biotecnologia de múltipla ovulação e transferência de embriões (MOTE), empregando a técnica de recuperação não-cirúrgica de embriões (RNCE) após tratamento hormonal de dilatação cervical na raça Lacaune. A principal vantagem da técnica é a possibilidade de realização de maior número de colheitas sucessivas numa mesma doadora se comparado à cirúrgica. Um ponto avaliado foi o grau de desalinhamento dos anéis cervicais na ultrassonografia. Tal avaliação demonstrou potencial em predizer o sucesso na transposição cervical para a RNCE. Outro estudo avaliou o período de permanência dos dispositivos intravaginais (impregnados com progesterona sintética ou análogos) por seis ou nove dias em tratamentos para indução do estro síncrono. O protocolo de nove dias obteve maior produção de embriões viáveis em ovelhas não superovuladas (2,9 versus 1.3) e superovuladas (3.5 versus 1.8) com 133mg do hormônio folículo estimulante - FSH. O protocolo de nove dias foi então utilizado como base para tratamento superovulatório comparando doses de 100 ou 200 mg do FSH. A dose de 100 mg foi insuficiente, mas a dose de 200 mg de FSH permitiu boa resposta superovulatória e a obtenção de 6,9 embriões viáveis por doadora. Os embriões obtidos foram então criopreservados por diferentes técnicas (congelamento lento e vitrificação) e transferidos em tempo-fixo para receptoras sincronizadas. Os embriões oriundos da RNCE após tratamento de dilatação cervical foram capazes de estabelecer resultados satisfatórios de gestação (39,4) e parição (20,9%) apenas com a técnica de congelamento lento. A presente tese comprova a viabilidade da realização de programas MOTE empregando a RNCE na raça Lacaune.

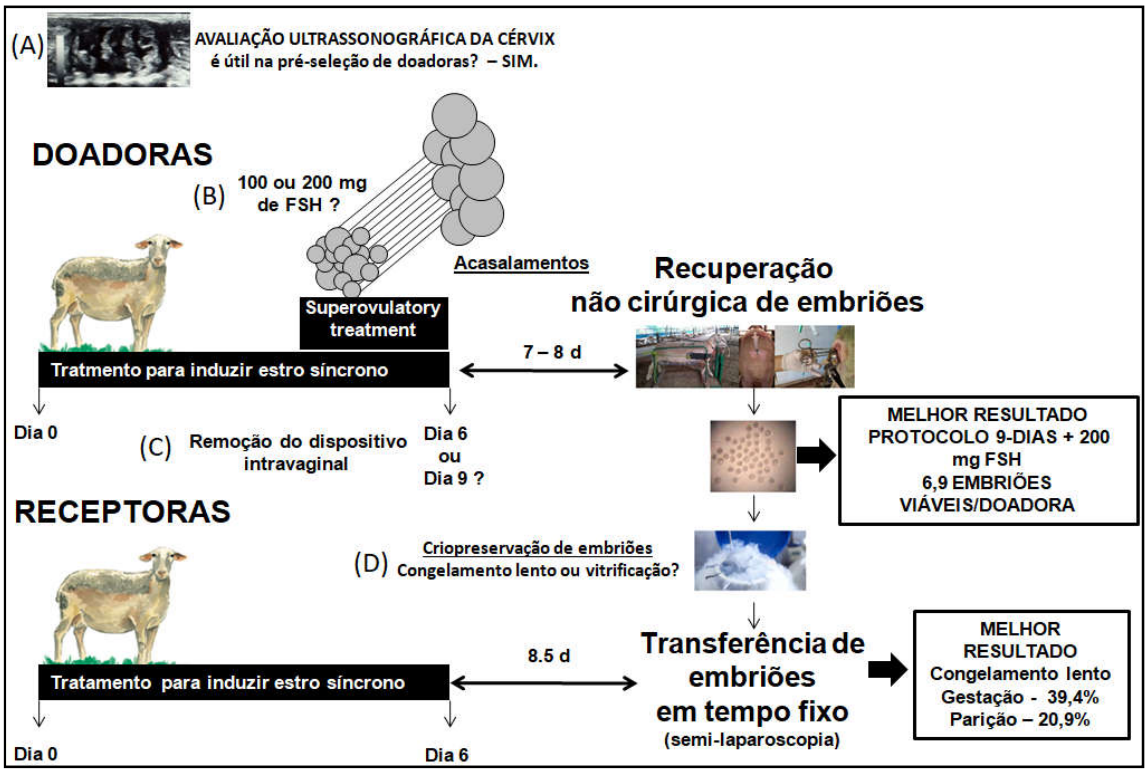
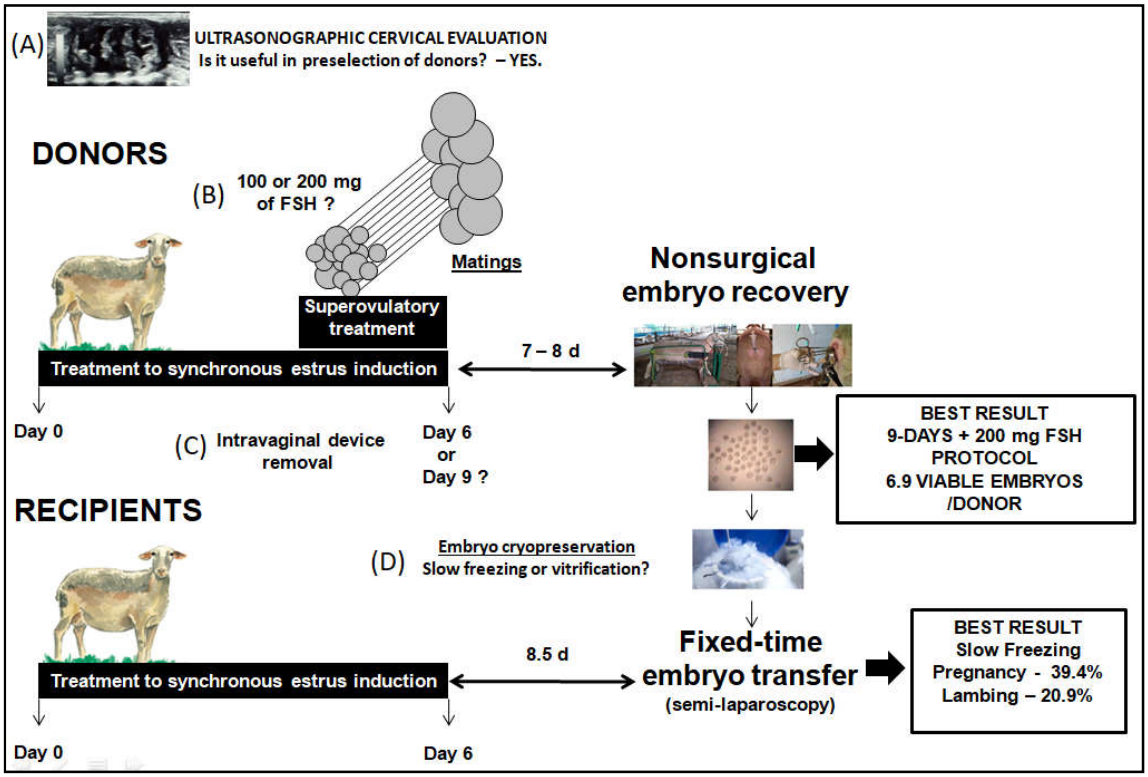


Diagrama simplificado do estudo de pontos estratégicos da biotecnologia de múltipla ovulação e transferência de embriões em ovelhas da raça Lacaune, empregando a técnica de recuperação não cirúrgica de embriões - RNCE. (A) Pré-seleção de doadoras para RNCE usando a ultrasonografia cervical (B) Ajuste da dosagem com 100 ou 200 mg de hormônio folículo estimulante (FSHp). (C) Ajuste do tempo de duração do protocolo de indução de estro síncrono, por 6 ou 9 dias. (D) Viabilidade dos embriões, obtidos por RNCE e criopreservados, após transferência em tempo-fixo.

Tese de Doutorado em Zootecnia na UFLA, defendida em 06/12/2019.

***In vivo* embryo production in Lacaune ewes collected by transcervical technique**
Elaborated by **Lucas Machado Figueira (UFLA)** and advised by Dra**. Nadja Gomes Alves (UFLA)** and **Dr. Jeferson Ferreira da Fonseca (EMBRAPA)**

In this thesis, several strategic points of biotechnology of multiple ovulation and embryo transfer were studied, using the non-surgical embryo recovery (NSER) technique after hormonal treatment of cervical dilation in the Lacaune breed. The main advantage of the technique is the possibility of making a greater number of successive recoveries in the same donor compared to the surgica technique. One point assessed was the degree of misalignment of the cervical rings on ultrasonography. It has potential to predict success in cervical transposition for NSER. Another study evaluated the duration of progestogen-treatment (intravaginal devices impregnated with synthetic progesterone or analogue) for six or nine days in treatments for synchronous estrus induction. The 9-day protocol obtained higher production of viable embryos, both in non-superovulated (2.9 versus 1.3) and in superovulated ewes (3.5 versus 1.8) with 133 mg of follicle stimulating hormone - FSH. The 9-day protocol was then used as a basis for superovulatory treatment comparing doses of 100 or 200 mg of FSH. The 100 mg dose was insufficient, but the 200 mg dose of FSH allowed a good superovulatory response and production of 6.9 viable embryos per donor. The embryos obtained were then cryopreserved by different techniques (slow freezing and vitrification) and fixed-time transferred to synchronized recipients. Embryos obtained from NSER after treatment of cervical dilation were able to establish satisfactory results of pregnancy (39.4) and lambing (20.9%) only with the slow freezing technique. This thesis proves the feasibility of carrying out MOET programs employing NSER in the Lacaune breed.



Simplified diagram of the study of strategic points in biotechnology of multiple ovulation and embryo transfer in Lacaune ewes, using the nunsurgical embryo recovery technique - NSER. (A) Preselection of donors for NSER using cervical ultrasonography. (B) Adjustment of the dosage with 100 or 200 mg of follicle stimulating hormone (FSH). (C) Adjust the duration of the synchronous estrus induction protocol, for 6 or 9 days. (D) Viability of after fixed-time transfer of embryos obtained by NSER and cryopreserved.

Doctoral Thesis in Animal Science at UFLA, presented onDecember 6, 2019

ABBREVIATION LIST

µg	micrograms
Ac	accuracy
BCS	body condition score
BW	body weight
CL	corpora lutea
cm	centimeters
CM	cervical map
DMCM	degree of misalignment on cervical map
DMUS	degree of misalignment on ultrasonography
eCG	equine chorionic gonadotropin
FN	false negative
FP	false positive
FSH	follicle-stimulating hormone
FTET	fixed-time embryo transfer
GnRH	gonadotropin-releasing hormone
h	hours
i.m.	intra-muscular
i.v.	intravenous
IU	international units
kg	kilograms
L	Liters
l.v.	laterovulvar
LF	large follicle
LSMEANS	least square means
m	meters
MAP	medroxyprogesterone acetate

mg	miligrams
MHz	megahertz
min	minutes
mm	millimeters
MOET	multiple ovulation and embryo recovery
ng	nanograms
NSER	non-surgical embryo recovery
P_4	progesterone
pFSH	porcine follicle-stimulating hormone
PGE	prostaglandin E
PGF2α	prostaglandin F2α
S	south
SEM	standard error of the means
SENS	sensitivity
SF	slow freezing
SPEC	specificity
TN	true negative
TP	true positive
US	ultrasonography
VT	vitrification
W	west
κ	Cohen's kappa index

SUMMARY

FIRST PART – OVERVIEW

1 INTRODUCTION

Dairy sheep farming is a traditional activity in different parts of the world, being the Lacaune one of the main breeds used and widespread due to the intensive genetic selection to milk production. The breed was introduced in Brazil in the 90's and currently is present in 96% of Brazil's dairy sheep farms (BIANCHI et al., 2016). With increasing effectiveness of non-surgical embryo recovery (NSER) in Brazil, the Brazilian dairy sheep farmers became interested to include Lacaune in multiple ovulation and embryo transfer (MOET).

Multiple ovulation and embryo transfer (MOET) programs has the potential to accelerate the multiplication of animals of high genetic merit in breeding programs. However, there are still technical limitations to their use in large-scale. Some of the main limitations refer to the the high cost relative to the animal value, wide variability in the superovulatory (SOV) response, and the limitation of successive collections resulting from adhesions formed after the surgical recovery of embryos. To overcome these problems, greater knowledge has been sought about intrinsic and extrinsic factors affecting SOV response and embryo production. Moreover, the NSER emerges as an alternative to surgical procedures, given the possibility of successive recoveries, as well as societal concerns about animal welfare. However, considering the particularities of the ovine cervix that hinder its transposition by pipettes or catheters breeds, it is necessary to evaluate the efficiency and repeatability of the NSER used in different breeds. On the other hand, since hormonal treatments for cervical dilation are necessary to produce efficient NSER, it is necessary to know if such hormones can affect the embryo viability after transfer in field conditions.

In this context, the implementation and / or enhancement of techniques that allow for good superovulatory responses and transcervical access in Lacaune ewes will make MOET based on NSER accessible to breeders and genetic improvement programs, increasing the generation of offspring in high genetic merit females and accelerating the multiplication of superior genotypes. The aim of this study was to evaluate MOET strategic points, such as selection of donors for NSER using ultrasonographic cervical evaluation, the adjustment of the estrus synchronization / induction and superovulation protocols to the breed, the post-transfer viability of embryos recovered by NSER and cryopreserved by different techniques.

2 THEORETICAL REFERENCES

2.1 *Lacaune breed of dairy sheep*

Lacaune is one of the main breeds of sheep raised for dairy farming. It is diffused worldwide due to the genetic gains achieved by the French improvement program. (BARILET et al., 2001). Lacaune animals were brought to Brazil during the 1990s and formed the basis of flocks specialized in milk production (BRITO et al., 2006). Currently, the Lacaune breed is present in 96% of Brazil's dairy sheep farms. Other pure breeds, such as the East Friesian, Bergamacia, and Santa Inês, or crossbreeds with East Friesian and Lacaune, are found too, but in smaller numbers. Overall, there are approximately 6,900 dairy ewes in the country, which produce 840,000 liters of milk annually (BIANCHI et al., 2016). Given the prevalence and importance of the Lacaune breed in sheep milk production in Brazil, the ARTICLE 1 of this thesis reviewed historical information on the breed, as well as its productive characteristics and the progress achieved by the French Lacaune genetic improvement program, which facilitated the global spread of the breed.

2.2 Multiple ovulation and embryo transfer

The MOET program is a combination of assisted reproduction methods that involves the production of multiple embryos by a female donor of high genetic merit, which will be transferred to several recipient females (GIBBONS; CUETO, 2013). MOET biotechnology has made significant contributions to the genetic improvement of sheep in several countries (MENCHACA et al., 2009), and also have been employed in the French program (TORRES; COGNIE; COLAS, 1987; BARIL, 1995; BARIL et al., 2001), allowing the production of more lambs per sheep when compared to normal physiological conditions. The MOET technique accelerates genetic improvement, making it possible to increase the transfer speed of superior genotypes in the flock (BARI et al., 1999, BARIL et al., 2001), as well as allowing conservation of the genotype of endangered species (LOPES-JÚNIOR et al., 2006, BETTENCOURT et al., 2008; FORCADA et al., 2011). Moreover, this reproductive biotechnology may prevent the transmission of some infectious viral (bluetongue, border disease, pulmonary adenomatosis, maedi/visna and caprine arthritis–encephalitis) and bacterial (brucellosis, campylobacteriosis, mycoplasmosis and chlamydial abortion) diseases.

The risks of disease transmission are minimal when the sanitary procedures are strictly followed (THIBIER; GUÉRIN, 2000).

However, MOET is possibly the most frustrating among the reproductive biotechnologies, because the results can vary unpredictably, from complete failure to total success (BALDASSARE; KARATZAS, 2004). In the last two decades the production of sheep embryos has been irregular, and according to IETS data, from 2016 production has been less than 40,000 embryos (FONSECA et al., 2019a). There are still some challenges to the achievement of greater efficiency. Among the main limitations for the wide application of MOET for commercial purposes and research is the variability in ovarian response and embryo yield after the superovulatory treatment (BARTLEWSKI et al., 2016). Another limiting factor is the embryo recovery technique: the most widely used technique is the laparotomy (FONSECA et al., 2016). However, this technique can lead to adhesion formation and reduces the number of embryos recovered after repeated surgeries(TORRES; SEVELLEC, 1987; BRUNO-GALARRAGA et al., 2014). The low use of MOET in sheep is also attributed to the relatively high cost of the technique when compared to the value of animals. Reduction of costs at any stage of MOET is likely to increase its use (BARIL et al., 2001). Stages of technique with greater potential for cost reduction are superovulatory treatment, progesterone/progestogen-based protocols to estrus synchronization, and embryo cryopreservation.

2.2.1 Superovulatory treatments

Superovulatory treatments have been used to increase the number of offspring of genetically superior females since the 1940's (CASSIDA; WARWICK; MEYER, 1944). The superovulation technique in ruminants is one of the main steps of MOET and consists in promoting multiple ovulations (supraphysiological number of ovulations, i.e. above the genetically established for the species and/or breed), by administering hormonal preparations that prevent atresia and ensure the development of several follicles until the ovulation. The first superovulatory treatments were based on the use of equine Chorionic Gonadotropin (eCG). However, the application of high doses of eCG results in low and extremely variable ovulatory responses (ARMSTRONG; EVANS, 1983). This is due to the long half-life of eCG, which may result in a high incidence of anovulatory follicles, which are then responsible for high estradiol secretion (EVANS; ARMSTRONG, 1984). The estrogenic condition may alter the transport of gametes through the genital tract, thereby reducing

embryo recovery rates. The formation of anovulatory follicles are also associated to premature luteal regression (OKADA et al., 2000) and lower mean efficiency of embryo recovery (CHAGAS E SILVA et al., 2003). The differences on embryo yield seem more evident outside of the breeding season (AZAWI; AL, MOLA, 2010). For these reasons, eCG has been replaced by the follicle-stimulating hormone (FSH).

Currently, FSH is the primary choice for hormonal ovarian superstimulation (BARTLEWSKI et al., 2016). Doses ranging from 150 to 300 mg FSH (porcine or ovine pituitary extract) are commonly used, either alone (SIMONETTI et al., 2008; BARTLEWSKI et al., 2009) or in combination with eCG (SIMONETTI et al., 2008; SALEHI et al., 2010; OLVEIRA et al., 2012; OLIVEIRA et al., 2014). According Gibbons and Cueto (2013) the treatment most used to promote multiple ovulation in sheep and goats is step-down application of FSH. Unlike eCG, FSH has a short biological half-life (3 to 4 hours), and needs multipleadministration (6-8 applications every 12 hours). The superovulatory treatment starts at the end of progestogen/progesterone treatment, around 60 hours after sponge removal. However, the dose regimen (number of applications and dosing protocol: constant or decreasing doses) are commonly questioned (BRASIL; MOREIRA; RAMOS, 2014).

The results of the technique are still highly variable (MENCHACA et al., 2010). The superovulatory response is dependent on several intrinsic and extrinsic factors, including breed, age, flock management, gonadotropic preparations and doses used, type of insemination, successive treatment intervals, among others (BARTLEWSKI et al., 2016). The breeding or non-breeding season may also affect the results. In the anovulatory season a greater variability in the results is observed (BARTLEWSKI et al., 2008). The progesterone profile induced by the intravaginal devices is also considered potentially responsible for the variable results in progesterone long-term treatments (OLIVEIRA; FONSECA; OLIVEIRA, 2013), so during long-term treatments (12-14d) device replacement was employed (OLIVEIRA et al., 2016). Even when some variation sources are minimized, or even eliminated, the response to superstimulatory gonadotropin treatment still remains variable, due to variability in the follicular response, premature luteal regression, and fertilization failures (MENCHACA et al. 2010). Advances in knowledge about follicular dynamics and superovulatory treatments in sheep have, however, resulted in gains in the control of influencing factors, and allowed the development of new treatments for improvement of embryo yield (MENCHACA et al., 2018).

In the last decade, some studies have evaluated the use of FSH doses lower than 150 mg in different breeds, as Merino (GIBBONS et al., 2010), Dorper (LOIOLA FILHO et al. 2015), and Santa Inês (MENEZES et al., 2014, RODRIGUEZ et al., 2019, MACIEL et al., 2019). Overall, these studies indicate that dose reduction can enable similar recovery of structures and viable embryos compared to higher doses, thus improving the cost benefit ratio.

2.1.1.1 Base-protocols for superovulation

The superovulatory treatment can be performed based on the natural estrus observation, or on estrus synchronization. Traditionally, to promote estrus synchronization, progestogen-soaked sponges or elastic rubber inserts (i.e., controlled intrauterine drug release [CIDR] devices) containing natural progesterone (P4) are left in the vagina for a long time (12–14 days) (ABECIA; FORCADA; GONZÁLEZ-BULNES, 2012). The protocols using progestins for 12 to 14 days (long-term) were established according to the luteal phase duration of the estrous cycle. They did not take into account the current knowledge of follicular growth (MENCHACA et al., 2010). These long-term protocols cannot avoid the presence of a large follicle, presumably dominant at the beginning of a gonadotropic treatment, and deleterious effects are observed on the superovulatory response and embryo yield (VEIGA-LOPEZ et al., 2005; MENCHACA et al., 2007). New approaches involving the synchronizing of a new follicular wave emergence (MENCHACA; PINCZAK; RUBIANES, 2002; MENCHACA, 2007; COGNIÉ, 2003; BARTLEWSKI, 2008; SOUZA-FABJAN et al., 2017) have been evaluated. The Day 0 protocol, starting FSH treatment from first follicular wave emergence at the time of ovulation, has improved ovarian response and embryo yield compared to traditional treatments, in both sheep and goats (MENCHACA et al., 2010). The adoption of progesterone protocols with different exposure time (MENCHACA et al., 2009) also has been evaluated in small ruminants, aiming at improving the results of superovulatory treatments. However, no differences were observed on superovulatory responses and embryo yield in ewes which received progesterone priming for 5 to 14 days. These authors concluded that the duration of the treatment could be more flexible, which has practical advantages for application in large-scale programs.

2.2.2 Physiological basis of cervical relaxation

The cervix is a dynamic structure which alters its conformation, and can assume varying degrees of relaxation/dilation, for example, at the time of parturition, in the follicular phase of estrous cycle, or induced by administering drugs that mimic endocrine mechanisms (SAFDAR; KOR, 2014; FALCHI, SCARAMUZZI, 2015).

At parturition, cervical relaxation is mediated by endocrine changes subsequent to increased fetal cortisol. The cortisol increases the activity of 17-hydroxylase and 17, 20-lyase in the placenta, promoting greater estradiol (E2) biosynthesis in relation to progesterone (P4). In turn, increase in the E2: P4 ratio stimulates the myometrial activity and culminates in parturition by increased expression of oxytocin receptors (WOOD, 1999). Oxytocin (OXY) is released intermittently during late pregnancy, having low or even undetectable plasma levels, but rising sharply during the expulsion phase of parturition (SCHAMS; SCHMIDT-POLEX; KRUSE, 1979).

During the follicular phase, such dilation occurs in response to increased FSH and E2 concentrations during the preovulatory follicular development, which induces receptors and enzymes that participate in prostaglandin E2 (PGE2) synthesis (FALCHI, SCARAMUZZI, 2015). During this period there is greater expression of receptors for E2, FSH, OXY and PPAR-γ (peroxisome proliferator-activated receptor), as well as prostaglandin-endoperoxide synthase 2 (COX-2) and phospholipase A2 enzymes that participate in PGE2 synthesis (MIZRACHI; SHEMESH, 1999; LEETHONGDEE et al., 2007, 2010; FALCHI, SCARAMUZZI et al., 2013).

The effectiveness of stimuli by estrogen is higher after a period of progesterone priming (PATE, 2018). Progesterone supports accumulation of arachidonic acid, prostaglandin endoperoxide synthase, and other molecules which are necessary for the synthesis of $PGF_{2\alpha}$ in the endometrium, and downregulates its own receptor, allowing for estrogen-stimulated expression of oxytocin receptors (PATE, 1998; SILVIA et al., 1991). These effects of progesterone appear to ensure that secretion of $PGF_{2\alpha}$ occurs at the appropriated time of luteolysis induction (SILVIA et al., 1991).

Oxytocin (OT) is released by the neurohypophysis under certain neural stimuli (FALCHI; SCARAMUZZI et al., 2015). The pituitary oxytocin release starts the episodic secretion of PGF2α from the uterus. The PGF2α is released into the uterine veins that are linked anatomically with the ovarian arteries (counter-current exchange system) ,and promotes the luteolityc cascade with degranulation of luteal OT stores. More oxytocin

promotes a positive feedback, increasing uterine PGF2α synthesis (EINER-JENSEN; HUNTER, 2005). By binding to its transmembrane receptor (G protein coupled-receptor) on target cells of the reproductive tract, oxytocin promotes conformational changes that trigger the cell signaling cascade. Opening of Ca^{++} ion channels in the endoplasmic reticulum increases their intracellular concentrations. This increase in cytoplasmic Ca^{++} is required to activate, via phosphorylation, the phospholipase A2 enzyme that mobilizes cell membrane phospholipids (arachidonic acid). Arachidonic acid is a substrate for the enzyme cyclooxygenase - 2 (COX-2) to produce prostaglandin H2, which in turn suffers the action of specific enzymes that convert it to prostaglandin E2 (PGE2). In addition, there is an increase in the cell nucleus of peroxisome proliferator-activated receptors, known as PPAR-γ, favoring COX-2 transcription (FALCHI; SCARAMUZZI et al., 2013, 2015).These evidences support that cervical dilation is mediated by PGE2 (LEETHONGDEE; KHALID; SCARAMUZZI et al., 2016).

The mechanism by which FSH stimulates cervical dilation is not completely elucidated. It seems to act in synergism with E2, favoring the production pathway of PGs, via increased COX-2 expression(FALCHI; SCARAMUZZI, 2015). In a study, the intracervical administration of gonadotropins (LH and FSH) selectively increased COX-2 expression, with greater increase in the caudal than in cranial portion of cervix. The FSH promoted its effect on smooth muscle fiber and stroma, while LH had its effect limited to smooth muscle fiber. These observations suggest that gonadotropins also participate in regulating cervical dilation during estrus (LEETHONGDEE; KHALID; SCARAMUZZI et al., 2016).

The PGE2 promotes smooth muscle relaxation by binding to EP1 and EP4 receptors, and the EP2 and EP4 receptors stimulate the synthesis of glycosaminoglycans (hyaluronic acid - HA) in the extracellular matrix by dispersing collagen fibers (KERSHAW et al. 2007, PEREIRA et al., 2007; JI et al., 2008). Such changes are important because they reduce the tensile strength of the cervix, allowing the cervical canal to dilate (FELTOVICH et al., 2005).

Maximum cervical relaxation is achieved around 72 hours after sponge removal in the estrus synchronization protocol. This moment is late for artificial insemination (LEETHONGDEE et al., 2007). Pharmacological induction of cervical relaxation would be an option for the improvement of the efficiency of transcervical artificial insemination, especially in sheep, where the anatomy and size of the cervix limit the degree of penetration (CANDAPPA & BARTLEWSKI, 2011). The mode of administration may also contribute to unpredictability of results of transcervical penetration observed in the literature (FALCHI et al., 2012). Success in cervical relaxation had already been attributed to intracervical HA

application, increasing applicator penetration degree in goats and sheep during estrus (PERRY et al., 2010; LEETHONGDEE et al., 2014). Local application of FSH and/or PGE2 analog (Misoprostol) were also effective in increasing insemination depth (LEETHONGDEE et al., 2007).

During the luteal phase of the estrous cycle, when progesterone concentrations are relatively higher than estradiol, there is inhibitory stimulus to HA synthesis and cervical relaxation, verified by decreased expression of EP2 receptors. (UCHIYAMA; SAKUTA; KANAYAMA, 2005; KERSHAW-YOUNG et al., 2009). However, cervical relaxation during the luteal phase is of interest, focusing on transcervical embryo recovery (FONSECA et al., 2016). Misoprostol (PGE analogue) in topical use on the cervical ostium allowed the collection in 94.8% of Dorper ewes (GUSMÃO et al., 2009) and 67% of Santa Inês ewes tested (GUSMÃO et al., 2007). However, misoprostol is not on the official drug list in 1/3 of the countries, including Brazil (LAVELANET; JOHNSON; GANATRA, 2020), limiting its use.

Induction of cervical relaxation during the luteal phase is also possible using a combination of estradiol and oxytocin (WULSTER-RADCLIFFE et al., 1999; FONSECA et al., 2016). Different estradiol esters (FONSECA et al., 2019b), estradiol administration routes (FONSECA et al., 2019c), oxytocin doses (MASOUDI et al., 2012), and oxytocin administration routes (PRELLWITZ et al, 2019) have been tested in an attempt to improve the protocols. Masoudi et al. (2012) evaluated a fixed dose of benzoate estradiol (100 µg) and different doses of oxytocin. Administration of estradiol benzoate alone did not achieve cervical dilation. However, when oxytocin (80 IU or more) was administered 12 hours after estradiol, cervical transposition was possible in a high percentage of the animals (75-100%) in three Iranian sheep breeds evaluated (MASOUDI et al., 2012). In Brazilian Santa Inês breed a high rate of transcervical penetration (81%) was reported, under the same hormonal combination, using higher benzoate estradiol (1 mg) and lower oxytocin (50 IU) doses (FONSECA et al., 2019c). However, considering the difference in transcervical penetration rates among breeds (KAABI et al., 2006), the efficiency and repeatability of NSER after cervical dilation treatment need to be carefully evaluated when applied to different breeds (FONSECA et al., 2016).

2.2.3 Non-surgical embryo recovery in sheep

In sheep, embryos can be recovered by surgical (laparotomy), laparoscopic or transcervical methods (FONSECA et al., 2016). The first embryo collections in goat and sheep were performed surgically by Warwick, Berry, and Horlach (1934) and the techniques later established by Hunter, Adams, and Rowson (1955).Few improvements in technique have been made and embryo collection in small ruminants is still performed predominantly in this manner, despite research efforts to improve and make accessible less invasive methods such as laparoscopic and transcervical (BARI et al., 1999; FONSECA et al., 2016). The surgical method demands prior fasting and use of anesthetic drugs that subject the animal to risks. Furthermore, adhesions, post-operative trauma and stress can arise from the procedure, limiting repeated embryo recoveries to two or three per animal (TORRES; SEVELLEC, 1987; BRUNO-GALARRAGA et al., 2014). -There are ongoing efforts to enable MOET programs based on non-surgical embryo recovery by cervical route, due to growing concerns about animal welfare and for better applicability of these biotechnologies (FONSECA et al., 2016, FONSECA et al., 2019a).

In goats this route is efficiently used, and Brazil is a world reference for this technique (FONSECA et al., 2013; FONSECA et al., 2014). However, sheep have greater limitations than goats due to the anatomy of the cervix (FONSECA et al., 2016). The ovine cervix is a fibrous tubular organ whose lumen is highly convoluted and tortuous by prominences and depressions in the mucosal membrane which form annular folds or rings in numbers of 3–7 (NAQVI et al., 2004; KERSHAW et al., 2005). In goats, there are more folds in the cervical lumen but they are aligned (HALBERT et al., 1990). The depth of cervical penetration using catheter/pipette is affected by the difficulty of rectal manipulation of the reproductive tract and complex anatomy of the cervical lumen, in which cervices with a higher degree of convolutions and interdigitations are less penetrable (KERSHAW et al., 2005). For this reason, the initial studies of Coonrod et al. (1986) achieved modest transcervical penetration rate (42% or 11/26). When NSER is accompanied by hormonal treatment to induce cervical dilation, higher transcervical penetration rates were observed. In Brazil, studies with Santa Inês (GUSMÃO et al., 2007) and Dorper (GUSMÃO et al., 2009) ewes, using misoprostol for cervical dilation treatment, reached 61% and 95% of transcervical penetration rate, respectively. Fonseca et al. (2019c) observed encouraging results (81%) in the Santa Inês breed using a protocol which combines estradiol benzoate, d-cloprostenol, and oxytocin.

These results show that hormonal treatments for cervical dilation are necessary to produce efficient NSER.

The use of non-invasive tools such as ultrasound is necessary in NSER for corpus luteum counting and evaluation of embryo recovery potential. Recently, it was demonstrated that B-mode and colour Doppler sonography are an useful tool for detecting luteal structures in superovulated ewes (OLIVEIRA et al., 2018; PINTO et al., 2018a). The use of Doppler US is an excellent tool for donor screening and evaluation of embryo recovery efficiency, and is in accordance with the NSER precepts, with attention to animal welfare and to the possibility of repeated collections (FONSECA et al., 2016). The diagnosis of animals with insufficient superovulatory response suggests the decision making by embryo collection or the continuity of pregnancy.

2.2.4 Embryo cryopreservation

Cryopreservation technology makes the long-term storage of embryos possible . This technology has been employed in commercial lines from the industry and research flocks, as well as in maintenance of rare genotypes worthy of conservation (BETTENCOURT et al., 2009). Efficient cryopreservation methods are also required to allow the delayed transfer of embryos (BARIL et al., 2001).

The most widely used technique for cryopreservation of sheep embryos is slow freezing, which requires an expensive biological freezer and intensive labour (DATTENA et al., 2004). Vitrification techniques are an alternative which offer a real possibility to reduce the cost of embryo transference (BARIL et al., 2001). The slow freezing technique is designed to maintain a delicate balance between cryoprotectants at low concentration (1–1.5 M) and aqueous embryo compartment, whereas the strategy of vitrification is a rapid solidification of liquid with high concentrations of cryoprotectants (6–7.5 M) to avoid ice crystal formation (GUIGNOT et al., 2006). Literature has shown no differences in pregnancy rates between the slow freezing (38-73%) and vitrification method (52-79%) applied to *in vivo*-derived embryos (reviewed by GIBBONS et al., 2011).

According to Gibbons et al., 2019, in spite of the research efforts, there is scarce extension of the vitrification technology in sheep embryos from the bench to the field. Despite the lower cost, the technique has not been extensively used because there is no standard procedure and because limited reports on fertility under field conditions are available (GREEN et al., 2009; GIBBONS et al., 2011).

2.2.5 Donor selection to non-surgical embryo recovery

The efficiency of MOET programs can be achieved through preselection of donors. Recently, Pinto et al. (2018b) demonstrated that the antral follicular count on ultrasonography made at a random moment of the estrous cycle (sponge insertion) or at the emergence of the first follicular wave had positive correlation with superovulatory response and embryo yield in sheep, and can be used alone or combined to Antimullerian hormone (AMH) dosage to select donor ewes. Another perspective is that the ovulatory response after single-shot eCG treatment has high correlation with further response to superovulatory treatment with FSH and can be used as preselection test for donors (BRUNO-GALARRAGA et al., 2015).

When the NSER is applied, the ease of cervical transposition can be predicted. The cervical transposition test at estrus has the ability to identify, with high sensitivity and accuracy, the potential of donor ewes to be submitted to non-surgical embryo recovery in MOET programs (SANTOS et al., 2019). However, it was observed that ~20% of animals showed discrepant results of cervical transcervical penetration after induction of cervical dilation or at estrus-induced (Fonseca et al., 2019a) suggesting that the degree of cervical dilation during synchronous induced estrus may be different from the physiological response to cervical dilation treatment for NSER.

In humans, the ease of embryo transfer in infertile women has been evaluated using the cervical ultrasonography (STANZIANO et al., 2017), but in small ruminants no type of ultrasound examination has yet been used for similar purposes. Therefore, new approaches to donor selection according to the cervical characteristics can still be tested.

3 GENERAL CONSIDERATIONS

Given the importance of the Lacaune breed for the exploitation of sheep milk in Brazil and worldwide, and the potential of reproductive biotechnologies to accelerate genetic improvement, it is necessary to fill the gaps to increase the efficiency of multiple ovulation and embryo transfer in sheep.

The results of the present study will support knowledge about the feasibility and efficiency of the non-surgical embryo recovery technique in the Lacaune breed, and open perspective for application in other sheep breeds.

Given the considerable amount of embryos to be produced, we highlight the possibility of study about estrus synchronization protocols, superovulation, cryopreservation, and the evaluation of embryo viability after transfer, since doubts still remain about their viability after hormonal coktail exposure used to promote cervical dilation.

Since studies indicate donor selection based on potential of superovulatory response (antral follicular counts on ultrasonography) can increase the efficiency of MOET programs, the preselection of donors for NSER by ease in which the cervix is transposed may also be used. Attempts at cervical penetration to estrus have already been employed for this purpose. We speculate that the use of ultrasound may also be useful for assessing the degree of cervical misalignment and predicting success in transcervcal penetration to NSER.

4 OBJECTIVES

The aim of this study was to evaluate strategic points to increase the efficiency of multiple ovulation and embryo transfer in Lacaune ewes, such as: the selection of donors for NSER using ultrasonographic cervical evaluation; the adjustment of the estrus synchronization/induction and superovulation protocols to the breed; the viability of the NSER technique in the Lacaune breed; and the post-transfer viability of embryos recovered by NSER and cryopreserved by different techniques..

4.1 Specific objectives

- to assess the degree of misalignment of cervical rings by ultrasonography, either at estrus or before NSER, to predict the penetration success at NSER.

- to evaluate the effects of the duration of progesterone-based estrus induction protocols (6 or 9 days) on preovulatory follicular dynamics, ovulatory response, and embryo yield after non-surgical embryo recovery.

- to evaluate the superovulatory response following porcine FSH step-down treatment in short- (6 days) or mid-term (9 days) progestogen-based protocols and the subsequent embryo yield after the NSER.

- to evaluate the effect of porcine FSH dose (pFSH, 100 mg versus 200 mg) on superovulatory responses and *in vivo* embryo yield after NSER procedure.

- to assess the survival capacity of non-surgically recovered and cryopreserved (vitrification or slow freezing) ovine embryos after fixed-time transfer.

5 CONCLUSIONS

The present thesis presents results that attest the feasibility of carrying out multiple ovulation and embryo transfer (MOET) programs based on non-surgical recovery (NSER) in Lacaune ewes. It was demonstrated that the adjustment of duration of the progestogen protocols to mid-term (9-days) in synchronous estrus induced and superovulated Lacaune ewes plus to customization of the pFSH doses brings progressive increases in the structures recovery rates and embryo yield after NSER. The average number of viable embryos thus produced was similar to world averages, in which the surgical technique is predominantly employed. The non-surgical technique applied to MOET programs can greatly increase the number of offspring of the same female of high genetic merit and contribute to the genetic improvement of Lacaune and other breeds in which the NSER can be successful performed. The results of pregnancy after embryo transfer were similar to those seen in field conditions, demonstrating the feasibility of the practical application of MOET employing NSER in commercial herds.

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SECOND PART – ARTICLES

ARTICLE 1 - Produção de leite ovino: a raça Lacaune (in portuguese)

Article following the norms and format of the Proccedings of 15º Workshop Goat Production in the Atlantic Forest Region.*

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ARTICLE 1 - Produção de leite ovino: a raça Lacaune (in portuguese)

Lucas Machado Figueira[1] ; Nadja Gomes Alves[2] ; Jeferson Ferreira da Fonseca[3]

1 Médico-veterinário, discente do Programa de Pós-Graduação em Zootecnia, Universidade Federal de Lavras, Lavras, MG

2 Médica-veterinária, docente do Programa de Pós-Graduação em Zootecnia, Universidade Federal de Lavras, Lavras, MG

3 Médico-veterinário, doutor em Zootecnia, pesquisador do Núcleo Regional Sudeste da Embrapa Caprinos e Ovinos, Coronel Pacheco, MG

Introdução

Animais da raça Lacaune (LA) foram introduzidos no Brasil durante a década de 1990, e formaram a base dos rebanhos especializados na produção de leite ovino (Brito et al., 2006). Atualmente, a Lacaune está presente em 96% das criações leiteiras do País. Outros animais de raças como a East Friesian (EF), Bergamácia e Santa Inês, puros ou cruzados com EF e LA, são encontrados também em menor número. No total são aproximadamente 6.900 matrizes leiteiras no País, com produção anual de leite de 840.000 litros (Bianchi et al., 2016). Diante da grande representatividade e importância da raça Lacaune para produção de leite ovino no país, o objetivo deste estudo foi levantar informações históricas de formação da raça, descrever suas características e os progressos alcançados pelo programa de melhoramento francês sob aspectos produtivos, que permitiram a ampla difusão da raça no mundo.

Origem e histórico da raça

A raça Lacaune foi desenvolvida em meados do século passado, nas regiões montanhosas ao sul da França, a partir de raças locais como a Pré-alpes do Sul (Quittet; Franck, 1983). As fazendas na área de produção do tradicional queijo Roquefort, que localizam-se no Maciço Central, nos departamentos de Aveyron, Tarn e adjacências, fizeram a opção pela criação e seleção de ovinos dessa raça. Em contraposição, outras regiões da França com forte tradição na produção de ovinos leiteiros, como os Montes Pirineus e a Ilha de Córsega, fizeram opção por outras raças, como a Basco-Bernese, Manech e Corsa. Nos anos 1950 e 1960 a raça era vista como de dupla aptidão, pois a terminação dos cordeiros até os 100-120 dias, permitia igualar as receitas provenientes das vendas de carne e leite (Barillet et al., 2001).

O progresso genético da raça acompanhou o ciclo de transformações na ovinocultura leiteira na França. A estagnação da produção na década de 1960, em virtude da baixa produção da ovelha Lacaune e da ordenha manual, exacerbou a necessidade de maiores investimentos. A melhoria genética dos plantéis e a implementação da mecanização de ordenha, foram ações propostas para atender a demanda crescente. Tais investimentos causaram redução do número de produtores de 8240 para 2517 e o aumento da produtividade de 56,8 milhões/litros/ano para 234 milhões/litros/ano, num período de apenas 40 anos (Barillet et al., 2001).

Ainda na década de 1960, duas centrais foram criadas para conduzir o programa de melhoramento genético dos plantéis Lacaune (Cooperativa OVI-TEST e Confederação do Roquefort). As centrais foram encarregadas de selecionar os melhores reprodutores, a partir de um sistema piramidal que continha os rebanhos núcleo de seleção (10%-20% dos animais nos rebanhos de alto mérito genético) e o rebanho base comercial (80%-90% restante) que também forneciam os dados zootécnicos para a realização dos testes de progênie (Baloche et al., 2014).

As primeiras tentativas do programa de melhoramento buscavam manter a dupla aptidão e utilizar cruzamentos com raças estrangeiras (East Friesian e Sarda). Entretanto, em virtude dos resultados insatisfatórios, a partir da década de 1980, tais estratégias foram descontinuadas. A seleção para a produção e composição do leite foi então priorizada, o que permitiu triplicar a produção de leite (Barillet et al., 2001).

O programa de melhoramento genético da raça Lacaune é atualmente o mais sofisticado e eficiente para ovelhas leiteiras no mundo (Thomas et al., 2014). A eficiência do programa fez da Lacaune uma das raças de maior destaque na produção leiteira. Isso foi possível graças a implementação da inseminação artificial. A cada ano cerca de 80% das fêmeas do rebanho núcleo e 50% do rebanho base são inseminadas (Barillet et al., 2001).

O intervalo entre gerações reduzido, de 4,2 anos (Baloche et al., 2014), favorece o rápido progresso genético de algumas características. Estima-se que o ganho anual para produção de leite seja de 6 kg, e para proteína e gordura 0,19 g/litro (Barillet, 2007). Também há uma pressão de seleção muito grande pela alta taxa de reposição de matrizes, que em alguns rebanhos alcança 35% (Fregeat, 2017).

Tal progresso despertou interesse de outros países e, a partir da década de 1990, exportações oficiais foram realizadas para 17 países. O Brasil foi um desses países, importando no ano de 1992 os animais que formariam a principal base dos rebanhos de ovinos leiteiros no País (Brito et al., 2006). À época, comparações entre raças foram feitas nos países importadores para avaliar o desempenho produtivo (leite e carne) e o Lacaune sobressaiu-se (Churra e Manchega - Espanha, e East Friesian - Canadá, Alemanha e Suíça) (Barillet et al., 2001).

O Rio Grande do Sul foi o estado brasileiro a receber os primeiros animais Lacaune e a implantar o primeiro laticínio especializado (Brito et al., 2006). A raça Lacaune foi então disseminada para outros estados do sul (Santa Catarina e Paraná), e alguns estados do Sudeste (Minas Gerais, Rio de Janeiro e São Paulo), além do Distrito Federal, basicamente a partir dos genótipos importados na década de 1990. Apenas em 2012, houve nova entrada de material

genético da raça, quando alguns produtores conseguiram trazer quantidade significativa de sêmen de boa procedência genética. Na Tabela 1, são apresentados dados sobre os rebanhos ovinos leiteiros no País, que podem ser facilmente extrapolados para a Raça Lacaune, em face da sua representatividade nos rebanhos (Bianchi et al., 2016).

Tabela 1. - Distribuição, efetivo e produtividade dos rebanhos ovinos leiteiros por estados federativos.

Estado/Distrito	Produtores	Matrizes	Laticínios	Produção anual de leite (l)
Rio Grande do Sul	7	2.000	4	270.000
Santa Catarina	4	2.400	3	315.000
Paraná	2	500	2	15.000
São Paulo	6	600	6	60.000
Rio de Janeiro	3	350	3	45.000
Minas Gerais	4	950	4	130.000
Distrito Federal	2	100	2	5.000
Total	28	6.900	24	840.000

Características raciais

Ovelhas da raça Lacaune são animais de temperamento dócil. A pelagem é predominantemente branca, com poucos traços de pigmentação. À idade adulta têm altura na cernelha de 70 cm a 80 cm. O peso médio das fêmeas adultas é de 70 kg a 80 kg (mínimo 60 kg) e o dos machos adultos de 95 kg a 100 kg (mínimo de 80 kg). São animais de corpo robusto, grande e comprido, com dorso reto e largo. O peitoral é profundo, se estendendo entre os membros anteriores. Os membros têm comprimento médio, sendo proporcionais ao corpo. A cabeça é fina e com chanfro comprido. As orelhas são compridas e implantadas lateralmente (mais horizontal é melhor). São animais naturalmente mochos e de mucosas róseas (Associação Brasileira de Criadores de Ovinos, 2018).

É desejável que o úbere tenha boa conformação e implantação. Os tetos devem ser de tamanho que permita a utilização da ordenha mecânica. O velo tem pouca extensão e cobre mais o dorso do animal superior e metade das faces laterais do pescoço e corpo, a anca e parte dos membros posteriores, deixando descoberto a cabeça, a nuca, o bordo inferior do pescoço, o peito, a parte inferior do tórax, o ventre, as axilas, as virilhas e membros. Em média os carneiros produzem mais lã que as ovelhas (2,5 kg x 1,5 kg) (Associação Brasileira de Criadores de Ovinos, 2018).

Aspectos produtivos

Leite

A produção leiteira da raça Lacaune no sistema tradicional francês costuma ultrapassar os 300 l (Fregeat, 2017), e para os melhores criatórios os 400 l/lactação (OVI-TEST, 2018). A Figura 1 mostra a evolução da produção leiteira na França, de acordo com o grau de seleção. Os dados de literatura têm apresentado bastante variabilidade na produção em função do grau de seleção e endogamia das linhagens que estão sendo trabalhadas nos diferentes países. Outro fator que interfere na produção de leite é o sistema de alimentação e manejo, que diferem bastante. No sistema tradicional, as forragens têm maior participação na dieta, enquanto outros sistemas mais intensivos em outros países adotam maior inclusão de concentrado (Thomas et al., 2014). Quanto ao manejo das ovelhas e suas crias, destacam-se três: (1) desmame do cordeiro aos 30 dias, quando se inicia o período de ordenha da ovelha. (2) aleitamento do cordeiro com mamada na ovelha nas primeiras 24h, e depois com substituto lácteo até os 30 dias. A ordenha é iniciada 24 h após o parto e em seguida uma ou duas vezes ao dia, até que a produção de leite seja de 0,25 kg/dia, quando é feito o processo de secagem. (3) Sistema misto, no qual o cordeiro é criado junto à mãe, sendo apartados durante a noite para reservar o leite para a ordenha matinal, até o desmame com 30 dias. (Thomas et al., 2004).

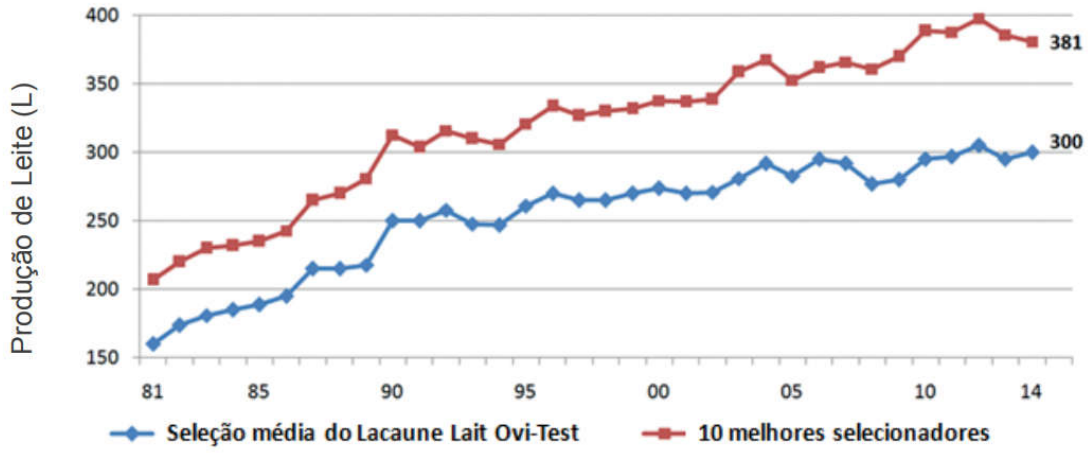

Figura 1. Comparação da produção leiteira dos dez melhores selecionadores da raça Lacaune com a produção média dos rebanhos em trabalho de seleção na França. Fonte: OVI-TEST (2018).

Um componente importante do sistema de produção é a eficiência alimentar. Such e Caja (1995) compararam ovelhas Lacaune e Manchega submetidas à mesma dieta e observaram produção de leite, corrigida para gordura e proteína, 89% maior nas ovelhas Lacaune, com eficiência alimentar bruta de 0,31 x 0,19. Diante da mesma dieta, ovelhas Manchega depositaram mais gordura corporal, enquanto ovelhas Lacaune mobilizaram gordura corporal, evidenciado por ligeira redução de escore de condição corporal.

A facilidade de ordenha é um aspecto importante na produção de leite ovino. O fluxo de leite durante a ordenha é variável entre raças. Such et al. (1999) compararam ovelhas de segunda lactação, Lacaune e Manchega, e verificaram menor leite residual no úbere em ovelhas Lacaune. Isso se deve ao maior reflexo de ejeção do leite. Ovelhas Lacaune demonstram melhor liberação de ocitocina após pré-estimulação e/ou início da ordenha, permitindo melhor fluxo do leite e menor leite residual, até quando comparadas a ovelhas de grande produtividade como a East Frisian (Bruckmaier et al., 1997).

A persistência de lactação é em torno de 180 dias de lactação (OVITEST, 2018). Ovelhas Lacaune que atingiram produção de leite 30% superior a East Friesian no pico da lactação, tiveram queda diária mais acentuada (8 g/dia^{-1} x 2 g/dia^{-1}), que determinou menor persistência (Ticiani et al., 2013). Em ovinos, a o declínio da lactação pós-pico pode ocorrer rapidamente em função da raça e genótipo, ou potencial leiteiro individual (Bencini; Pulina, 1997).

Ovelhas da raça Lacaune mostraram maior constância na produção de leite após sucessivas lactações comparadas a raças de menor produção leiteira (Tsigai e Improved Valachian). O decréscimo na produção leiteira não foi superior a 4% entre lactações (Oravcová et al., 2006). Tal característica permite menor reposição de matrizes e maior longevidade dos animais nos rebanhos.

Outro aspecto notável da raça Lacaune é o menor comprometimento da produção de leite quando o intervalo entre ordenhas é ampliado. No trabalho de Castillo et al. (2008), houve redução da produção de leite mais pronunciada em ovelhas Manchegas (-20%) do que em ovelhas Lacaune (-11%), quando o intervalo entre ordenhas foi estendido de oito para 20h. Quando ovelhas Lacaune são ordenhadas uma única vez ao dia, há redução em torno de 15%, mas muitos produtores de queijo veem vantagens na utilização desse manejo. Deve-se ter em mente que a redução do número de ordenhas afeta diversos componentes do sistema produtivo: (1) extensão do período produtivo, (2) aumento do rebanho, (3) aumento de área de pastagens e (4) validade dos dados para o melhoramento genético. Sendo assim, tais fatores

devem ser levados em consideração para as adequações do sistema produtivo (Vanbergue et al., 2013).

Outras características relacionadas à produção leiteira da raça Lacaune que vêm recebendo maior atenção nos últimos 10 anos de programa de seleção são: resistência à mastite, a morfologia da mama e a facilidade de ordenha (OVI-TEST, 2018).

Composição do leite

Proteína e gordura são os componentes do leite que mais variam em função da alimentação do animal, que responde por até 50% dessas variações (Fredeen, 1996). Portanto, comparações entre raças devem ser realizadas controlando variações na dieta. Em sistema de confinamento nos Estados Unidos, a raça Lacaune teve produção de leite e persistência da lactação ligeiramente inferior a East Friesian, mas com maior produção de proteína e gordura, que tornaram a produção total de sólidos do leite similar (Thomas et al., 2004).

Entretanto, Ticiani et al. (2013) comparando as raças Lacaune e East Friesian em sistema de pastejo semi-intensivo na Região Sul do País observaram maior produção de leite (1,67 kg/dia^{-1} x 1,35 kg/dia^{-1}), gordura (114,80 g/dia-1 x 102,85 g/dia^{-1}) e proteína (82,82 g/dia^{-1} x 73,10 g/dia^{-1}) na Lacaune, mas menores teores de gordura (6,86 x 7,31) e proteína (4,93 x 5,18) do que na East Friesian. No trabalho de Brito et al.(2006), ovelhas Lacaune em sistema confinado, também na Região Sul do país, apresentaram menores teores de gordura e proteína de 5,79% e 4,46%, respectivamente. Esses dados divergentes mostram que a produção e composição do leite nas diferentes raças têm grande variabilidade em função do manejo alimentar adotado e da representatividade dos genótipos. Assim sendo, as comparações precisam ser cautelosas.

Ademais, raças altamente selecionadas para produção de leite (Awassi, East Friesian, Lacaune and Sarda) apresentam correlação negativa entre volume de produção de leite e componentes do leite, apresentando menores concentrações de gordura e proteína, num efeito de diluição (Bencini; Pulina, 1997) conforme demonstrado na Tabela 2.

Tabela 2. - Concentrações (%) de proteína e gordura em diferentes raças de ovelhas.

Raça	Proteína	Gordura	Fonte
Aragat	5.49	5.70	Anifantakis (1986)
Awassi	6.05	5.84	Mavrogenis & Louca (1980)
Babass	5.29	5.84	Dilanian (1969)
Boutsiko	6.04	7.68	Voutsina et al. (1988
Bulgaria population	5.83	8.10	Baltadjieva et al. (1982)
Chios	6.00	6.60	Mavrogenis & Louca (1980)
Clun Forest	5.90	5.80	Poultron & Ashton (1970)
Comisana	7.30	9.10	Muscio et al. (1987)
Dorset	6.50	6.10	Sakul & Boyland (1992)
East Friesian	6.21	6.64	Anifantakis (1986)
Egyptian population	5.84	8.30	Askar et al. (1984)
Fat-tailed	6.40	6.26	Mavrogenis & Louca (1980)
Finn	5.40	6.00	Sakul & Boyland (1992)
Greece population	5.74	6.88	Baltadjieva et al. (1982)
Karagouniki	6.60	8.70	Anifantakis (1986)
Karakul	5.57	7.36	Anifantakis (1986)
Lacaune	5.81	7.14	Delacroix-Buchet et al. (1994)
Massese	5.48	6.79	Casoli et al. (1989)
Merino	4.85	8.48	Bencini & Purvis (1990)
New Zealand Romney	5.50	5.30	Barnicoat (1952)
Rambouillet	5.90	6.10	Sakul & Boyland (1992)
Romanov	6.10	5.90	Sakul & Boyland (1992)
Sarda	5.89	6.61	ARA (1995)
Suffolk	5.80	6.60	Sakul & Boyland (1992)
Sumava	6.47	7.93	Flam et al. (1970)
Targhee	4.51	9.05	Reynolds & Brown (1991)
Tzigai	5.45	7.41	Margetin (1996)
Vlachiki	6.52	9.05	Anifantakis (1986)
Welsh Mountain	5.40	6.20	Owen (1957)

A composição do leite é uma das principais características trabalhadas no programa de melhoramento genético francês, tendo em vista que o teor de sólidos totais determina o rendimento no beneficiamentto do leite em queijos e outros produtos. A Figura 2 mostra a evolução na produção de proteína e gordura ao longo de 20 anos de seleção pela Cooperativa OVI-TEST.

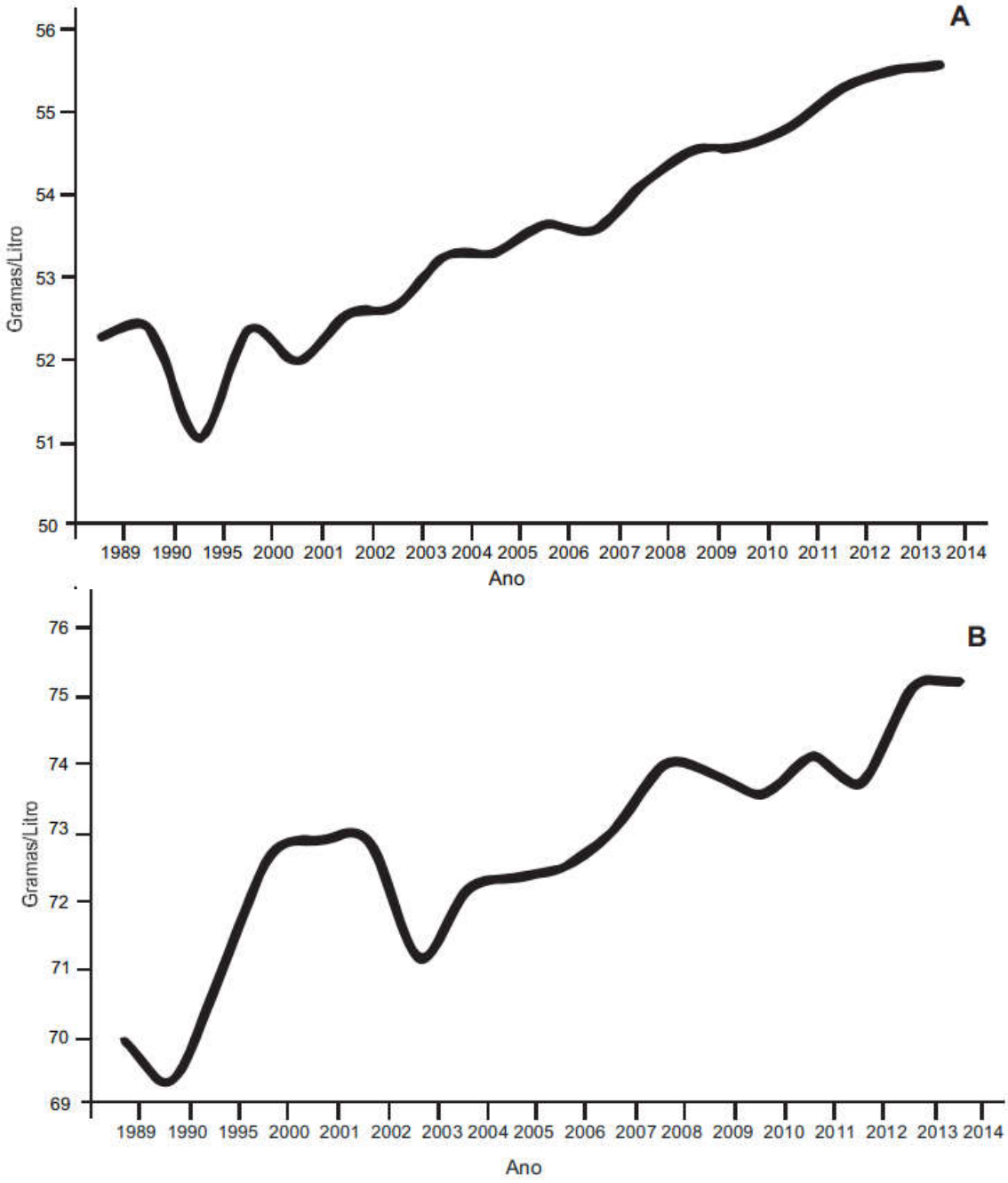

Figura 2. Evolução da produção de proteína (A) e gordura (B) no leite em animais selecionados da raça Lacaune no programa de melhoramento genético da Cooperativa OVI-TEST.

Fonte: Adaptado de OVI-TEST (2008).

Carne

Em virtude da dupla aptidão, uma linhagem divergente foi criada com foco na seleção para corte. Esse trabalho, iniciado em 1975 pela Cooperativa OVI-TEST, conta agora com 26 rebanhos núcleo de seleção, que totalizam mais de 11 mil animais. O foco inicial do trabalho era a prolificidade, já que esse é um traço de baixa herdabilidade. Em 20 anos, foi possível aumentar de 1,28 para 1,98 o número de crias por ovelha (Martin et al., 2014). Na seleção para o corte o objetivo principal é a melhoria das características maternas, crescimento efetivo de produtividade (prolificidade) e adequação da carne para o abate: crescimento, engorda e conformidade (Genelex, 2017). O acasalamento das borregas já aos 11 meses de idade, juntamente com o manejo reprodutivo intensivo (três partos a cada dois anos) permitem reduzir o intervalo entre gerações (Martin et al., 2014).

Cordeiros são tipicamente abatidos após o desmame (~10 kg de peso vivo) ou após um período de engorda, quando alcançam de 20 a 30 kg nas regiões do Mediterrâneo (Esquivelzeta et al., 2017) e 30 kg a 40 kg no Brasil. Cordeiros da raça Lacaune (linhagem leite), criados em confinamento até os 37 kg, tiveram rendimento comercial de carcaça médio de 46,33%, e ganho de peso diário de cerca de 330 g/dia^{-1} equivalendo-se ao verificado para raças de corte (Cesco, 2015).

Diante do desenvolvimento da linhagem de corte, a aquisição de animais sem pedigree pode envolver risco em obter animais não especializados para leite. Um diferencial é o peso dos animais adultos da linhagem de corte, que costuma ser superior. As fêmeas têm de 70 kg-90 kg e os machos de 100 kg -150 kg (Genelex, 2017).

Cruzamentos

Alguns cruzamentos do Lacaune com diversas raças já foram realizados e alguns ganhos de produtividade no mestiço foram observados (Ricordeau; Flamant, 1969; Thomas et al., 2004; Esquivelzeta et al., 2017). Um cruzamento de particular interesse nacional é Lacaune x Santa Inês. O incremento de 50% na produção do mestiço sobre o Santa Inês puro justifica a sua utilização nos sistemas de criação de ovinos em que se objetiva o aumento da produção leiteira (Ferreira et al., 2011).

Perspectivas futuras no melhoramento

A partir do ano 2000, houve aumento do número de carneiros genotipados no programa de melhoramento. Entretanto, o alto custo da genotipagem e o menor valor individual dos animais ainda limitam a ampla utilização da predição genômica em ovinos, a exemplo do que ocorre com bovinos leiteiros (Baloche, 2014). Num cenário futuro de redução de custos, talvez o uso da genotipagem seja ampliado. A predição genômica em animais jovens reduz o número de carneiros e os altos custos de manutenção desses no programa de melhoramento.

Há 15 anos os genótipos têm sido resistentes à Paraplexia Enzóotica ou Scrapie ovino (OVI-TEST, 2018), num trabalho contínuo de seleção. Como desafios futuros ao programa de seleção, estão: (1) ordenha uma vez ao dia, (2) aumento do período de lactação, (3) longevidade, (4) resistência a parasitas, (5) melhoras contínuas na composição do leite (Fregeat, 2017).

Considerações finais

Diante dos resultados do programa de melhoramento e da pressão de seleção em seus rebanhos, podemos pressupor que os genótipos importados pelos diversos países, na década de 1990, têm produtividade menor que os genótipos trabalhados atualmente na França. A implantação de um programa de melhoramento local se faz necessário, juntamente à viabilização da importação de sêmen e embriões da França, tendo em vista a melhoria da produtividade dos plantéis nacionais. A raça Lacaune demonstra enorme potencial para continuar contribuindo com a expansão da ovinocultura leiteira no País.

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ARTICLE 2 - Ultrasonographic cervical evaluation: an additional tool to select ewes for non-surgical embryo recovery

Article following the norms and format of the journal Animal Reproduction Science.

ARTICLE 2 - Ultrasonographic cervical evaluation: an additional tool to select ewes for non-surgical embryo recovery

Lucas Machado Figueira[1,2 b], Nadja Gomes Alves[1a], Joanna Maria Gonçalves Souza-Fabjan[2], Gabriel Brun Vergani[3], Maria Emilia Franco Oliveira[3], Renato Ribeiro de Lima[1], Jeferson Ferreira Fonseca[4]

1Universidade Federal de Lavras - Av. Doutor Sylvio Menicucci, 1001 - Kennedy, CEP 37200-000, Lavras-MG, Brazil

2 Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, Vital Brazil, CEP 24220-008, Niterói-RJ, Brazil

3 Universidade Estadual Paulista "Júlio de Mesquita Filho", Via Prof. Paulo Donato Castellane s/n, CEP 14884-900, Jaboticabal-SP, Brazil

4 Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, km 4, CP D10, CEP 62011-000, Sobral-CE, Brazil

ABSTRACT

The objective of this study was to check the usefulness of cervical ultrasonography mapping as a predictor for successful non-surgical embryo recovery (NSER). Synchronous estrus-induced (Trial 1; *n* = 28) or superovulated (Trial 2; *n* = 25) Lacaune ewes had their cervix evaluated by transrectal ultrasonography 12 h after estrus onset (Trial 1) or 30 min before NSER (Trial 2). Cervical rings were evaluated and the arrangement between them was classified by degree of misalignment on ultrasonography (DMUS): DMUS-1 – cervix rectilinear, DMUS-2 – intermediate, and DMUS-3 – highly asymmetrical. For prediction, DMUS-1 and DMUS-2 were considered to be suitable for NSER performed at days 6 to 7 after estrus, following cervical dilation. Similar ranking was also attributed to degree of misalignment on the cervical map made during the NSER. In Trial 1, cervical retraction was not possible in three ewes of the DMUS-3 group. Transcervical penetration reached high rates (80% to 100%) in the DMUS-1 and DMUS-2 groups in both Trials, but in Trial 2, one ewe in DMUS-3 did not have the cervix completely transposed. In Trial 1, the prediction performance for success at transcervical penetration showed low sensitivity (45%) and no specificity due to the high incidence of false negatives (52%). However, in Trial 2, sensitivity and specificity were 100%. Ultrasound assessment made before NSER seems to be more predictive of the conditions found for transcervical penetration than those made at estrus. Cervical ultrasonography allows for the classification and selection of ewes eligible for NSER.

Keywords: Cervix; Penetration; Sheep; Ultrasound.

1. Introduction

The anatomy of the cervix in sheep is highly variable among breeds (Kaabi et al., 2006) and is the major limitation for the application of reproductive biotechnologies by non-surgical methods (Kershaw et al., 2005; Fonseca et al., 2016). The depth of cervical penetration is affected by the difficulty of rectal manipulation of the reproductive tract and complex anatomy of the cervical lumen, in which cervices with a higher degree of convolutions and interdigitations are less penetrable (Kershaw et al., 2005).

Pharmacological induction of cervical dilation is a crucial point to efficiently perform non-surgical embryo recovery - NSER (Gusmão et al., 2009; Candappa and Bartlewski et al., 2014). Satisfactory results of transcervical penetration have been achieved with the application of misoprostol (PGE analogue) before embryo recovery in Santa Inês (67% - Gusmão et al., 2007) and Dorper ewes (95% - Gusmão et al., 2009). Another treatment combines estradiol-17β plus oxytocin to stimulate PGE synthesis, which is involved in the physiology of cervical dilation (Leethongdee et al., 2016). This combination allows uterine flushing and ova/embryo recovery rates like those observed with laparoscopic embryo recovery (Wulster-Radcliffe, Costine, Lewis, 1999). The combination of d-cloprostenol, estradiol benzoate and oxytocin was tested in Brazilian sheep breeds (Fonseca et al., 2019a; Fonseca et al., 2019b; Fonseca et al., 2019c), achieving high rates of transcervical penetration, uterine flushing, and media recovered. However, the feasibility of cervical access and manipulation (i.e., immobilization, retraction, and penetrability) should be tested in individual donor ewes before any embryo collection attempt, aiming at better effectiveness in the application of the technique in multiple ovulation and embryo transfer (MOET) programs (Fonseca, 2017; Fonseca et al., 2019a; Fonseca et al., 2019c).

An attempt at cervical penetration performed between 10 and 12 hours after the onset of estrus (natural, induced, or synchronized) with a Hegar dilator allows the creation of a cervical map, in which the geographic-anatomical arrangement of the rings is recorded (Fonseca, 2017; Fonseca et al., 2019c). The records of transcervical penetration allow for individuals to be ranked by the degree of ease, making it possible to select donors, thus obtaining success in the NSER (Fonseca, 2017; Fonseca et al., 2019b). However, it was observed that ~20% of animals showed discrepant results after induction of cervical dilation or at estrus-induced (Fonseca et al., 2019a), suggesting that the degree of cervical dilation during synchronous induced estrus may be different from the physiological response to cervical dilation treatment for NSER.

The use of modern US equipment can ensure the optimal visualization of details of the cervix in small ruminants, thus allowing new diagnostic and interpretative approaches. We hypothesized that cervical ultrasonography evaluation can be an additional tool for predicting transcervical penetration and successful non-surgical uterine flushing in ewes. The aim of the present study was to assess the degree of misalignment of cervical rings by ultrasonography either at estrus or before NSER to predict the penetration success at NSER in Lacaune ewes.

2. Materials and methods

2.1. Experimental conditions

This research was reviewed and approved by the Animal Care Committee of Embrapa Dairy Cattle (Protocol 2512100516/2016 – ATTACHMENT A) and conducted during the non-breeding season (September – October, Trial 1) and breeding season (April, Trial 2), in a commercial farm located in Soledade de Minas, Minas Gerais State, Brazil (latitude 22°3' S, longitude 45º2' W and altitude of 938 m). The animals were kept indoors in collective pens with access to external brightness and temperature variations. The diet consisted of corn silage and balanced concentrate offered according to the nutritional requirements (National Research Council-NRC, 2007), provided in two meals, with offer of mineral salt (DeHeus®, Rio Claro, Brazil) and water *ad libitum*.

2.1.1. Trial 1

A total of 28 Lacaune ewes were selected. The ewes were in good sanitary and clinical condition with ages between 15–93 months, body weight (BW) of 67.2 ± 2.0 kg (mean ± SEM) and body condition score of 3.4 ± 0.1 (BCS, scale 0 - 5, being 0 = emaciated to 5 = obese, according to Suiter, 1994). Ewes received hormonal treatments to induce synchronous estrus, which consisted of intravaginal sponges (60 mg medroxyprogesterone acetate, Progespon®, Zoetis, Campinas, Brazil) for nine (n = 14) or six days (n = 14). At 24 h before sponge withdrawal, in both treatments, 400 IU of eCG (Folligon 5000 IU®, Intervet, São Paulo, Brazil) and 37.5 µg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) i.m. were administered.

2.1.2. Trial 2

A total of 24 ewes (66.4 ± 0.9 kg of BW and BCS 3.5 ± 0.1) were selected as embryo donors and received a 0.36 g progesterone intravaginal device (Primer PR®, Tecnopec, São Paulo, Brazil) for nine days, 37.5 µg of d-cloprostenol i.m. (Prolise®, Tecnopec, São Paulo, Brazil) 24 h before device withdrawal, and 50 µg of gonadorelin (GnRH analogue, Gestran®, Tecnopec, São Paulo, Brazil) 24 h after device withdrawal. Superovulatory treatments consisted of 100 mg (n = 13) or 200 mg (n = 12) of porcine FSH (Folltropin®-V; Bioniche Animal Health, Belleville, Canada), administered i.m. (twice a day) for three consecutive days, in decreasing doses (25, 25, 15, 15, 10, and 10%), starting at 60 h before device withdrawal.

2.2. Estrus detection

After sponge/device withdrawal, estrous behavior was monitored for healthy and fertile rams (ratio ram:ewes = 1:6) twice a day (0800 and 1800), for at least 30 min per each ewe. Ewes that exhibited a standing response were considered to be in estrus.

2.3. Ultrasonographic cervical evaluation

Assessment of cervix was always performed by the same technician by transrectal ultrasonography (8.0 MHz, Mindray M5VET®, Shenzen, China) 12 h after the onset of estrus (Trial 1) or 16 h after treatment to cervical dilation for NSER (Trial 2). A slow scan was conducted to evaluate the sequential arrangement of cervical rings, which were classified in three degrees of misalignment on ultrasonography (DMUS): DMUS-1 – cervix rectilinear, DMUS-2 – intermediate, and DMUS-3 – highly asymmetrical cervix. US images of cervix with different DMUS are shown in Figure 1.

This nomenclature was proposed due to similarities between the classifications in three degrees of cervical rings misalignment in anatomical samples (Kershaw et al., 2005) and the "cervical maps" made during *in vivo* cervical penetration (Fonseca, 2017; Fonseca et al., 2019c). The cervical maps were made by the same technician for achieving transcervical uterine access in ewes submitted to NSER. The ostium of each ring was recorded according to its position on the "clock" and its distance from the central axis. The number of rings was counted only in Trial 1, due to time constraints in Trial 2.

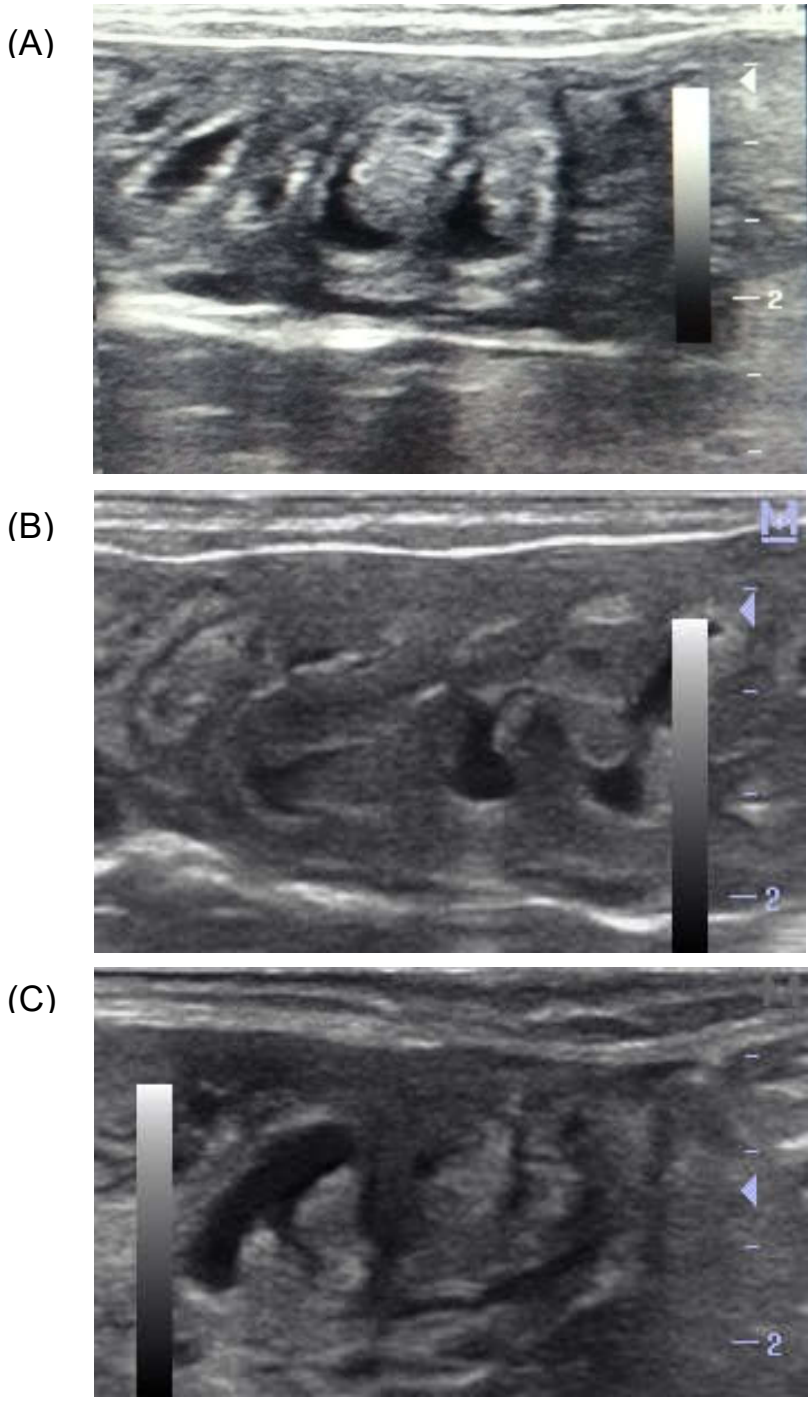

Fig. 1. Cervical ultrasonography images according to the classification of degree of cervical misalignment on ultrasonography (DMUS) in Lacaune ewes, (A) DMUS-1: cervix rectilinear, (B) DMUS-2: intermediate, and (C) DMUS-3: highly asymmetrical. Images show longitudinal section of the cervix in the caudal cranial direction (left to right).

2.4. Transervical penetration and uterine flushing by NSER technique

Cervical retraction and cervical penetration attempt were performed in both trials approximately 6–7 days after onset of estrus by the transcervical method (Fonseca et al., 2016). Only ewes that expressed estrus were used. The ewes received treatment to induce cervical dilation in both trials. This treatment consisted of a latero-vulvar injection of 37.5 μg of d-cloprostenol and an i.m. injection of 1 mg of estradiol benzoate (Sincrodiol®, OuroFino, Cravinhos, Brazil) 16 h and 50 IU of oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) administered i.v. 20 min before uterine flushing. Immediately after oxytocin, 40 mg of hyoscine-N-butylbromide and 5 g of sodium dipyrone (Buscofin®, Agener Union, Embu-Guaçu, Brazil) were administered, by both i.v. and i.m. routes, in equal parts. In addition, 0.1 mg/Kg of BW acepromazine maleate (Acepran 1%®, Vetnil, Louveira, Brazil) i.m. was administered 20 minutes prior to cervical manipulation. The sedation continued for 45 min, enough time for carrying out the procedure. Animals in a standing position were restrained in a sheep cart and received 2 mL of 2% lidocaine epidural block (S5-C1) (Lidovet®, Bravet, Rio de Janeiro, Brazil) before cervical manipulation. After insertion of a vaginal speculum, a sterile gauze soaked with 5 mL of 2% lidocaine (without vasoconstrictors) was gently placed ventrally to the cervical opening with the aid of an Allis forceps (26 cm) and left in place for the duration of the procedure. Cervical retraction, cervical penetration, and uterine flushing were always performed by the same technician.

During transcervical penetration the number and anatomical arrangement of rings were recorded for creation of a cervical map (CM) in the ewes that had the cervix completely transposed. The degree of misalignment of the cervical map (DMCM) was assigned in three degrees as described earlier in section 2.3.

2.5. Statistical analysis

The data were analyzed separately by Trial using SAS® software (Statistical Analysis System, version 9.3, SAS Inst., Inc., Cary, NC, USA). Percentage of ewes according to the degree of misalignment and number of rings were analyzed by Chi-square test for a one-way table (goodness of fit) to evaluate if the data were significantly different from the expected *value* (equal proportions for each class). Cervical retraction and transcervical penetration rates were analyzed by Chi-square or Fisher's exact test. Time for transcervical penetration was tested by generalized linear models using Gamma distribution and log link function. The

models included degree of misalignment, number of rings counted by the technician, and hormonal treatment (for synchronize estrus –Trial 1 or superovulation – Trial 2) as fixed effect. Post-hoc multiple comparison was performed using Tukey's test. The number of rings counted by US or CM was compared by paired *t*-test. The comparison of the number of rings counted by US and CM considered the degree of variation (null–zero, slight ± 1 ring and discrepant ± 2 rings) and estimative evaluation (coincided: zero, underestimated: −2 or −1 rings and overestimated: +1 or +2 rings). Associations between variables were evaluated using Pearson or Spearman's correlation. Results were expressed as percentages or least square means ± standard error of the means (LSMEANS ± SEM). Statistical significance was declared at $P < 0.05$.

The performance (efficacy) of the ultrasonographic cervical evaluation as a tool to identify ewes eligible for NSER was evaluated based on the sensitivity (SENS=TP/[TP+FN]), specificity (SPEC=TN/[FP+TN]), positive predictive value (PPV=TP/[TP+FP]), negative predictive value (NPV=TN/[FN+TN]), accuracy (Ac=[TP+TN]/n), where TN=true negative, FN= false negative, TP= true positive, and FP=false positive. Cohen's kappa index was also used for evaluation of test performance and considered the nomenclature proposed by Landis and Koch (1977): poor ($\kappa < 0.00$), slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$), or almost perfect ($0.81 < \kappa < 1.00$). For these analyses, the DMUS-1 (cervix rectilinear) and DMUS-2 (intermediate) were considered to be suitable and DMUS-3 (highly asymmetrical cervix) non-suitable for penetration. The transcervical penetration was considered to be successful (value equals to 1) if all cervical rings were transposed, and it failed otherwise (value equals to 0).

3. Results

3.1. Trial 1

The percentage of ewes according to DMUS or number of rings on US did not present equal proportions ($P < 0.05$ – Table 1). A positive relative deviation (+0.76) was observed in DMUS-3 and a negative relative deviation (−0.38) in DMUS-1 and DMUS-2. Considering the number of rings on US there was a positive relative deviation (+0.75) to ewes that had six rings and negative relative deviation to those that had five (−0.50) or seven rings (−0.25). Regarding the DMCM and the number of rings on CM, no difference ($P > 0.05$) was observed in the frequencies. The cervical retraction and transcervical penetration rates, as well as the time for transcervical penetration, did not differ ($P > 0.05$) among the degrees of

misalignment or number of rings on US. The time for transcervical penetration was lower ($P < 0.05$) in DMCM-1 compared to DMCM-2 and DMCM-3, but no difference ($P > 0.05$) was found between DMCM-2 and DMCM-3. Therefore, time for transcervical penetration was positively correlated only with DMCM ($r = 0.67$, $P < 0.01$), and no correlation was observed with DMUS ($r = 0.01$, $P = 0.96$).

Table 1
Cervical retraction and transcervical penetration rates (%) and time for transcervical penetration (LSMEANS ± SEM) according to the degree of misalignment and number of rings determined by ultrasonography (DMUS) or cervical map (DMCM) evaluation in synchronous estrus-induced Lacaune ewes (Trial 1) submitted to cervical dilation treatment.[1]

End points	Percentage of animals (%)	Cervical retraction rate (%)	Transcervical penetration rate (%)	Time for transcervical penetration (min)
Ultrasonography				
DMUS-1	20.8 (5/24)*	100.0 (5/5)	100.0 (5/5)	4.8 ± 1.5 (5)
DMUS-2	20.8 (5/24)*	100.0 (5/5)	80.0 (4/5)	4.6 ± 1.4 (4)
DMUS-3	58.3 (14/24)*	78.6 (11/14)	100.0 (11/11)	5.9 ± 1.4 (11)
5 rings	16.7 (4/24)*	100.0 (4/4)	100.0 (4/4)	5.4 ± 1.7 (4)
6 rings	58.3 (14/24)*	92.9 (13/14)	92.3 (12/13)	4.2 ± 0.9 (12)
7 rings	25.0 (6/24)*	66.7 (4/6)	100.0 (4/4)	5.7 ± 1.8 (4)
Overall	100 (24/24)	87.5 (21/24)	95.2 (20/21)	5.6 ± 0.8 (20)
Cervical map				
DMCM-1	55.0 (11/20)	-	-	3.3 ± 0.6[b] (11)
DMCM-2	20.0 (4/20)	-	-	7.5 ± 2.0[a] (4)
DMCM-3	25.0 (5/20)	-	-	8.7 ± 2.2[a] (5)
5 rings	10.0 (2/20)	-	-	7.6 ± 2.7 (2)
6 rings	50.0 (10/20)	-	-	6.2 ± 0.9 (10)
7 rings	25.0 (5/20)	-	-	5.1 ± 1.4 (5)
8 rings	15.0 (3/20)	-	-	5.5 ± 1.6 (3)
Overall	100.0 (20/20)	-	-	5.6 ± 0.8 (20)

[1]Cervical dilation treatment: 37.5 μg of d-cloprostenol latero-vulvar and 1 mg of estradiol benzoate intramuscular 16 h, and 50 IU of oxytocin intravenous 20 min before non-surgical embryo recovery.
* Chi-square test for one-way tables ($P < 0.05$).
a,b Means with different superscript letters within columns (Tukey´s test; $P < 0.05$).
( ) Number of animals

The number of rings counted by US ($r = 0.00$, $P = 1.00$) or CM ($r = -0.19$, $P = 0.42$) also were not correlated with time for transcervical penetration. The two methods of assessing cervical misalignment, DMUS and DMCM, were not correlated ($r = -0.15$, $P = 0.51$).

The number of rings was higher ($P < 0.05$) on CM (6.5 ± 0.2, range 6 to 9) than in US counting (6.0 ± 0.1, range 5 to 8), without correlation ($r = 0.37$, $P = 0.11$) between methods due to over- or underestimation. As shown in Table 2, in 5% of cases the counting by US over- and in 45% underestimated the number of rings on CM. The number of rings coincided

in both techniques in 50% of cases and, considering one ring's difference to be an acceptable estimate of error (slight variation), the US count allowed for the number of rings to be estimated in 85% of cases.

Table 2
Frequency and percentage of synchronous estrus-induced Lacaune ewes according to the degree of variation (null, slight, or discrepant) or estimative evaluation (coincided, overestimated, or underestimated) between the cervical rings counting by ultrasonography assessment and the count performed by the technician during the non-surgical embryo recovery procedure.

Parameters	Frequency	Percentage (%)
Variation		
Null (Zero)	10	50.0
Slight (-1 or +1 ring)	7	35.0
Discrepant (-2 or +2 rings)	3	15.0
Estimate		
Coincided (equal)	10	50.0
Underestimated (-2 and -1 rings)	9	45.0
Overestimated (+1 and +2 rings)	1	5.0

Evaluating the performance of US for prediction of transcervical penetration in Trial 1, high number of false negatives was observed, which dramatically reduced specificity and negative predictive value, resulting in low accuracy and poor Cohen´s kappa index (Table 3).

Table 3
True positive (TP), false positive (FP), true negative (TN), sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), accuracy (AC), and Cohen's kappa index (κ) of the prediction of transcervical penetration by ultrasonography of cervix performed at 12h after onset of induced estrus (Trial 1) or 16 h after cervical dilation treatment (Trial 2) in Lacaune ewes.

Trials	TP	FP	TN	FN	SENS	SPEC	PPV	NPV	AC	κ
Trial 1	9	1	0	11	45%	0%	90%	0%	43%	-0.09
Trial 2	16	0	1	0	100%	100%	100%	100%	100%	1.00

3.2. Trial 2

The percentage of ewes according to DMUS, DMCM, and number of rings counted by the technician showed equal proportions ($P > 0.05$; Table 4). There were no ewes with DMCM-3. All females had the cervix successful retracted in Trial 2. Transcervical penetration was not possible in only one ewe classified as DMUS-3. In this case, there was a

suspicion of folding of the catheter and flushing in the cervix, which was evidenced by the absence of recovered structures and by the transparency of the recovered medium. On the US, it was possible to observe the greater distance between the last two cervical rings, which may have facilitated the fold of the catheter.

The time for transcervical penetration did not differ ($P > 0.05$) between DMUS-1 and DMUS-2. The time for transcervical penetration did not differ ($P > 0.05$) between DMCM-1 compared to DMCM-2 (Table 4). There was moderate positive correlation between DMUS and DMCM ($r = 0.59$, $P = 0.02$). However, DMUS and DMCM were not correlated to the time for transcervical penetration ($r = 0.04$, $P = 0.87$ and $r = 0.15$, $P = 0.56$, respectively). Number of rings counted by the technician also was not correlated with the time for transcervical penetration ($r = 0.01$, $P = 0.95$).

Table 4
Cervical retraction and transcervical penetration rates (%) and time for transcervical penetration (LSMEANS ± SEM) according to the degree of cervical misalignment determined by ultrasonography (DMUS) or cervical map (DMCM) evaluation in superovulated Lacaune ewes (Trial 2) submitted to cervical dilation treatment.[1]

End points	Percentage of animals	Cervical retraction rate	Transcervical penetration rate	Time for transcervical penetration
Ultrasonography				
DMUS-1	29.4 (5/17)	100.0 (5/5)	100.0 (5/5)	3.8 ± 0.9 (5)
DMUS-2	64.7 (11/17)	100.0 (11/11)	100.0 (11/11)	4.0 ± 0.6 (11)
DMUS-3	5.9 (1/17)	100.0 (1/1)	0.0 (0/1)	-
Overall	100 (17/17)	100.0 (17/17)	94.1 (16/17)	3.9 ± 0.6
Cervical map				
DMCM-1	37.5 (6/16)	-	-	4.5 ± 1.1
DMCM-2	62.5 (10/16)	-	-	4.2 ± 0.8
DMCM-3	0.0 (0/16)	-	-	-
6 rings	12.5 (2/16)	-	-	3.4 ± 1.1
7 rings	37.5 (6/16)	-	-	4.8 ± 1.0
8 rings	43.8 (7/16)	-	-	3.0 ± 0.5
9 rings	6.2 (1/16)	-	-	7.3 ± 3.5
Overall	100.0 (16/16)	-	-	3.9 ± 0.6

[1]Cervical dilation treatment: 37.5 μg of d-cloprostenol latero-vulvar and 1 mg of estradiol benzoate intramuscular 16 h, and 50 IU of oxytocin intravenous 20 min before non-surgical embryo recovery.
( ) Number of animals

Performance of US for prediction of transcervical penetration was enhanced in Trial 2. There were no false positives and negatives, resulting in 100% positive and negative predictive values, excellent accuracy, and almost perfect Cohen's kappa index (Table 3).

4. Discussion

This is the first study performed in small ruminants that assessed the use of ultrasonography as a tool for cervical evaluation and prediction of penetration at estrus or before NSER (after cervical dilation by hormonal treatment). Cervical ultrasonography was able to identify nuances of the lumen and cervical rings, allowing estimation of the misalignment degree. The presence of mucus in the vaginal lumen helps to locate the cervical external *os* and to start ring counting, but does not allow detailing external cervical *os* type. The B-mode image quality is essential for obtaining detailed cervical images. However, the activation of the Color Doppler mode does not seem to be particularly advantageous, although it allows distinguishing blood vessels from the cervical lumen. It is expected that continuous improvements in US equipment will allow acquisition of better images, further facilitating diagnostic approaches of the genital tract (Medan and Abd El-Aty, 2010).

According to Fonseca et al. (2019c), transcervical penetration will be easier in ewes with DMCM-1 and DMCM-2, and it would be more difficult or even impossible in ewes with DMCM-3. In anatomic samples, Kershaw et al. (2005) also observed lower penetrability in grade 3 cervices. In the present study, the penetrability in DMUS-3 ewes probably was improved by stretching after retraction and manipulation of the cervix with the thumb and index finger.

The high transcervical penetration rate also did not allow obtaining statistical differences between DMUS or DMCM. The high transcervical penetration rate in DMUS-1 (100%) and the high PPV observed in both Trials (90% and 100%) show the value of US for choosing animals eligible for NSER, but the low specificity and NPV (0%) in Trial 1 suggest that ewes should not be discarded by US alone. The cervical penetration attempt with a Hegar dilator at estrus (Fonseca et al., 2019a; Santos et al., 2019) can be performed in association with a US diagnostic mainly in DMUS-3 ewes. An interesting finding was that restriction to cervical retraction occurred only in three ewes with DMUS-3 in Trial 1. In Trial 2, one ewe classified as DMUS-3 was not transposed to uterine flushing. Therefore, the DMUS-3 or DMCM-3 ewes when used as a donor could be preventively submitted to fasting for surgical collection in case of non-penetration on the day of embryo recoveries.

The time required to complete cervical penetration is another indicator of the difficulty of the NSER procedure (Fonseca et al., 2019c) Only DMCM in Trial 1 presented a high positive correlation with time for transcervical penetration. This method seems more

predictive of difficulty observed by the technician in successive attempts and can be used as a screening test at estrus, as suggested by Santos et al. (2018).

The anatomy of the ovine cervix is variable between breeds, and this affects the degree of ease in cervical penetration. The Lacaune seems to be one of the breeds in which cervical anatomy offers less limitation to catheter penetration, since it presented high pregnancy rates to cervical insemination compared to other breeds (reviewed by Kaabi et al., 2006). Therefore, it is possible that ultrasonographic cervical evaluation should be more effective in breeds with lower effectiveness in the cervical penetration at NSER.

It is possible that there are differences in the cervical dilation intensity observed at estrus (Trial 1) and after treatment for cervical dilation (Trial 2), since in the first Trial DMUS-3 was more frequent, while in the second Trial DMUS-3 was less frequent. Cervical penetration screening test with a Hegar dilator at estrus fails in 20% of cases, producing a false positive or false negative when a second attempt at NSER is performed (Santos et al., 2019). Fonseca et al. (2019a) also observed differences in the success of transcervical penetration between these two moments. Therefore, US evaluation after treatment for cervical dilation seems more representative of conditions for penetration at NSER than estrus time.

The number of rings does not seem to be as restrictive of cervical retraction and transcervical penetration as the degree of misalignment. Cervices with flap-like folds were penetrated easier than those with ring-like folds (Kaabi et al., 2006), but neither US nor CM methods allow this kind of distinction. In this study, errors associated with the count of number of rings by US and CM methods may have resulted in over- or underestimation. Although both methods still lack validation with subsequent dissection of anatomical samples, it is curious to note that in 50% of the cases the counts coincided and in 85% the difference between the counts did not exceed one ring. This suggests that it is possible to at least estimate with a slight margin of error.

From a practical point of view, the combination of DMUS-3 and/or DMCM-3 at estrus with high body weight, vaginal narrowing, and/or insufficient ovulatory response would be the primary criteria for disqualifying a donor candidate in a MOET program based on NSER. Furthermore, ranking of animals by score could also help to determine the order of collections, leaving out or leaving to the end the animals supposedly more difficult, if it is necessary to perform the surgical procedure.

Finally, the high SENS and Cohen´s kappa index observed in Trial 2 also could be the result of better training of the technician. Ultrasonographic evaluation is dependent on technician experience; therefore, training is an important factor for correct application of this

tool. US is a powerful tool to reduce the variability of results by donors selecting (Bruno Galarraga et al., 2015; Pinto et al., 2018a) and measuring of the efficiency in MOET programs based on NSER by CL counting (Oliveira et al., 2018; Pinto et al., 2018b). Likewise, ultrasonographic cervical evaluation can also become a valuable tool to classify and select eligible animals for NSER.

5. Conclusions

Ultrasonographic cervical evaluation is more predictive of transcervical penetration after hormonal treatment to induce cervical dilation for NSER than at estrus-induced. The ultrasonographic cervical evaluation can be considered an additional tool to select ewes eligible for NSER.

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ARTICLE 3 -Preovulatory follicular dynamics, ovulatory response and embryo yield in Lacaune ewes subjected to synchronous estrus induction protocols and non-surgical embryo recovery

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ARTICLE 3 - Preovulatory follicular dynamics, ovulatory response and embryo yield in Lacaune ewes subjected to synchronous estrus induction protocols and non-surgical embryo recovery

Lucas Machado Figueira[1,2], Nadja Gomes Alves[1], Joanna Maria Gonçalves Souza-Fabjan[2], Maria Emilia Franco Oliveira[3], Renato Ribeiro de Lima[1], Guilherme Nunes de Souza[2,4], Jeferson Ferreira Fonseca[5]

[1]Universidade Federal de Lavras, Av. Doutor Sylvio Menicucci, 1001 - Kennedy, CEP 37200-000, Lavras - MG, Brazil

[2]Universidade Federal Fluminense Federal, Rua Vital Brazil Filho, 64, CEP 24220-008, Niterói-RJ, Brazil.

[3]Universidade Estadual Paulista "Júlio de Mesquita Filho", Via de acesso Prof. Paulo Donato Castelane, s/n, Zona Rural, CEP 14884-900, Jaboticabal, SP, Brazil.

[4]Embrapa Gado de Leite, Rua Eugênio do Nascimento, 610 - Dom Bosco CEP 36038-330, Juiz de Fora – MG, Brazil.

[5]Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, km 4, CP D10, CEP 62011-000, Sobral-CE, Brazil.

A B S T R A C T

The objective of this study was to assess the effect of the duration of progesterone-based estrus induction protocols on preovulatory follicular dynamics, ovulatory response and embryo yield after non-surgical embryo recovery (NSER) in Lacaune ewes. Females received acetate medroxyprogesterone intravaginal sponges for six (G-6; n=14) or nine (G-9; n=14) days plus d-cloprostenol and eCG 24 h before sponge removal (Day 0). Preovulatory follicular dynamics and the luteal characteristics were evaluated by B-mode and Color-Doppler ultrasonography. NSER was performed five to six days after ovulation. Estrous behavior rate was 85.7% for both groups. Percentage of ewes that ovulated was 92.9% in G-6 and 100% in G-9. The day of wave emergence (relative to Day 0) did not differ ($P > 0.05$) between G-6 (-3.0 ± 0.5) and G-9 (-4.2 ± 0.5). The number of follicles of size 4.1-5.0 mm was higher ($P < 0.05$) in G-9 (1.4 ± 0.2) compared to G-6 (0.8 ± 0.2) during the Days - 4 to 0. At NSER, the transcervical penetration rate was 95.2% (20/21) and its duration time was lower ($P < 0.05$) in G-9 (3.4 ± 0.6 min) than G-6 (7.2 ± 1.3 min). The number of ovulations and viable embryos were higher ($P < 0.05$) in G-9 (2.9 ± 0.3 and 1.3 ± 0.4, respectively) than in G-6 (1.9 ± 0.3 and 0.4 ± 0.2, respectively). In conclusion, the 9-day protocol promoted higher ovulation rate and embryo yield; moreover, the cervical dilation treatment allowed NSER in high percentage of Lacaune ewes.

Keywords: Cervical dilation; Dairy sheep; Transcervical embryo recovery; Uterine flushing

1. Introduction

Lacaune is one of the main breeds of sheep raised for dairy farming. It is diffused worldwide due to the genetic gains achieved by the French improvement program [1]. In order to accelerate herd multiplication and genetic improvement, it is essential to use of reproductive biotechnologies, as estrus induction [2,3], artificial insemination [1] and multiple ovulation and embryo transfer - MOET [4,5].

Due to the sheep seasonal status [6] estrus induction protocols are required in non-breeding season [7]. These treatments when applied for long-term (12 to 14 days) results in satisfactory estrus induction, but variable fertility [8,9]. Short-term (5-7 days) protocols are an alternative that offer similar or better fertility rates [10], with lower ovulation of persistent dominant follicles [11], minor hormonal residual effect [12], less likely to lose intravaginal sponge/device [13], as well as to induce the ovulation of new follicles from the first follicular wave [14], given that there are a distinctive pattern of follicular wave dynamics during the treatment period [15]. There are few studies on progesterone-based estrus induction protocols of different durations in dairy ewes. In Awassi breed, ewes treated with progesterone (P_4) devices for 6, 9 or 12 days had similar pregnancy and lambing rates [16], while in dairy Lacaune ewes there was a tendency to highest lambing rate after long-term (12 days) compared to short-term (6 days) progesterone treatment [2]. Fine adjustment of progesterone/progestogen exposure time in dairy Lacaune ewes may improve the reproductive results from stimulation of different ovulatory follicular wave which grows under different hormonal conditions and to provide better support to other reproductive biotechnologies; but there is still no information in this regard.

In ewes, the laparotomy is the technique of choice worldwide for embryo recovery [17]. This method demands prior fasting and anesthetic drugs that subject the animal to risks. Furthermore, adhesions, post-operative trauma and stress can arise from the procedure, limiting successive recoveries. Therefore, there are efforts to enable MOET programs based on non-surgical embryo recovery (NSER) by cervical route due to growing concerns about animal welfare and for better applicability of these biotechnologies [18,19]. In Brazil, studies with Santa Inês ewes reached lower transcervical penetration rates of 61% [20] and 81% [19], compared to 95% in Dorper ewes [21]. However, considering the difference in transcervical penetration rates among breeds [22], the efficiency and repeatability of the NSER need to be carefully evaluated when used in different breeds [18]. The NSER has not yet been reported in the Lacaune ewes.

The main objective of this study was to evaluate the effects of the duration of progesterone-based estrus induction protocols (6 or 9 days) on preovulatory follicular dynamic, ovulatory response and embryo yield after non-surgical embryo recovery in Lacaune ewes.

2. Material and methods

2.1. Experimental conditions

This research was approved by the Animal Care Committee of Embrapa Dairy Cattle (protocol 2512100516/2016 – ATTACHMENT A). The study was conducted during the period of lengthening daylengths [15] (September – October) in a commercial farm located in Soledade de Minas, Minas Gerais State, Brazil (latitude 22°3' S, longitude 45º 2' W and altitude of 938 m). Dairy ewes of Lacaune breed (n=28) in good sanitary and clinical condition were used. The animals were kept in collective pens and were fed twice a day with diet based on silage corn and balanced concentrated to meet their nutritional requirements [23]. Mineralized salt (DeHeus®, Rio Claro, Brazil) and water were offered *ad libitum*. The ewes were 15 to 93 months old, had 67.2 ± 2.0 kg (mean ± SEM) of body weight, 3.4 ± 0.1 units of body condition score (BCS - scale from 1 to 5, where 1 = emaciated and 5 = obese) [24] and 89.1 ± 6.2 days in milk at the beginning of the experiment.

2.2. Induction of synchronous estrus, estrus detection and mating

Ewes were randomly allocated into two experimental groups according to their parity (14 primiparous and 14 multiparous) and body weight (G-6: 69.7 ± 2.9 and G-9: 63.9 ± 2.0 kg). Synchronized estrus induction treatment consisted of intravaginal sponges containing 60 mg of medroxyprogesterone acetate (MAP, Progespon®, Zoetis, Campinas, Brazil) for six (G-6; n=14) or nine (G-9; n=14) days. The MAP sponges were inserted on a random day of estrous cycle or anovulatory period. At 24 h before sponge removal, in both treatments, 400 IU of eCG i.m. (Folligon 5000IU®, Intervet, São Paulo, Brazil) and 37.5 µg of d-cloprostenol (synthetic analogue of $PGF_{2\alpha}$, Prolise®, Tecnopec, São Paulo, Brazil) latero-vulvar (l.v.) were administered. Sponge removal day was considered Day 0 for data analyses.

After sponge removal, estrous behavior was recorded by using healthy and fertile rams (ratio of 1 ram:6 ewes) twice a day (08:00 and 18:00). The rams were rotated in both

treatments to minimize variations on the male fertility. Ewes remained with the ram at least 30 min in each observation period if had no mounting acceptance. Estrus onset was defined as the time when the ewe first stood to be mounted by the ram. Mating was repeated every 12 h until no mounting acceptance.

2.3. Ultrasonographic evaluations

B-mode transrectal ovarian ultrasonography (M5VET, Mindray®, Shenzen, China, 8.0 MHz) was carried out daily during exogenous progestogen treatment to determine the presence of corpora lutea (CL) and the follicular population. On MAP sponge insert day the Color-Doppler mode ultrasonography was performed in animal with CL to evaluate the luteal functionality [25]. In each exam, the number, position, and diameter of ovarian follicles (≥ 2 mm in diameter) were recorded on an individual ovarian map to follow the sequential follicular development. These data were used for retrospective evaluation of follicular dynamics. The day of emergence of an antral follicle was the day that the follicle was 2 mm in diameter, with an increase to ≥3 mm on the next day. The day of emergence of the ovulatory antral follicular wave was assigned to the day of emergence of the largest follicle of the antral follicular wave. After sponge withdrawal, evaluations were performed twice a day to measure the ovarian antral follicles and tracking the moment of ovulations, which was considered as the average period between the last exam at which the first preovulatory follicle was observed and the first exam at which it was no longer seen. The growth rate was calculated for the two largest ovulatory follicles, which was considered as the difference between maximum and minimum diameter of the ovulatory follicle divided by duration of growth phase. The duration of the follicular growth phase was the time taken (in days) to grow from 2 to 3 mm until the ovulatory diameter.

Seven days after sponge removal an ultrasound evaluation was performed using B-mode and Color-Doppler mode ultrasonography for counting and measuring the CL, and to calculate the total and vascularized luteal tissue areas. The area (cm^2) of each CL was measured using the ultrasound device calipers (ellipse and trace tools). The luteal area and the cavity were measured using ellipse tool, and the luteal tissue area was obtained by subtracting the area of the cavity of the total luteal area. The vascularized luteal area was defined using the trace tool. Values were summed in ewes with > 1 CL for the analysis of total luteal tissue area and total vascularized luteal area. A percentage of vascularized luteal tissue area were also established (total vascularized luteal area (cm^2) / total luteal tissue area (cm^2) * 100).

This assessment allowed determining the direction of the first uterine flushing, ipsilateral to the ovary with higher CL count or more vascularized. Thirty days after embryo recovery, genital tract was evaluated by US again, to verify the health of cervix, uterus and ovaries (absence of altered echotexture suggestive of abnormality).

2.4. Cervical dilation and transposing

All naturally-mated ewes (n=24) were submitted to the cervical dilation protocol 4-5 days after ovulation, containing: 1 mg of estradiol benzoate (Sincrodiol®, Ouro Fino, Cravinhos, Brazil) i.m. and 37.5 µg of d-cloprostenol (synthetic analog of PGF2α, Prolise®, Tecnopec, São Paulo, Brazil) l.v. were administered at 16 h before NSER. Twenty minutes before cervical penetration attempt, 50 IU of oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) intravenously (i.v.) was also administered (Fig. 1). Immediately after oxytocin, 40 mg of hyoscine-N-butylbromide and 5 g of sodium dipyrone (Buscofin®, Agener Union, Embu-Guaçu, Brazil) were administered by both i.v. and i.m. routes, respectively. In addition, 1 mg/kg of acepromazine maleate (Acepran 1%®, Vetnil, Louveira, Brazil) i.m. was administered immediately after oxytocin. This sedation continued for 45 min, enough time for carrying out the procedure.

After sedation, the ewes were kept in standing position and contained in an appropriate cart that avoids lateral and dorsal-ventral movements. Transcervical embryo recovery was performed by the same experienced technician, as described by Fonseca et al. (2016). The external *os* of each cervix was classified into one of six type, with few modifications as earlier described [26], based on folds of tissue around it. The classification was as follows: duckbill – two opposing folds, the flap – one-fold, rosette – cluster of folds, slit – no projection, papilla – small rounded protuberance and mixed – no pattern or combination of two types.

During cervical penetration attempt the number and geographic-anatomical arrangement of rings were recorded to creation of cervical map. The animals were then classified according to the degree of cervical misalignment (one - rectilinear cervix, two - intermediate and three - highly asymmetrical) [27]. The time of transcervical penetration (considered the time to transpose the cervical rings with Hegar dilator and catheter) and uterine flushing (considered the time to flushing the first uterine horn added to the time for the second transcervical penetration with catheter and flushing of the second uterine horn) were recorded and summed to determine the total time of embryo recovery procedure. At the end of

procedure, the ratio of fluid recovered in a graduated cylinder and fluid inputted by graduated syringe was calculated for evaluation of fluid recovery rate.

All the structures recovered (non-fertilized oocytes, embryos of different developmental stages and zona pellucida) were listed and the embryos were transferred to maintenance medium (Holding Plus®, Cultilab, Campinas, Brazil). Embryo evaluation was performed using a stereomicroscope with magnification: X 40 (Nova®, model XTD-20, Piracicaba, Brazil), followed the same principles used for cattle, being Grade I: excellent or good; Grade II: fair; Grade III: poor and Grade IV: dead or degenerating [28]. Embryos grade I, II and III were considered as viable embryos.

2.5. Statistical analyses

Parametric data as day of emergence of the ovulatory antral follicular wave, duration of follicular growth phase, ovulatory diameter and growth rate of the largest and the second largest ovulatory antral follicle, estrus duration, interval from sponge removal to estrus onset, interval from sponge removal to ovulation, interval from estrus onset to ovulation, number of ovulations, number of CL, total luteal tissue area, total vascularized luteal area, percentage of vascularized area, time of transcervical penetration, time of uterine flushing and total time of embryo recovery procedure were analyzed by generalized linear models, with Gamma distribution and log link function using GLIMMIX procedure in SAS (SAS, Cary, NC). The model included body weight as covariate, and treatment, category (primiparous and pluriparous) and interaction as fixed effects. The numbers of rings, recovered structures, unfertilized oocytes, morulae, blastocysts, viable embryos, and cervix misalignment score were also analyzed by using GLIMMIX procedure but considering Poisson distribution and log link function. The model included treatment and category as fixed effects.

Data from follicular populations were analyzed from Day -4 to Day 0 relative to sponge removal. Population of antral follicles (number) were divided into four categories according to follicular diameters ($\leq$ 3.0 mm, 3.1-4.0 mm, 4.1-5.0 mm and $>$ 5.0 mm) were studied by repeated measure analysis considering generalized linear mixed models with Poisson distribution and log link function by using GLIMMIX procedure. It was considered the autoregressive and unstructured matrix to model the residual covariances. The model included category, day, treatment, ewe (as random variable) and interactions where necessary. Multiple comparisons were made by Tukey test.

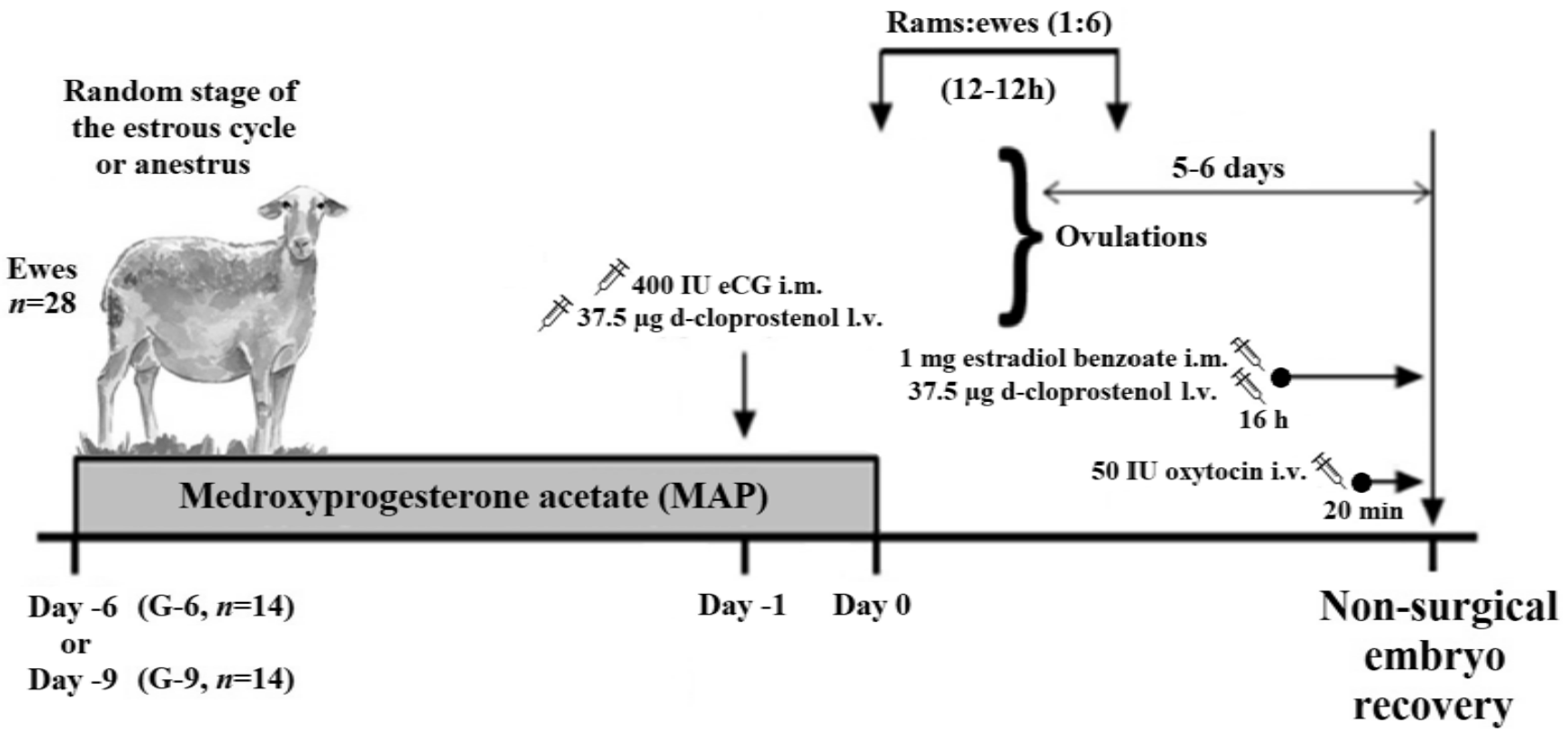


Fig. 1. Schematic representation of the experimental procedures used to assess two synchronous estrus induction protocols for six or nine days of progestogen exposure and checked the feasibility of non-surgical embryo recovery in the Lacaune breed; eCG: equine chorionic gonadotropin; i.m.: intramuscular; i.v.: intravenous, l.v.: laterovulvar.

Percentage data as multiple ovulations (ewes that ovulated ≥ 3 CL), successful recoveries (% of ewes in which at least one structure was recovered), viable embryos rate (obtained dividing number of viable embryos by total number of structures recovered) and unfertilized rate (obtained dividing number of unfertilized by total number of structures recovered) were analyzed as using GLIMMIX procedure, with binomial distribution and logit link function. The model included treatment and category as fixed effects.

Descriptive statistics of estrous behavior, ovulation, cervical retraction and transposition rates, ewes successfully flushed, fluid recovery rate and recovery rate (obtained by dividing the total number of recovered structures by total number of corpora lutea) were presented. Results of generalized linear model analyses are shown as least square means ± standard error of the means (LSMEANS ± SEM) and differences were considered significant from $P < 0.05$. Rates were expressed as percentages.

3. Results

3.1. Reproductive behavior and ovarian response

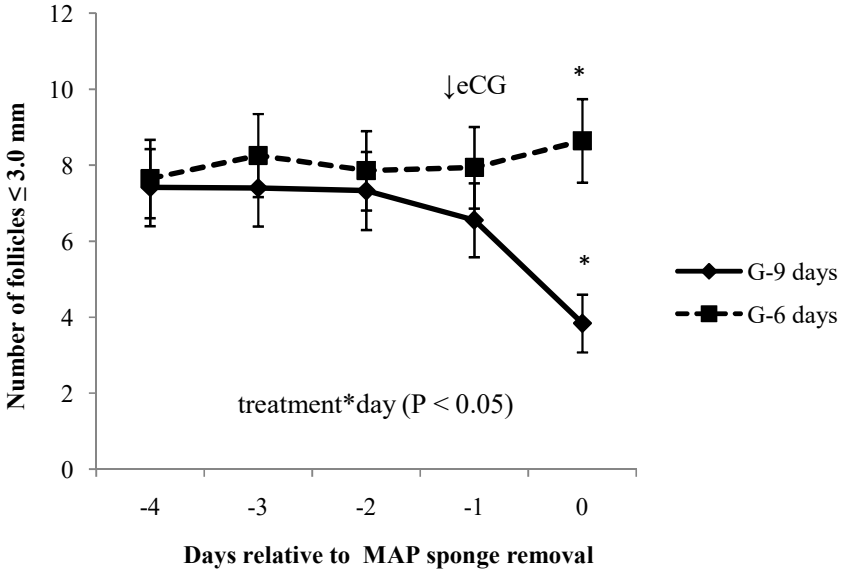
The percentage of ewes bearing CL at the beginning of the estrus induction protocols was the same (57.1% or 8/14) for both treatment groups. The emergence day of ovulatory antral follicular wave relative to Day 0 (MAP sponge removal day) did not differ ($P > 0.05$) between G-6 (range -6 to -1) and G-9 (range -7 to -1) as showed in Table 1. The duration of progestogen treatment for six (G-6) or nine (G-9) days did not affect ($P > 0.05$) ovulatory follicular development (ovulatory diameters, durations of growth phase; and growth rates of the two largest follicles), intervals from sponge removal to estrus onset and to ovulation, estrus duration and interval from estrus onset to ovulation (Table 1). The number of ovulations and percentage of multiple ovulations was higher ($P < 0.05$) in G-9 compared to G-6.

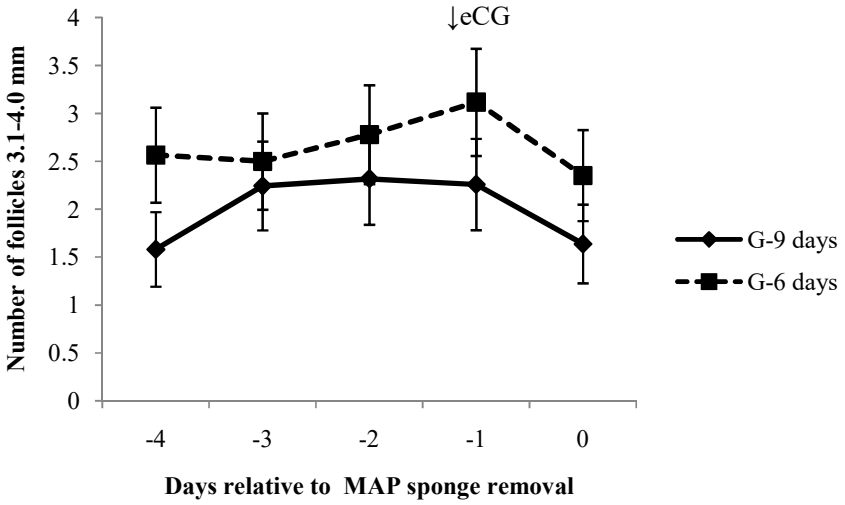
Antral follicular counts according to the diameter categories in the last days of MAP treatment in G-6 and G-9 treatment groups are shown in Fig. 2. There was treatment x day interaction, with decrease ($P < 0.05$) in follicle number ≤ 3.0 mm on Day 0 in relation to previous day only in G-9. The number of follicles of 3.1-4.0 mm did not differ ($P > 0.05$) between treatments or days. On the other hand, there was a treatment effect ($P < 0.05$) on the follicle number with 4.1-5.0 mm, with higher count in G-9 (1.4 ± 0.2) than in G-6 (0.8 ± 0.2).

Table 1
Ovulatory follicular dynamic and estrus end points (LSMEANS ± SEM) in Lacaune ewes subjected to estrus induction protocols* for six (G-6) or nine (G-9) days during non-breeding season.

End points	G-6 days	G-9 days	P-value
Emergence day of ovulatory antral follicular wave[a] (days)	-3.0 ± 0.5	-4.2 ± 0.5	0.10
Duration of follicular growth phase of the largest preovulatory follicle (days)	5.1 ± 0.4	6.1 ± 0.4	0.11
Ovulatory diameter of the largest preovulatory follicle (mm)	6.9 ± 0.3 (13)	7.1 ± 0.3 (14)	0.66
Growth rate of the largest preovulatory follicle (mm/day)	0.9 ± 0.1 (13)	0.7 ± 0.1 (14)	0.13
Duration of follicular growth phase of the second largest preovulatory follicle (days)	4.3 ± 0.5 (10)	5.0 ± 0.5 (13)	0.25
Ovulatory diameter of the second largest preovulatory follicle (mm)	6.4 ± 0.3 (10)	6.1 ± 0.3 (13)	0.47
Growth rate of the second largest preovulatory follicle (mm/day)	0.8 ± 0.1 (10)	0.7 ± 0.1 (13)	0.26
Estrus duration (h)	25.3 ± 2.6 (12)	21.5 ± 2.2 (12)	0.27
Interval from sponge removal to estrus onset (h)	36.7 ± 3.3 (12)	37.2 ± 3.4 (12)	0.91
Interval from sponge removal to ovulation (h)	57.1 ± 4.7 (13)	54.9 ± 4.3 (14)	0.74
Interval from estrus onset to ovulation (h)	21.9 ± 2.0 (13)	22.8 ± 2.0 (14)	0.75
Number of ovulations	2.0 ± 0.2 (13)	2.9 ± 0.3 (14)	0.02
% of multiple ovulations (≥3 CL)	21.4 ± 11.0 (14)	64.3 ± 12.8 (14)	0.03

*protocol with medroxyprogesterone acetate (60 mg) sponge plus 37.5 µg of d-cloprostenol and 400 IU of eCG 24 h before sponge withdrawal.
[a] Relative to Day 0 (day of MAP sponge withdrawal).
( ) Number of animals.


12
10
8
6
4
2
0
Number of follicles ≤ 3.0 mm
↓eCG
*
*
treatment*day (P < 0.05)
-4
-3
-2
-1
0
Days relative to MAP sponge removal
G-9 days
G-6 days


4
3.5
3
2.5
2
1.5
1
0.5
0
Number of follicles 3.1-4.0 mm
↓eCG
-4
-3
-2
-1
0
Days relative to MAP sponge removal
G-9 days
G-6 days

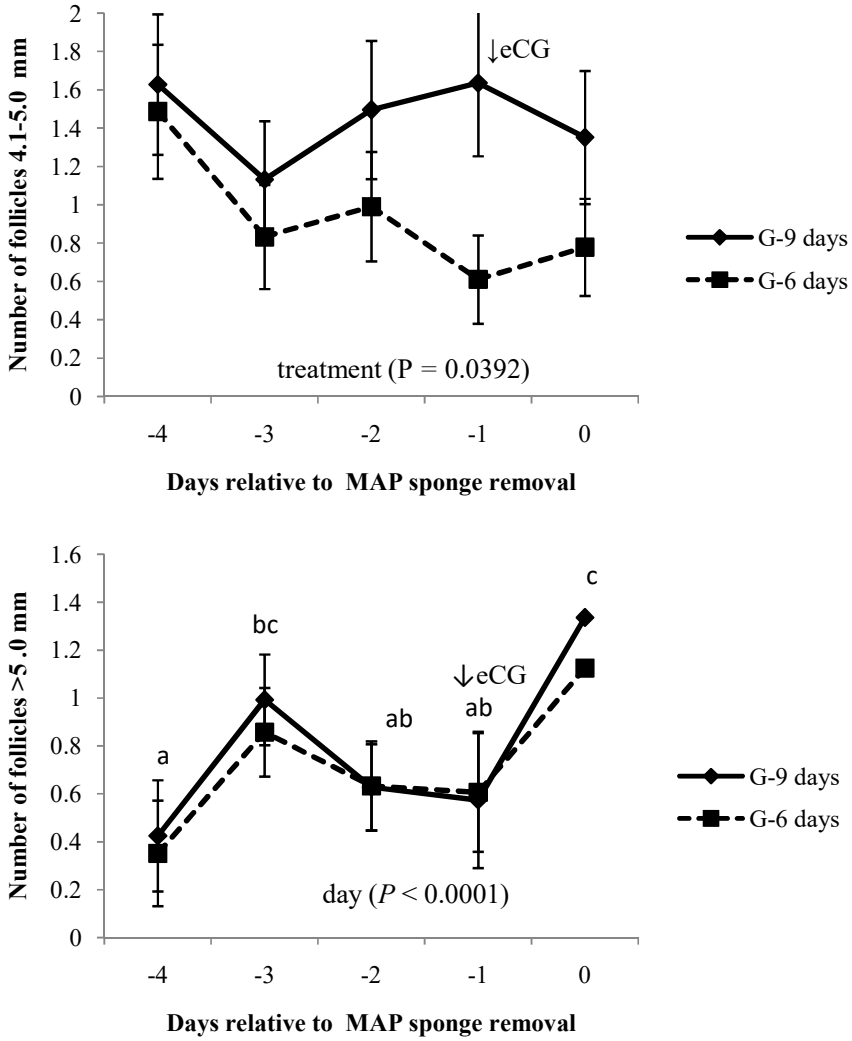


Fig. 2. Follicular counts (LSMEANS ± SEM) grouped by diameter categories (≤ 3.0 mm,3.1-4.0 mm, 4.1-5.0 mm, > 5.0 mm) on the relative days to MAP sponge withdrawal (Day 0) in Lacaune ewes subjected to synchronized estrus induction protocols with medroxyprogesterone acetate sponge for six (G-6, discontinuous line ----) or nine (G-9, continuous line——) days plus 37.5 µg of d-cloprostenol and 400 IU of eCG 24 h before sponge removal.*In each point asterisk indicates difference between treatments ($P < 0.05$). [ab]Values with different letters differ between days ($P < 0.05$).

There was day effect on the number of follicles > 5 mm which increased (P < 0.05) on Days -3 and 0 in both treatments compared to other days.

After MAP sponge removal, the percentages of estrous behavior in those ewes bearing CL and in those without CL at the beginning of the study were 87.5% and 83.3% in G-6 and 100% and 66.7% in G-9, respectively. Overall, estrous behavior rate was 85.7% for both treatment groups. Percentage of ewes that ovulated was 92.9% in G-6 and 100.0% in G-9. Ovulations of follicles coming from the two last waves occur in 15.4% (2/13) of cases in G-6 and in 35.7% (5/14) in G-9. The interval between the emergence of these last waves was 4.0 ± 0.3 days. Percentage of estrus was lower than ovulation because three ewes (one from

G-6 and two from G-9) had silent ovulation. These animals presented preovulatory follicles > 5 mm at the time of eCG treatment and ovulated few hours after sponge removal (24.0 ± 8.0 h). In G-6, one ewe had persistent dominant follicles and did not ovulate. The G-9 treatment induced better ovulatory response as measured by the number of ovulations and percentage of multiple ovulations (≥ 3 CL, Table 1), besides of higher (P < 0.05) total luteal tissue area in relation to G-6 (Table 2). The vascularized luteal area and percentage of vascularized luteal tissue area did not differ between groups.

Table 2
Number of corpora lutea, total luteal tissue area, vascularized total luteal area and percentage of vascularized luteal tissue area (LSMEANS ± SEM) on Day 5 after ovulations in Lacaune ewes subjected to synchronized estrus induction protocols* for six (G-6) or nine (G-9) days during non-breeding season.

End points	G-6 days (n=13)	G-9 days (n=14)	P-value
Corpora lutea (n)	2.0 ± 0.2	2.9 ± 0.3	0.02
Total luteal tissue area (cm^2)	1.5 ± 0.2	2.2 ± 0.2	0.02
Vascularized luteal area (cm^2)	0.4 ± 0.1	0.6 ± 0.1	0.20
% of vascularized luteal tissue area	29.0 ± 4.0	29.0 ± 3.0	0.94

*Protocol with medroxyprogesterone acetate sponge plus 37.5 µg of d-cloprostenol and 400 IU of eCG 24 h before sponge withdrawal.

3.2. Transcervical penetration and embryo recovery

Embryo recovery was conducted 5- 6 days after ovulation (5.4 ± 0.4 days), only in the 24 ewes that showed signs of estrus. The percentage of ewes for each type of cervical *os* was: duckbill (26%), rose (26%), flap (21%), slit (4%), papilla (9%) and mixed (9%). The external cervical *os* was not adequately visualized for clamped and retraction in 12.5% (3/24) of ewes. Cervical retraction rates reached 91.6% (11/12) in G-6 and 83.3 (10/12) in G-9. Cervical penetration attempt was performed in those ewes that had the cervix retracted and the

transposition rate achieved was 100.0 (11/11) in G-6 and 90.0 (9/10) in G-9. In the only ewe that transcervical penetration was not possible with the catheter, the dilator Hegar was successfully transposed. In three animals of the G-6 treatment group, the cervix was penetrated to uterine flushing of the first horn but in the second transposing attempt to flushing the other horn was not achieved. Considering only ewes that had cervix visualized and adequately retracted there was 72.7% (8/11) in G-6 and 100% (9/9) in G-9 of ewes successful flushed (two uterine horns flushed). The percentage of successful recoveries in G-9 was almost the double than in G-6 treatment group (Table 3).

Table 3
End points of cervical transposition and uterine flushing (LSMEANS ± SEM)in Lacaune ewes submitted to treatment to induce cervical dilation before non-surgical embryo recovery (NSER) performed 5 to 6 days after ovulations in response to synchronized estrus induction protocols* for six (G-6) or nine (G-9) during the non-breeding season.

End points	G-6 days	G-9 days	P-value
Number of cervical rings of ewes transposed	6.5 ± 0.8 (11)	6.3 ± 0.8 (9)	0.84
Successful recoveries[a] (%)	45.5 (5/11)	88.9 (8/9)	0.13
Degree of cervical misalignment[b]	2.3 ± 0.5 (11)	1.0 ± 0.3 (9)	0.01
Time of cervical transposition (min)	7.2 ± 1.3 (11)	3.4 ± 0.6 (9)	0.01
Time of uterine flushing (min)	18.3 ± 1.3 (8)	20.2 ± 1.3 (9)	0.34
Total time of embryo recovery procedure (min)	26.5 ± 2.2 (8)	23.8 ± 2.1 (9)	0.34

*Protocol with medroxyprogesterone acetate sponge plus 37.5 µg of d-cloprostenol and 400 IU of eCG 24 h before sponge withdrawal.
( ) Number of animals.
[a]Percentage of ewes in which at least one structure was recovered.
[b]Degree of cervical misalignment (one - rectilinear cervix, two - intermediate and three - highly asymmetrical).

Although the number of rings did not differ ($P > 0.05$) between treatments, the cervical misalignment score was higher ($P < 0.05$) in G-6, increasing ($P < 0.05$) the time of transcervical penetration in this group compared to the G-9. There was no effect of treatment ($P > 0.05$) on the time of uterine flushing or for total time of embryo recovery procedure (Table 3). The time spent to cervical transposition did not differ ($P > 0.05$) between animals presenting cervix with six or less rings (6.1 ± 1.0 min) and those with more than six rings (4.0 ± 1.0 min).

The number of recovered structures and number of blastocysts tended ($P = 0.07$) to be higher in G-9 and the number of viable embryos was higher ($P < 0.05$) in this treatment compared to the G-6. The number of morulae, number of unfertilized oocytes, unfertilized oocytes rate and the viable embryo rate did not differ between treatments ($P > 0.05$, Table 4). The efficiency of embryos collections by transcervical method was measured by recovery of

fluid and structures (recovery rate). The fluid recovery rate was 99.7% in G-6 and 99.9% in G-9. Recovery rate in G-6 and G-9 were 30.4% (7/23) and 48.3% (14/29), respectively. All recovered embryos (100.0%) were classified as Grade I in both treatments.

After embryo recovery, all ewes walked back to pens and immediately searched for water or food. No ewe presented any uterine abnormality at ultrasonography performed 30 days after embryo recovery.

Table 4
End points of embryo yield (LSMEANS ± SEM) in Lacaune ewes subjected to synchronized estrus induction protocols* for six (G-6) or nine (G-9) days and non-surgical embryo recovery (NSER) performed 5-6 days after ovulations.

End points	G-6 (n=11)	G-9(n=10)	P-value
Recovered structures[a] (n)	0.6 ± 0.2	1.6 ± 0.4	0.07
Unfertilized oocytes (n)	0.2 ± 0.2	0.1 ± 0.1	0.45
Morulae (n)	0.1 ± 0.1	0.3 ± 0.2	0.27
Blastocysts (n)	0.3 ± 0.2	1.0 ± 0.3	0.07
Viable embryos[b] (n)	0.4 ± 0.2	1.3 ± 0.4	0.03
Viable embryo rate (%)	62.3 ± 0.2	87.7 ± 0.1	0.23
Unfertilized rate (%)	39.4 ± 23.9	6.5 ± 8.0	0.20

*Protocol with medroxyprogesterone acetate sponge plus 37.5 µg of d-cloprostenol and 400 IU of eCG 24 h before sponge withdrawal.
[a]Structures definition: non-fertilized oocytes, embryos of different developmental stages and/or zona pellucida.
[b]Viable embryos were considered embryos Grade I, II and III.
( ) Number of animals.

4. Discussion

The ability to set or adjust estrus induction/synchronization protocols according the breed, animal categories and condition of cyclicity of females would be favorable to increase the efficiency of the outcomes, to increase the cost-benefit and then extend the use of this reproductive biotechnology in commercial herds. In addition, the ability to perform NSER by transcervical route in all sheep breeds would be extremely beneficial in terms of both ethical and practical senses. The approach used in this study, i.e., to apply an estrus induction treatment, is a non-expensive way to test transcervical penetration after cervical dilation treatment, as we did in other breeds [19, 29]

The majority (57.1%) of Lacaune ewes had functional CL at the beginning of the protocols of estrus induction, which indicates in the period of lengthening daylengths [15] a large part of the animals may still be cyclic in tropical climate. A similar (56.5%) percentage

of Santa Inês ewes with CL was previously observed during the same climate [15]. Cyclicity in approximately half of Lacaune ewes raised in high latitude and temperate climate on Switzerland [2] suggests this breed is not strictly photoperiod-dependent, as previously reported in a lower latitude on Greece [30].

The emergence day of ovulatory follicular wave occurred in the Day -3 (range -6 to -1) in G-6 and Day -4 (range -7 to -1) in G-9. However, it was observed wide range among ewes in both treatment groups. The progesterone treatment is not originally designed to synchronize follicular waves, however, there is a distinctive pattern of antral follicular wave dynamics during the treatment period affected mainly by the number of emerging follicular waves and ovarian status at the beginning of protocol [15]. This fact may justify the variation observed among the animals. In Santa Inês breed, the emergence of the first and second antral follicular waves occur approximately on Days 2.0 and 5.9 in relation to the CIDR insertion (Day 0), respectively [15]. If the antral follicular wave emergence pattern is the same for sheep of the Santa Inês and Lacaune breeds, it may be indicated that the G-6 and G-9 ewes had ovulations of follicles from the first and second antral follicular waves, respectively. Hormonal strategies to synchronize follicular wave emergence in sheep is not as effective as reported in cattle. It was demonstrated that the use of P_4 devices associated with cloprostenol at the beginning of the protocol seems to be appropriate to synchronize follicular emergence (in average, 56.6 h after device insert) in Santa Inês ewes without any benefit of adding GnRH agonists or estradiol benzoate [14]. In the present study, the d-cloprostenol dose was only administrated 24 h before MAP sponge removal, but perhaps if applied at the start of treatment it could reduce the variation for the follicular wave emergence day.

The fluctuation in the number of antral follicles according to the size categories which were observed in the last five days of the protocols correspond to the development of the ovulatory follicular wave. This reflects the progressive growth of small follicles from one size class to another in a wave-like fashion [31]. On the day of MAP sponge withdrawal, the number of small follicles ($\leq$ 3 mm) was lower in G-9 possibly because the ovulatory follicular wave emerged earlier in these ewes (i.e. at least one day earlier compared to G-6 ewes). The higher number of follicles between 4.1 and 5.0 mm in the G-9 perhaps could be related to the expected lower concentrations of progestogen in the last days of this hormonal protocol than in the G-6 treatment. It is known that serum progestogen concentrations decline over time [32] and could fall to subluteal values after the 6^{th} day; [33, 8]. A reduction of 50% was observed from third day on the serum MAP concentrations in ewes treated for long-term with 60 mg MAP sponges outside the breeding season, and suggested that a limited quantity of

progestogen is absorbed by the epithelium or that the clearance rate of the serum MAP is high [21]. These authors observed that ewes treated with halved sponges (30 mg) had an increase on the fecundity compared to 60 mg.

In ewes, the clearance of progesterone is increased from elevated feed intake [34]. Due the high dry matter intake of the experimental animals to achieve nutrient requirement to milk production, it is possible to suggest that high clearance rate may have been exacerbating subluteal conditions. Therefore, lower concentrations of progestogens possibly had less suppression on endogenous LH concentrations, which could favor the development of a greater number of follicles in the dominant phase in G-9 compared to those ewes in G-6. This may also justify the higher number of ovulations in the G-9 ewes due the lower dominance effect. It is known that the LH pulse frequency increases in the absence of a CL and with decreases of progestogen release from device over time [35,36], but the LH surge does not occur during treatment [8]. The gradual increase of LH pulsatility and/or increase in serum FSH concentrations at proestrus are possibly the responsible for ovulations of follicles coming from the two last waves in prolific breed [37]. The percentage of ovulation coming from the last two waves in the G-9 was increased (~ 36%) and close to that observed by these authors (~ 50%). Dairy Lacaune is considered a low prolific breed [38], and the ovulation rate of prolific selected parents and non-prolific Lacaune breed are 2.35 and 1.67, respectively [39]. In double ovulatory cycles the ovulatory follicles emerge as part of the same follicular wave, but in few cases also as a part of different waves [8]. The higher frequency of ovulatory follicles from distinct waves in the G-9 was in some cases associated to multiple ovulations (triple, quadruple and even quintuple), which in turn must have occurred due to the lower dominance effect. Lassoued et al. [40] also observed a higher number of medium follicles on the days prior to synchronized estrus in prolific strains compared to non-prolific strains. Therefore, the G-9 protocol with eCG administered 24 h before sponge withdrawal appears to mimic the hormonal and ovarian events that occur in prolific sheep breeds and, seems to have a positive effect in the number of ovulations of the lactating Lacaune ewes.

Both hormonal treatments were efficient to induce synchronous-estrus and promoted ovulation in high percentage of females, like observed in Lacaune ewes after P_4 treatments for 6 or 12 days in high latitude [2]. The duration of progesterone-based estrus induction protocols had no effect on the time of estrus and ovulation. The highest number of ovulations was responsible for the greater total luteal tissue area in the G-9 ewes compared to those from G-6, however, the characteristics of luteal vascularization (i.e., vascularized luteal area and percentage of vascularized luteal area), indicative of the luteal angiogenesis, were not altered

by the MAP permanence. It is known that the production and release of progesterone is dependent on an active angiogenic process that occurs during the first few days after ovulation [41] and that the total luteal area and progesterone concentration in sheep are positively correlated to each other between days 3 and 15 after ovulation [42]. In ewes, both the luteal area and the vascularized luteal area were positively correlated with progesterone during luteogenesis [25], suggesting that maybe G-9 ewes had higher progesterone concentrations.

This is the first report of NSER in Lacaune ewes. The transcervical penetration at the initial luteal phase after estrus synchronization is a reliable screening test to determine the procedure efficiency and to select donors for MOET program [19]. The cervical dilation protocol based on estradiol, oxytocin and cloprostenol was efficient in Lacaune ewes, allowing uterine flushing and embryo recovery in a high percentage of animals with ~ 100% of fluid recovery rate. Despite the use of a lower oxytocin dose, compared to Masoudi et al. [43], the effectiveness was greater (measured as the % of transcervical penetration) and may reflect previous exposure to higher dose of estradiol benzoate and different physiological or anatomical conditions between breeds. Estradiol has several effects that may contribute to the regulation of uterine PGF2α secretion, maximizing its responsiveness to oxytocin and may modulate pulsatile secretion of neurohypophyseal oxytocin [44].

It has been well established that changes in progesterone: estradiol ratio are important in the physiological and morphological changes of cervix observed during parturition [45]. The effectiveness of estrogen being greater after a period of progesterone priming [46], which promotes accumulation of arachidonic acid, prostaglandin endoperoxide synthase, and other molecules necessary for the synthesis of $PGF_{2\alpha}$ in the endometrium [44] and ultimately downregulates its own receptor, allowing for estrogen-stimulated expression of oxytocin receptors [46]. These effects of progesterone appear to ensure that secretion of $PGF_{2\alpha}$ occurs at the appropriated time of induce luteolysis [44]. Therefore, adequate progesterone profile prior treatment to cervical dilation seems necessary for efficient NSER.

It can be supposed that an expected increase in circulating oxytocin in the group with more CL and luteal tissue (G-9) may play an important role in the lower cervical misalignment after cervical retraction and shorter time for transcervical penetration, suggestive of better cervical dilation. The CL contains large amounts of mRNA and protein for oxytocin synthesis and their concentrations stored in secretory granules reach peak between days 5 and 9 after estrus [47] and decline thereafter [48]. Thus, the application of d-cloprostenol before NSER may have induced luteolytic cascade and greater release of

oxytocin secretory granules by luteal cells in G-9 treatment. This synergic effect of exogenous and endogenous hormones seems to be important for NSER technique efficiency beyond the cervical anatomy. Physiology of cervical dilation depends on stimulating PGE synthesis in response to concentrations of reproductive hormones [49].

Recovery rates (~ 40.4%) in present study were similar to those observed in non-superovulated Santa Inês ewes (41.2%) [19], and lower than 65% observed in superovulated animals [50,51] by the transcervical method. The reasons for low recovery rates observed in non-superovulated ewes are unclear. It seems to involve CL health status, since in ewes with < 1 ng plasma progesterone concentrations at the time of collection the embryo recovery rate was only 20% *vs* 50% in ewes with > 1 ng [19]. In the current study, the percentage of successful recoveries (at least one structure recovered) was almost doubled in the group with higher ovulation rate. Another study conducted by our team found similar (~65%) recovery rates in Lacaune superovulated ewes (article in preparation). Indeed, there are apparently limitations for achieving high rates of embryo recovery by transcervical method in non-superovulated animals.

A higher number of recovered structures and viable embryos was found in the G-9 group, probably due to the higher number of ovulations. The percentage of viable embryos was 100% for both groups, suggesting there was no deleterious effect of hormonal treatment to cervical dilation on embryo quality. This finding agrees with Figueira et al. [52], who observed good pregnancy and lambing rates after transfer of frozen-thawed embryos retrieved by NSER (using the same cervical dilation treatment).

5. Conclusions

In conclusion, both synchronous-estrus induction treatments showed a high rate of estrus response and ovulation, but MAP sponge for 9 days increased the number of follicles between 4.1 – 5.0 mm and promoted higher number of ovulations and embryo yield. Finally, the protocol combining d-cloprostenol, estradiol benzoate and oxytocin allowed high percentage of transcervical penetration followed by NSER in Lacaune ewes.

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ARTICLE 4 - Embryo yield is associated with the duration of progestogen treatment in superovulated lactating Lacaune ewes subjected to non-surgical embryo recovery

Article following the norms and format of the journal Theriogenology.*

ARTICLE 4 - Embryo yield is associated with the duration of progestogen duration in superovulated lactating Lacaune ewes subjected to non-surgical embryo recovery

Lucas Machado Figueira[a,b], Nadja Gomes Alves[a,**], Ana Lucia Rosa e Silva Maia[b], Joanna Maria Gonçalves Souza-Fabjan[b], Ribrio Ivan Tavares Pereira Batista[b], Maria Clara da Cruz Moraes[b], Renato Ribeiro de Lima[a], Maria Emilia Franco Oliveira[c], Jeferson Ferreira da Fonseca[d,*]

[a] Universidade Federal de Lavras - Av. Doutor Sylvio Menicucci, 1001 - Kennedy, CEP 37200-900, Lavras-MG, Brazil

[b] Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, Vital Brazil, CEP 24220-008, Niterói-RJ, Brazil

[c] Universidade Estadual Paulista "Júlio de Mesquita Filho, Via Prof. Paulo Donato Castellane s/n, CEP 14884-900, Jaboticabal-SP, Brazil

[d] Embrapa Caprinos e Ovinos , Estrada Sobral/ Groaíras, km 4, CP D10, CEP 62011-000, Sobral-CE, Brazil

A B S T R A C T

This study assessed the effect of the duration of progestogen protocol on ovarian parameters and embryo yield in superovulated Lacaune ewes collected by non-surgical embryo recovery (NSER). Twenty-three lactating ewes were subjected to superovulatory treatment 30 d apart, in a cross-over design. All ewes received 60 mg of MAP intravaginal sponges for six (G-6 group) and nine (G-9 group) days. A total dose of 133 mg pFSH was given in six decreasing doses (twice a day) starting at 60 h before sponge removal. Ultrasound exam was performed to assess the ovarian structures at the first pFSH injection and one day before NSER, which was performed 6 to 7 days after estrus onset. The NSER procedure was conducted only in the ewes that expressed estrus and were mated. At the first pFSH injection there was no difference ($P > 0.05$) in the total number of follicles between G-6 (15.7 ± 1.0) and G-9 (15.6 ± 0.8). The percentage of ewes in estrus was 78.2% (18/23) in both groups, and the percentage of responding donors (≥ 3 corpora lutea - CL) did not differ ($P > 0.05$) between G-6 (78.2%, 18/23) and G-9 (69.5%, 16/23). The number of CL in ewes collected did not differ ($P > 0.05$) between G-6 (7.0 ± 1.2) and G-9 (8.1 ± 1.6), as well as the transcervical penetration rate in G-6 (94.4% or 17/18) and G-9 (83.3% or 15/18). The structures recovery rate (structures/CL) did not differ ($P > 0.05$) between G-6 (54.5%) and G-9 (68.0%), but there was a lower ($P < 0.05$) number of viable embryos in G-6 (1.8 ± 0.7) compared to G-9 (3.5 ± 1.1) group. In conclusion, the use of progestogen device for 9 days was beneficial for viable embryo yield in superovulated Lacaune ewes.

Keywords: Dairy sheep, MOET, Superovulation, Transcervical, Uterine flushing.

1. Introduction

With increasing effectiveness of non-surgical embryo recovery (NSER) in Brazil [1], Brazilian dairy sheep farmers became interested to include Lacaune sheep in multiple ovulation and embryo transfer (MOET) programs. This biotechnique is a well-known contributor to accelerate the genetic improvement in sheep [2]. However, the variability of ovarian response to superovulatory treatments limits the wide application of MOET in commercial programs and reproductive research [3]. A breed component itself has been identified as an intrinsic factor responsible for variations [4,5], emphasizing the need for specific treatments for each breed [6]. Despite of the worldwide importance of Lacaune breed for the dairy sheep industry, only few studies applying MOET were reported in this breed [4,7].

The non-surgical embryo recovery (NSER) technique has been improved in the last years, as an alternative to surgical procedures [8]. Recently, encouraging results of transcervical penetration rates for NSER in 73 to 81% of Santa Inês [9,10] and 95% of Lacaune ewes [11] were reported, using hormonal treatment to induce cervical dilation, which combines d-cloprostenol, estradiol benzoate and oxytocin. However, these studies evaluated non-superovulated ewes. Therefore, the suitability of this protocol for NSER in different breeds, seasons of the year, and in superovulated ewes remains to be assessed [8].

Traditional superovulatory protocols with progesterone priming during 12 to 14 days (long-term) were established according to the luteal phase duration of the estrous cycle. However, the progestin profile induced by the intravaginal devices is considered potentially responsible for the variable results in long-term treatments [12]. The use of progestins protocols with short exposure (5 to 7 days) enabled comparable performance to long-term protocols in Merino breed, as well as brings practical advantages [2] and use of a single pessary. Recently, our group observed differences on preovulatory follicular dynamics in progestogen-based estrus induction protocols for six or nine days in the Lacaune breed [11]. Interestingly, we observed that the higher frequency of ovulatory follicles from different waves in the 9-days protocol associated to 400 IU of eCG was in some cases associated with multiple ovulations (up to five), which in turn, occurred probably due to the lower dominance effect. The ovulatory response after single-shot eCG treatment has high correlation with further response to superovulatory treatment with FSH [13] and can be used as preselection test for donors. Thus, we hypothesized that this lower dominance effect observed in the 9-

days progestogen protocol may be beneficial for ovarian superstimulation in the Lacaune breed.

The objective of present study was to evaluate the superovulatory response following pFSH step-down treatment in short- (6 days) or mid-term (9 days) progestogen-based protocols and the subsequent embryo yield after the NSER in lactating Lacaune ewes.

2. Materials and Methods

2.1. Ethics and animal care

The Animal Care Committee of Embrapa Dairy Cattle approved the study design (Protocol number # 2512100516/2016 – ATTACHMENT A), and it was conducted under the principles of the Brazilian Society of Laboratory Animal Science, which regulates experimental conditions involving animals.

2.2. Local, animals and experimental conditions

This study was conducted in the anestrous season, from October to December on a commercial farm located in Soledade de Minas (latitude 22°3'S, longitude 45º2'W and altitude of 938 meters above mean sea level), in Minas Gerais State, Brazil.

Twenty-three lactating, clinically health Lacaune ewes (119.9 ± 6.7 days in milk and producing 1.0 ± 0.1 kg of milk/day/ewe, mean ± SEM) were used. These ewes were primiparous (n=12) and multiparous (n=11), ranged from 16 to 95 months of age, with 69.4 ± 2.5 kg of body weight (BW) and 3.6 ± 0.1 of body condition score (BCS; scale 0 - 5, being 0 = emaciated and 5 = too fat) [14].

The animals were kept in collective pens and fed corn silage. A balanced concentrated supplement was offered twice a day to complete their nutritional requirements [15]. Mineralized salt (DeHeus®, Rio Claro, Brazil) and drinking water were available *ad libitum*.

2.3. Experimental design

This study was performed in a crossover experimental design with an interval of 30-days between replicates. In the first replicate, the ewes were randomly allocated into two different groups. Ewes received treatment with intravaginal sponges containing

medroxyprogesterone acetate (MAP; 60 mg; Progespon®, Zoetis, Campinas, Brazil) for six (G-6) or nine (G-9) days. Twenty-four hours before sponge removal, 37.5 µg of d-cloprostenol (Sincrocio®, OuroFino, Cravinhos, Brazil) were administered intramuscularly (i.m.). Ewes were treated with pFSH (133 mg; Folltropin®, Bioniche, Belleville, ON, Canada) i.m. twice daily (12 h intervals), in six decreasing doses (25, 25, 15, 15, 10 and 10%). To achieve this dose, 10 mL of saline solution was added to 20 mL of the diluents. The pFSH treatment started 60 h (2.5 days) before the sponge removal (i.e., in G-6 and G-9 group the superovulatory treatment began 3.5 days and 6.5 days after the sponge insertion, respectively).

The estrous behavior was recorded during four days from sponge removal, by using healthy and fertile rams (maximum ratio of 1 ram: 4 ewes) twice a day (morning – 08:00 and evening - 18:00). The rams were rotated in both treatments to minimize male fertility variations. Ewes remained with the ram at least 30 min in each observation period if no mounting acceptance occurred. Estrus onset was defined as the time when the ewe first stood to be mounted by the ram. Mating was repeated every 12 h until no mounting acceptance.

2.4. Ovarian ultrasonography

The ovarian ultrasonography (US) was conducted at the time of the first pFSH injection in all animals by the same experienced operator using a B-mode ultrasonographic scanner (Mindray®, M5Vet, Shenzen, China) with a linear transrectal transducer (8.0 MHz) fitted to a plastic rod that allowed its manipulation inside the rectum. These examinations aimed to count the number of follicles and CL. On sixth and seventh days after sponge removal, another US evaluation was performed using Color-Doppler mode for CL count and luteal blood flow assessment, which provides high accuracy on counting luteal structures [16,17].

2.5. Non-surgical embryo recovery (NSER) and embryo yield evaluation

Embryo recovery was conducted only in ewes that expressed estrus and were mated, on Day 6 – 7 after onset of estrus (6.8 ± 0.9 days), adopting the Embrapa Protocol of NSER [8]. The cervical penetration and the uterine flushing were performed after the hormonal treatment to induce cervical dilation as described by Figueira et al. [11]. This treatment consisted of 1 mg of estradiol benzoate (Sincrodiol®, Ourofino, Cravinhos, Brazil) i.m. and

37.5 µg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) laterovulvarly (l.v.) 16 h before NSER. An injection of 50 IU oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) intravenously (i.v.) was administered 20 min before the procedure (Fig. 1). Immediately after oxytocin, the combination of 40 mg of hyoscine-N-butylbromide and 5 g of sodium dipyrone (Buscofin®, Agener Union, Embu-Guaçu, Brazil) were administered i.v. and i.m. routes, in equal parts. In addition, 1 mg/kg BW of acepromazine maleate (Acepran 1%®, Vetnil, Louveira, Brazil) i.m. was administered 20 min prior to cervical manipulation, as previously described [18]. This sedation continued for 45 min, enough time for carrying out the procedure. Animals in a standing position were restrained in a cart and received 2 mL of 2% lidocaine epidural block (S5-C1) (Lidovet®, Bravet, Rio de Janeiro, Brazil). Cervical immobilization, uterine access and embryo recovery were performed by the same experienced technician, as described by Fonseca et al. [8]. The ultrasound assessment allows determination of the direction of the first uterine flushing ipsilateral to the ovary with greater count of vascularized CL.

All the structures recovered were listed and the embryos were transferred to the maintenance medium (Holding Plus®,Cultilab, Campinas, Brazil). Embryo evaluation was performed using a stereomicroscope with magnification of 40X (Nova®, model XTD-20, Piracicaba, Brazil) and following International Embryo Transfer Society recommendations [19]. The quality score of the embryos was Grade 1 (excellent or good); Grade 2 (fair); Grade 3 (poor); and Grade 4 (dead or degenerated). Embryos Grade 1, 2 and 3 were considered viable. The following indices were calculated to evaluate the efficiency of the NSER: transcervical penetration rate - number of ewes in which the cervix was completely transposed for uterine flushing / number of ewes in which cervical penetration was attempted x 100; successful recovery rate - number of ewes with at least one structure recovered / number of ewes in which the cervix was completely transposed for uterine flushing x 100; structures recovery rate - total number of recovered structures (non-fertilized oocytes, embryos of different developmental stages, and zona pellucida) / number of CL x 100; viable embryo rate - number of viable embryos / total number of recovered structures x 100; and the non-fertilized rate -number of non-fertilized oocytes / total number of structures recovered x 100.

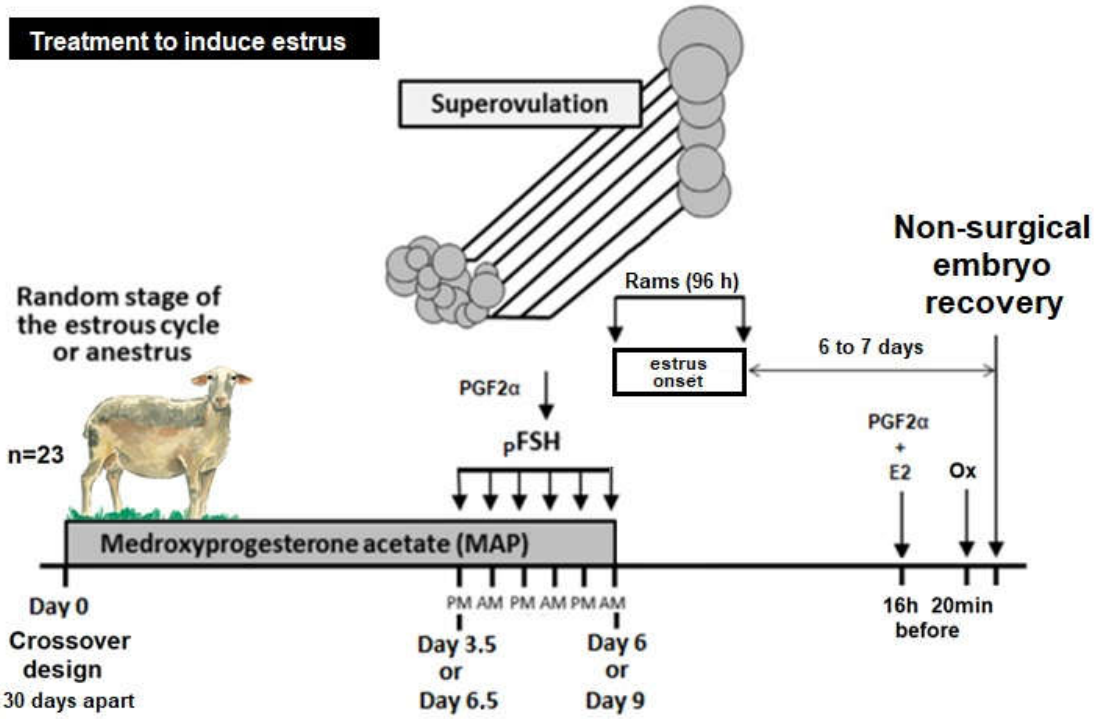

Fig. 1. Schematic representation of treatments used to induce estrus, to promote superovulation and to induce cervical dilation for non-surgical embryo recovery in lactating Lacaune ewes. pFSH: 133 mg of porcine follicle-stimulating hormone, PGF2α: 37.5 µg of d-cloprostenol, E2:1 mg of estradiol benzoate, OX:50 IU of oxytocin.

2.6. Statistical analyses

The statistical analyses were performed using SAS® software version 9.3 (SAS Inst., Inc., Cary, USA). The statistical models to study estrous behavior, superovulation, transcervical penetration, and embryo recovery responses from crossover experimental design were like proposed by Kaps and Lamberson [20]. Models included order (applied treatments sequence), treatment, and replicate as fixed effects and ewe nested in order as random effect.

Quantitative data as estrus duration, interval from sponge removal to estrus, time of cervical penetration and total time of procedure were analyzed by generalized linear models, with Gamma distribution and log link function using GLIMMIX procedure. The follicular counts grouped by diameter categories (≤ 3.0 mm, 3.1-4.0 mm, 4.1-5.0 mm, and > 5.0mm) and the numbers of CL, non-vascularized CL, recovered structures, non-fertilized oocytes,

luteinized anovulatory follicles, and viable embryos were also analyzed by using GLIMMIX procedure, but considering Poisson distribution and log link function. The relationship between variables was assessed by using the Pearson´s correlation.

Qualitative data as donors in estrus, percentage of responding donors (≥3 normal CL), ewes with follicles >5.0 mm and ewes bearing CL at the beginning of pFSH treatment, transcervical penetration rate, responding donors, successful recovery rate, structures recovery rate, viable embryos rate, and non-fertilized rate were analyzed by using GLIMMIX procedure, considering binomial distribution and logit link function. Results are shown as means ± standard error of the means (MEANS ± SEM). Differences are declared significant at $P < 0.05$.

3. Results

3.1. Ovarian population at the beginning of pFSH treatment

The ovarian population at the beginning of the superovulatory treatment is summarized in Table 1. There were no differences ($P > 0.05$) between groups for the follicular diameter categories analyzed. The percentage of ewes with large follicles (> 5.0 mm) or bearing CL at the time of first pFSH injection did not differ ($P > 0.05$) between G-6 and G-9.

3.2. Reproductive behavior and ovarian response

Parameters related to estrous behavior such as the percentage of donors in estrus, the interval from sponge removal to estrus and the estrus duration did not differ ($P > 0.05$) between groups (Table 2); five ewes (21.7%) in each treatment group did not respond.

Superovulatory responses, such as the percentage of responding donors (≥3 CL), the number of CL, vascularized CL, and non-vascularized CL did not differ between treatment groups ($P > 0.05$; Table 2). The number of luteinized anovulatory follicles was twice as much in G-6 ($P < 0.10$) compared to G-9 ewes.

Table 1

Ovarian population (means ± SEM) at the beginning of pFSH treatment in lactating Lacaune ewes treated with progestogen-based estrus induction protocol for six (G-6) or nine (G-9) days associated to 133 mg of pFSH[a] in a crossover design (two replicates – 30 days apart).

End points	Treatment		
	G-6 (n=23)	G-9 (n=23)	P-value
Number of follicles ≤ 3.0 mm	12.5 ± 0.9	11.8 ± 0.8	0.54
Number of follicles 3.1-4.0 mm	1.3 ± 0.2	2.0 ± 0.5	0.07
Number of follicles 4.1-5.0 mm	0.7 ± 0.2	1.0 ± 0.2	0.22
Number of follicles >5.0 mm	1.2 ± 0.2	0.7 ± 0.2	0.17
Total number of antral follicles	15.7 ± 1.0	15.6 ± 0.8	0.99
Ewes bearing large follicles (%)	73.9 (17/23)	52.2 (12/23)	0.15
Ewes bearing corpora lutea (%)	34.7 (8/23)	43.5 (10/23)	0.52

[a] Six decreasing injections (25, 25, 15, 15, 10 and 10% of total dose) twice a day. The first pFSH injection occurred at 60 h before sponge removal.

Table 2

End points (means ± SEM) related to estrous behavior expression, superovulatory responses and non-surgical embryo recovery (NSER) in Lacaune ewes treated with progestogen-based estrus induction protocol for six (G-6) or nine (G-9) days associated to 133 mg of pFSH[a] in a crossover design (two replicates - 30 days apart).

	Duration of device permanence		
End Points	G-6	G-9	P-value
Ewes (n)	23	23	-
Estrus response (%)	78.3 (18/23)	78.3 (18/23)	1.00
Interval from sponge removal to estrus (h)	38.1 ± 5.2 (18)	36.0 ± 3.1 (18)	0.48
Estrus duration (h)	27.3 ± 3.7 (18)	29.7 ± 3.1 (18)	0.65
Responding donors (≥ 3 CL) (%)[b]	78.2 (18/23)	69.5 (16/23)	0.60
CL count (n)	7.0 ± 1.1 (23)	6.6 ± 1.4 (23)	0.52
Vascularized CL (n)	6.3 ±1.1(23)	6.4 ± 1.4 (23)	0.81
Non-vascularized CL (n)	0.4 ± 0.2 (23)	0.3 ± 0.2 (23)	0.53
Luteinized anovulatory follicles (n)	1.1 ± 0.3 (23)	0.5 ± 0.2 (23)	0.09
Successful cervical transposing rate (%)[c]	94.4 (17/18)	83.3 (15/18)	0.30
Duration of cervical transposing (min)	5.8 ± 1.2 (17)	4.6 ± 0.9 (15)	0.47
Duration of uterine flushing (min)	18.5 ± 0.8 (17)	19.0 ± 1.0 (15)	0.64
Ewes with at least one structure recovered (%)	82.4 (14/17)	80.0 (12/15)	0.78
CL count in ewes collected (n)	6.5 ± 1.2(17)	8.1 ± 1.6 (15)	0.83
Structures recovered (n)	3.5 ± 1.0 (17)	5.5 ± 1.6 (15)	0.01
Structures recovery rate (%)	54.5 (60/110)	68.0 (83/122)	0.59
Viable embryos (n)	1.8 ± 0.7 (17)	3.5 ± 1.1 (15)	0.03
Viable embryo rate (%)	50.0 (30/60)	62.7 (52/83)	0.49
Non-fertilized oocytes (n)	1.6 ± 0.6 (17)	1.7 ± 1.2 (15)	0.24
Non-fertilized rate (%)	45.0 (27/60)	30.1 (25/83)	0.06

[a] Six decreasing injections (25, 25, 15, 15, 10 and 10% of total dose) twice a day. The first pFSH injection occurred at 60 h before sponge removal.

[b] Including ewes that did not express estrous behavior.

[c] NSER was performed in all ewes that expressed estrous behavior.

( ) Number of animals.

3.3. Correlations of follicular populations at time of the first pFSH with superovulatory response and embryo yield

There were moderate correlations between ovarian population at the time of first pFSH and superovulatory response. The number of CL was positively correlated ($P < 0.05$) with total number of antral follicles ($r= 0.36$), number of follicles ≤ 3.0 mm ($r = 0.42$) and > 5.0 mm ($r = 0.38$) and was negatively correlated ($P < 0.05$) with the number of follicles of 4.1-5.0 mm ($r = -0.33$). The follicles > 5.0 mm were also positively correlated ($r = 0.37$) with the number of recovered structures. Moreover, numbers of follicles ≤ 3.0 mm and > 5.0 mm were positively correlated with the number of non-fertilized oocytes ($r = 0.34$ and 0.38, respectively). Neither follicular categories nor total follicle count was correlated ($P > 0.05$) with embryo yield, measured as viable embryos.

3.4. Transcervical penetration and embryo recovery

Regarding the transcervical penetration, successful recovery, and structure recovery rates there was no difference ($P > 0.05$) between the treatment groups (Table 2). The time of transcervical penetration and the time of uterine flushing also did not differ ($P > 0.05$). There was high fluid recovery efficiency (%): 99% in G-6 and 97.2% in G-9. The number of recovered structures and the number of viable embryos were higher ($P < 0.05$) in the G-9 compared to G-6.

4. Discussion

This study is an important step towards understanding the factors influencing superovulatory response during the anestrous season in Lacaune ewes raised under tropical conditions, aimed at establishing the basis for an efficient MOET program in this breed. Additionally, it proved the repeatability and efficiency of used protocol to induce cervical dilation for NSER in superovulated lactating Lacaune ewes, given the high transcervical penetration rates and satisfactory embryo recovery were achieved.

In the current study, there were no significant differences on the ovarian population between groups at the 1stFSH injection, and there was similar superovulatory response in both protocols. However, there was significantly lower number of viable embryos in G-6 compared to G-9 group. Other authors did not observe differences on superovulatory and embryo yield

responses comparing different progesterone exposure times (ranging from 5 to 14 days) in superovulation protocols in Merino breed [2]. In G-6 group, there was double number of luteinized anovulatory follicles, suggesting failures in the ovulatory process. Together, these data indicate insufficient progesterone priming in G-6 protocol. It is known that ovarian and endocrine changes after superovulatory treatments can cause abnormalities in follicular development and fertilization processes, decreasing the number and quality of recovered embryos [21,22,23]. Furthermore, it was demonstrated additional benefit of exposure to high progesterone concentrations on oocyte fertilization rate and embryo quality, which suggests an important role in the preovulatory follicular development under superovulatory treatment [24]. Progesterone priming also affect the expression of angiogenic factors in large preovulatory follicles, ensuring adequate luteal development and function [25]. It was demonstrated that Lacaune ewes are benefited with increased duration of progestogen exposure comparing synchronous estrus induction protocols of 6 and 12 days [26]. These authors observed better luteal function in the 12-d protocol, measured as progesterone concentrations at 14 days after device removal. Therefore, the mid-term (9-days) protocol seems to provide better hormonal milieu to *in vivo* embryo production in Lacaune ewes than short-term (6-days) protocol.

The lower embryo yield in short-term group (6-days) can be partially explained by the fact that 74% of ewes presented large ovarian antral follicles at the beginning of pFSH treatment, almost 50% more than mid-term group (9-days), and similar to 75-80% observed in traditional protocols (long-term,12-14 d) [27-28]. It is known that large ovarian follicles exert a dominant effect and can impair the superovulatory response in small ruminants [29]. Despite any controversy over the absence dominance effect of large follicles during non-breeding season [30], there were a moderate number of animals bearing CL at beginning of the first FSH treatment in the two replicates, and perhaps the dominance effect of the large follicles may have contributed to observed responses outside the breeding season.

There was a moderate positive correlation between the number of small follicles ($\leq$ 3.0 mm in diameter) at the first pFSH injection and superovulatory response, similar to reported by other authors [31,32]. This follicular class is representative of follicular population potentially responsive to FSH and capable of growing to ovulatory size [3]. Interestingly, follicles larger than 5 mm were also positively correlated with the superovulatory response, and either follicles of diameter size $\leq$ 3.0 mm and > 5.0 mm were positively correlated with non-fertilized oocytes. The reason why there was no correlation between the number of small follicles and viable embryos is that, although follicles of 2 mm

in diameter can grow and ovulate in response to superovulatory treatment, they may not be sufficiently mature to develop into viable embryos [27].

The combination of short or mid-term progestogen exposure with reduced dose of pFSH (133 mg) resulted on low efficiency of embryo yield in Lacaune breed. In a study with the same breed [4], greater superovulatory response (~12 CL) and embryo production (~7) were observed after treatment with 16 mg Armour equivalent of pFSH plus 500 IU of eCG at the end of long-term (14 d) progestogen protocol. However, the use of higher doses of FSH can promote more ovulations but not necessarily ensure high correlation with viable embryo production [33]. For instance, in Dorper breed, it was observed better response with the lower pFSH dose, when comparing 128 vs 200 mg [34]. Curiously, in superovulatory treatment combining pFSH and eCG, the 133 mg dose produced a lower number and rate of viable embryos than 100 and 200 mg in Santa Inês breed [35]. Therefore, further studies are needed to improve cost-benefit by adjusting the doses employed for the Lacaune.

Despite high fluid recovery efficiency, the structures recovery rate after NSER had similar results to those 65% reached in previous studies [36,37]. In those studies, the structure recovery rate was evaluated dividing the number of recovered structures (ova and embryos) by the number of CL counted by laparoscopy. Recently, studies evaluated Color-Doppler ultrasound imaging as a substitute for laparoscopy to count the CL in superovulated sheep, with great accuracy to identify animals that did not respond adequately, and which are not feasible for collection [16,17]. This deserves special attention, because there appear to be limitations for achieving high structure recovery rates at NSER in non-superovulated animals [10,11]. Therefore, the use of Doppler US is an excellent tool for the donor screening and evaluation of embryo recovery efficiency, and it is in accordance with the NSER precepts, with attention to animal welfare and the possibility of successive collections [8]. The recovery rate after NSER was slightly lower than rates reported by laparotomy recovery in the same breed (77%) [4] and in Santa Ines breed (77%) [38], but comparable to observed in others breeds, as Corriedale (49 to 68%) [39] and Dorper (57%) [34].

Finally, it should be highlighted that earlier results reported in non-superovulated Lacaune ewes with the same duration of progestogen treatment [11] appeared to be a good screening test of donors, as previously proposed [13]. The average total structures and viable embryos recovered (respectively) in non-superovulated Lacaune favored the 9-days (1.6 and 1.4) when compared to 6-days (0.6 and 0.4) progestogen treatment [11]. Results of the present study confirmed this perspective with significant superior total structures and viable embryos,

respectively, recovered in 9-days (5.5 and 3.5) than in 6-days progestogen (3.5 and 1.8) protocols for superovulated lactating Lacaune ewes.

5. Conclusions

Lacaune ewes superovulated in 9-days progestogen-based estrus induction protocol presented better embryo yield after NSER than in 6-days protocol. The recovery rates observed with NSER were satisfactory, justifying the use of this technique in genetic improvement programs, due to the possibility of successive collections and concerns regarding animal welfare issues. However, considering that 20% of donors did not show estrus, 20 to 30% did not superovulate, and overall superovulatory response (CL count 6.6 to 7.0) was not high, it appeared that 133 mg FSH was not efficient to promote great superovulation in lactating Lacaune ewes.

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ARTICLE 5 - Non-surgical embryo recovery in Lacaune ewes superovulated with different doses of pFSH

Article following the norms and format of the journal Small Ruminant Research.*

ARTICLE 5 - Non-surgical embryo recovery in Lacaune ewes superovulated with different doses of pFSH

Lucas Machado FIGUEIRA[a,b],Nadja Gomes ALVES[a,**], Ana Lucia Rosa e Silva MAIA[b], Joanna Maria Gonçalves SOUZA-FABJAN[b]; Ribrio Ivan Tavares Pereira BATISTA[b], Aline Matos ARRAIS[c]; Renato Ribeiro de LIMA[a], Maria Emilia Franco OLIVEIRA[d,e], Jeferson Ferreira FONSECA[e,*]

[a] Universidade Federal de Lavras - Av. Doutor Sylvio Menicucci, 1001, Kennedy, CEP 37200-900, Lavras - MG, Brazil.

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

[c]Universidade Federal Rural do Rio de Janeiro,Rodovia BR 465, Km 07, s / n Zona Rural, CEP 23890-000, Seropédica - RJ, Brazil.

[d] Universidade Estadual Paulista "Júlio de Mesquita Filho", Via de acesso Prof. Paulo Donato Castelane, s/n, Zona Rural, CEP 14884-900, Jaboticabal - SP, Brazil.

[e] Embrapa Caprinos e Ovinos, Estrada Sobral/Groaíras, km 4, CP D10, CEP 62011-000, Sobral-CE, Brazil.

A B S T R A C T

This study assessed the effect of different doses of pFSH for superovulation and embryo yield in lactating Lacaune ewes. Ewes (n=24) received progesterone intravaginal device for nine days to estrus synchronization and decreasing doses of 100 mg (G-100, n=24) or 200 mg (G-200, n=24) of pFSH starting 60 h before device removal, in a crossover design (60 days apart). Estrus was monitored twice a day and ewes were naturally mated. On Day 6 after device removal, the luteinized structures were counted by ultrasonography. Ewes with ≥ 3 luteinized structures were subjected to hormonal cervical dilation prior to non-surgical embryo recovery (NSER; Day 7). Estrus response and CL count were inferior ($P < 0.05$) in G-100 (66.7% and 2.6 ± 0.7) than in G-200 (91.7% and 11.6 ± 1.2) ewes. NSER was successfully performed in 73.4% (11/15) in G-100 and in 90.9% (20/22) in G-200 ewes ($P > 0.05$). The number of recovered structures and viable embryos were higher ($P < 0.05$) in G-200 (8.7 ± 1.1 and 6.9 ± 1.1) than in G-100 (1.3 ± 0.5 and 1.0 ± 0.5), respectively. The structures recovery rate was higher ($P < 0.05$) in G-200 (67.8%) than in G-100 (27.6%). The dose of 200 mg of pFSH resulted in great superovulatory response and embryo yield after NSER in lactating Lacaune ewes while 100 mg dose proved to be insufficient. Furthermore, good structures recovery rate was observed only in the group of ewes with great superovulatory response.

Keywords: Dairy sheep, Gonadotropins, MOET, Ovine, Transcervical, Uterine flushing.

1. Introduction

Multiple ovulation and embryo transfer (MOET) has been employed in the Lacaune genetic improvement program in France (Torres et al., 1987; Baril, 1995; Baril et al., 2001) for accelerated multiplication of superior genotypes. However, there are still technical limitations to the large-scale use of MOET. The main limitations refer to the wide variability in the superovulatory response (Bartlewski et al., 2016), to adhesions formed after surgical recovery of embryos (Torres and Sevellec et al., 1987), and to the relatively high cost of the technique when compared to the value of animals.

Superovulatory response can be affected by intrinsic factors as breed (Torres et al., 1987; González-Bulnes et al., 2004), emphasizing the need for specific treatments for each breed (Oliveira et al., 2012), and by extrinsic factors (Menchaca et al., 2010; Bartlewski et al., 2016). Among the external factors that can be easily manipulated are the gonadotropins preparations, dosage regimen, and doses used. Currently, the FSH (porcine or ovine pituitary extract) is the primary choice for hormonal ovarian superstimulation (Bartlewski et al., 2016). The 150 to 300 mg FSH doses are commonly used (Simonetti et al., 2008; Bartlewski et al., 2009) or in combination with eCG (Simonetti et al., 2008; Salehi et al., 2010; Oliveira et al., 2012; Oliveira et al., 2014). However, some authors have evaluated the use of FSH dose lower than 150 mg in Merino (Gibbons et al., 2010); Dorper (Loiola Filho et al., 2015), and Santa Inês (Menezes et al., 2014, Maciel et al., 2019, Rodriguez et al., 2019) breeds. Overall, these studies indicated that the dose reduction enable similar recovery of structures and viable embryos compared to higher doses. When a very high dose is administered, greater number of follicles can develop, but decreased oocyte fertilization and embryo quality may occur. Higher doses can also induce anovulatory follicles (D'Alessandro et al., 1996), resulting in luteal premature regression (Okada et al., 2000) and lower efficiency of embryo recovery (Chagas e Silva et al., 2003).

Non-surgical embryo recovery (NSER) is a less invasive method than laparotomy (Oliveira et al., 2018a) and has been improved in sheep mainly from hormonal treatment to induce cervical dilation (Fonseca et al., 2016). However, the variability in cervical anatomy observed among breeds can impair its penetrability (Kaabi et al., 2006) affecting embryo recovery success. In Lacaune breed, high rates of transcervical penetration (91 to 95%) were reported in non-superovulated (Figueira et al., 2019a) and superovulated ewes (Figueira et al., 2018). Although almost all uterine flushing medium is recovered at every transcervical collection, the structures recovery rate seems lower when poor ovulatory response occurs

(Fonseca et al., 2019a; Figueira et al., 2019). Since the hormonal cocktails used to induce cervical dilation (Masoudi et al., 2012; Fonseca et al., 2019a; Figueira et al., 2019a) activate the luteolytic cascade (McKracken et al., 1984), it is possible that the luteal function prior to the cervical dilation treatment affects the subsequent softening. Nonetheless, this information still needs confirmation. Similarly, the relationship between gonadotropin doses and the embryo recovery by transcervical route deserves to be investigated. Gonadotropin dose reduction can reduce costs in employing superovulation protocols and make MOET programs more attractive for commercial purposes, but their impact on NSER needs to be evaluated. The aim of this study was to evaluate the effect of porcine FSH dose (pFSH, 100 mg versus 200 mg) on superovulatory responses and *in vivo* embryo yield after NSER procedure in lactating Lacaune ewes.

2. Materials and Methods

2.1. Ethics and animal care

The Animal Care Committee of Embrapa Dairy Cattle approved the study design (protocol number # 2512100516/2016 – ATTACHMENT A), and it was conducted under the principles of the Brazilian Society of Laboratory Animal Science, which regulates conditions for experiments involving animals.

2.2. Experimental conditions

This study was conducted during the breeding season, from April (Autumn) to June (early Winter) months, on a commercial farm located in Soledade de Minas (latitude 22°3'S, longitude 45º2'W, and altitude of 938 meters), in the Minas Gerais State, Brazil.

The animals were kept in collective pens and fed with corn silage. Balanced concentrated supplement was provided twice a day to complete their nutritional requirements (National Research Council, 2007). Mineralized salt (DeHeus®, Rio Claro, Brazil), and drinking water was available *ad libitum*.

2.3. Experimental design

This study was performed in a crossover experimental design with 60-days apart between the first (April) and second (June) embryo recoveries (two replicates). Twenty-four lactating Lacaune ewes, two primiparous and 22 pluriparous were selected. At the beginning of experiment, the ewes were 129.2 ± 7.1 (mean ± SEM) days in milk and producing 1.2 ± 1.0 kg. These females had 66.4 ± 1.3 kg of body weight (BW) and 3.5 ± 0.04 of body condition score (BCS -scale from 1 to 5, where 1 = emaciated and 5 = obese; Suiter, 1994).

The intravaginal devices containing 0.36 g progesterone (Primer PR®, Tecnopec, São Paulo, Brazil) were maintained for nine days to estrus synchronization. Twenty-four hours before device removal an injection of 37.5 µg d-cloprostenol laterovulvarly (l.v.) was administered. Ewes were randomly allocated into two superovulatory treatments that consisted of 100 mg (G-100) or 200 mg (G-200) of pFSH (Folltropin®-V; Bioniche Animal Health, Belleville, Canada). Superovulatory treatment was started 60 h before device removal and was administered twice a day (08:00 and 20:00), in six decreasing doses (25, 25, 15, 15, 10 and 10% of total dose). At 24 h after device removal, 50 µg of gonadorelin (Gestran®, Tecnopec, São Paulo, Brazil) were i.m. administrated. A simplified schematic representation of experimental procedures is shown in Figure 1.

The estrous behavior was recorded up to 96 h after device removal by using healthy and fertile rams (1:4 ram:ewes ratio), twice a day (08:00 and 20:00). Estrus onset was defined as the time when the ewe first stood to be mounted by the ram. Mating was repeated every 12 h until no mounting acceptance.

2.4. Ovarian ultrasonography

Transrectal ovarian ultrasonography (M5VET, Mindray®, Shenzen, China, with linear transrectal probe to 8.0 MHz) was performed to count the number of antral follicles and corpora lutea (CL) on the ovaries on day of the first (D 6.5 after device insertion) and second pFSH injection (D7). The ovarian follicles were measured using electronic calipers and were classified in four categories (≤ 3.0 mm, 3.1-4.0 mm, 4.1-5.0 mm, and > 5.0 mm). On Day 6 after device removal an ultrasonography evaluation was performed using color Doppler mode for counting luteinized structures, as CL and luteinized anovulatory follicles (LAF). The LAF were defined as structures greater than 5.0 mm with luteinized wall and cavity size over that 50% of diameter. This approach is based on studies from Bartlewski et al. (2017), who

considered LAF as structures ≥ 5 mm and lacking ovulatory stigmata and form Oliveira et al. (2018) that demonstrated that B and Color Doppler mode ultrasonographic techniques can be used to quantify LAF in superovulated ewes. The LAF rate was calculated by dividing the percentage of LAF by number of luteinized structures. This assessment allows determining the direction of the first uterine flushing ipsilateral to the ovary with higher CL count.

2.5. Embryo recovery and morphological evaluation

Embryo recovery was conducted on Day 7 after device removal in the ewes with number of CL ≥ 3, adopting the protocol of cervical dilation previously used in Lacaune ewes (Figueira et al., 2019). The time of transcervical penetration (considered the time to transpose the cervical rings with Hegar dilator and catheter) and uterine flushing (considered the time to flushing the first uterine horn added to the time for the second cervical penetration with catheter and flushing of the second uterine horn) were recorded and summed to determine the total time of embryo recovery procedure. These times were taken as NSER efficiency measures. All the structures recovered (non-fertilized oocytes, embryos of different developmental stages, and zona pellucida) were listed and the embryos were transferred to maintenance medium (Holding Plus®, Cultilab, Campinas, Brazil). Embryo evaluation was performed using a stereomicroscope with magnification of X 40 (Nova®, model XTD-20, Piracicaba, Brazil) and followed the same principles used for cattle (Stringfellow and Givens, 2010). Grade 1, 2, and 3 embryos were considered as viable embryos. Grade 1 and 2 were considered freezable embryos and were cryopreserved using by either vitrification (Gibbons et al., 2011) or slow freezing (Fonseca et al., 2018) techniques for the study of viability post-transfer (Figueira et al., 2019b). The following indices were calculated to assess the efficiency of the NSER: transcervical penetration rate - number of ewes in which the cervix was completely transposed for uterine flushing / number of ewes in which cervical penetration was attempted x 100; successful recovery rate - number of ewes with at least one structure recovered / number of ewes in which the cervix was completely transposed for uterine flushing x 100; structures recovery rate - total number of recovered structures / number of CL x 100; and viable embryo rate - number of viable embryos by the total number of recovered structures x 100.

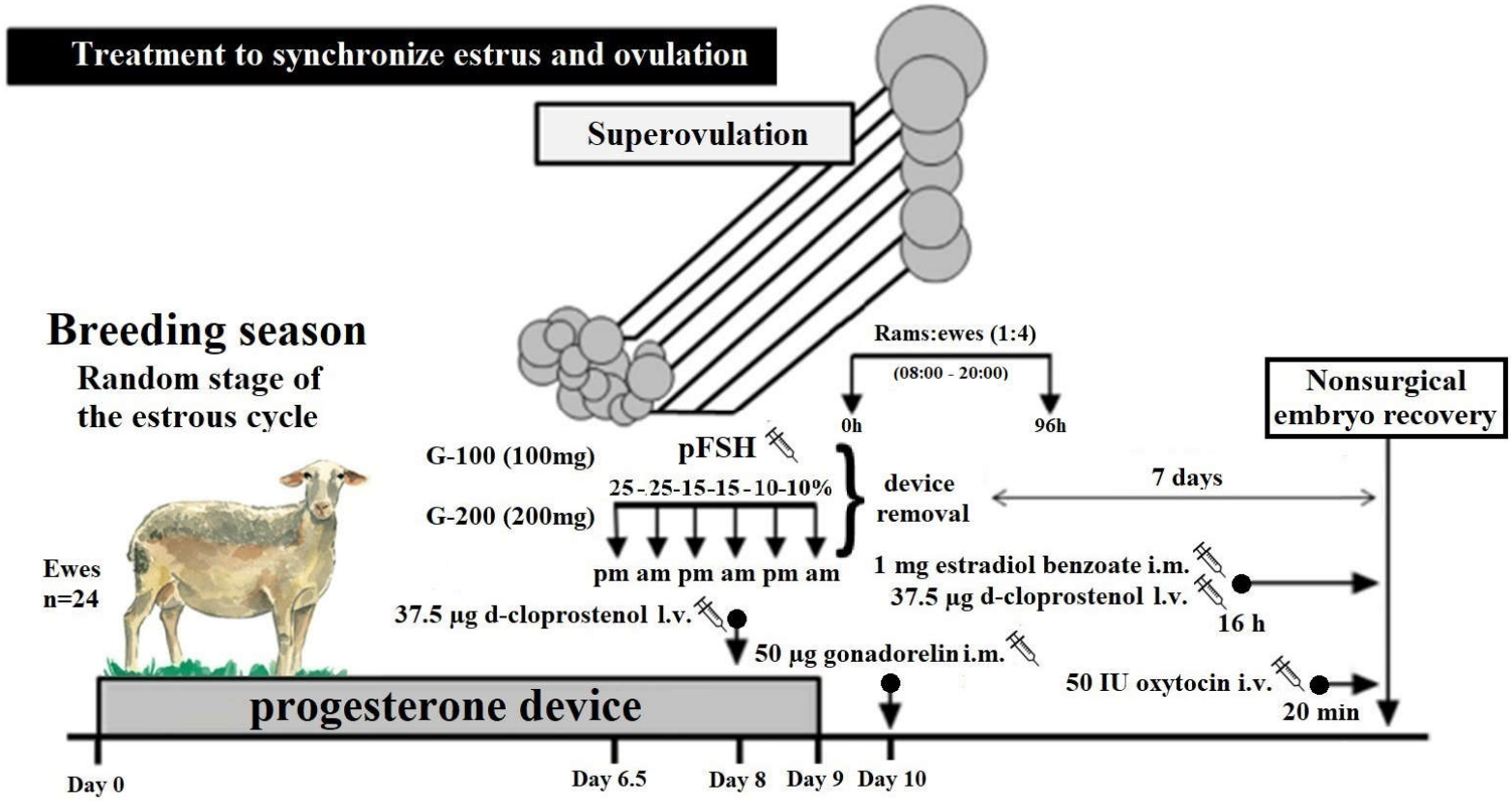

Fig. 1. Schematic representation of the procedures used in a crossover experimental design (two replicates with 60-days apart) to assess different superovulatory protocols with 100 (G-100) and 200 (G-200) mg of pFSH and the non-surgical embryo recovery (NSER, after cervical dilation treatment*) performed seven days after the device removal in Lacaune ewes; i.m. intramuscular; i.v. intravenous; l.v.: laterovulvar.

2.6. Statistical analyses

The data were analyzed using SAS software (Statistical Analysis System®, version 9.3, SAS Inst., Inc., Cary, NC, USA).The statistical models to study the responses from crossover experimental design were similar to proposed by Kaps and Lamberson (2017). The models included order (sequence of applied treatments), treatment (groups) and replicate as fixed effects and ewe nested in order as random effect.

Quantitative data as estrus duration, interval from device removal to estrus, time of cervical penetration, and total time of procedure were analyzed by generalized linear models, with Gamma distribution and log link function using GLIMMIX procedure. The numbers of CL, LAF, total follicles, and follicles according to the diameter categories (≤ 3.0 mm, 3.1-4.0 mm, 4.1-5.0 mm, > 5.0 mm), recovered structures, and viable embryos were also analyzed by using GLIMMIX procedure, but considering Poisson distribution and log link function. The relationship between variables was assessed by using the Pearson´s correlation.

Qualitative data as ewes bearing follicles > 5.0 mm and ewes bearing CL at the beginning of pFSH treatment, and viable embryos rate were analyzed as using GLIMMIX procedure, with binomial distribution and logit link function. The variables as donors in estrus, percentage of responding donors (≥ 3 CL), transcervical penetration rate, successful recovery rate, and structures recovery rate were analyzed by Chi-square test.

Results are shown as means ± standard error of the means (MEAN ± SEM). Differences were considered significant as $P < 0.05$.

3. Results

There was no difference ($P > 0.05$) in the percentage of ewes bearing CL or follicles > 5.0 mm at the beginning of the pFSH treatment between groups (Table 1). The total number of follicles and follicular count in each diameter category (≤ 3.0 mm, 3.1-4.0 mm, 4.1-5.0 mm, > 5.0 mm) did not differ ($P > 0.05$) between groups at the time of the first pFSH injection (Fig. 2). At the time of the second pFSH injection, there were a greater number of follicles of size 3.1-4.0 mm in the G-200 group, without differences in the other follicular diameter categories ($P > 0.05$) between groups.

Table 1
Ovarian activity (MEANS ± SEM) at the first and second injections of 100 (G-100) or 200 (G-200) mg of pFSH* (six decreasing doses, starting 60 h before device removal) during progesterone-based protocol for nine days in Lacaune ewes, in a crossover experimental design with two replicates.

Parameters	Groups		P-value
	G-100 (*n*=24)	G-200 (*n*=24)	
First pFSH injection			
Total number of antral follicles	10.9 ± 0.9	11.2 ± 0.9	0.80
Ewes bearing follicles > 5.0 mm (%)	62.5 ± 10.1	45.8 ± 10.4	0.23
Ewes bearing corpora lutea (%)	37.5 ± 10.1	66.7 ± 9.8	0.06
Second pFSH injection			
Total number of antral follicles	11.8 ± 1.0	13.5 ± 0.9	0.10
Ewes bearing follicles > 5.0 mm (%)	62.5 ± 10.1	62.5 ± 10.1	0.96
Ewes bearing corpora lutea (%)	37.5 ± 10.1	58.3 ± 10.3	0.16

*Administered in six decreasing doses (25, 25, 15, 15, 10 e 10 %) starting at 60 h (first dose) and 48 h (second dose) before device removal.
[ab]Denotes significant differences between treatments (P < 0.05).

The percentage of ewes in estrus was higher (P < 0.05) in G-200 compared to G-100 group (Table 2). There was a group effect (P < 0.05) for estrus duration, with higher values observed in G-200 group. Greater responsiveness of donors (≥ 3 CL) to the superovulatory protocol was observed with the 200 mg dose. Thus, there were higher (P < 0.05) numbers of luteinized structures, CL and LAF in G-200 compared to G-100 group. However, the LAF rate was higher (P < 0.05) in the G-100 compared to G-200 group.

Table 2
End points (MEANS ± SEM or %) of estrous behavior and ovarian response in Lacaune ewes treated with 100 (G-100) or 200 (G-200) mg of pFSH* during progesterone-based protocol for nine days, in a crossover experimental design with two replicates.

Parameters	Groups		P-value
	G-100 (n=24)	G-200 (n=24)	
Percentage of ewes in estrus (%)	66.7 (16/24)	100.0 (24/24)	<0.01
% of responding donors (with ≥ 3 CL)	45.8 (11/24)	91.7 (22/24)	0.02
Interval from device removal to estrus (h)	31.5 ± 1.9	26.5 ± 1.6	0.07
Estrus duration (h)	24.0 ± 3.4	32.0 ± 2.5	0.03
Luteinized structures (n)	4.2 ± 0.8	13.8 ± 1.3	<0.01
Corpora lutea (n)	2.6 ± 0.7	11.6 ± 1.2	<0.01
Luteinized anovulatory follicles (n)	1.4 ± 0.3	2.2 ± 0.6	0.04
% of luteinized anovulatory follicles	34.0 (34/100)	16.0% (53/331)	<0.01

*Step-down – six decreasing doses (25, 25, 15, 15, 10 and 10 %), starting 60 h before device removal.
[ab]Denotes significant differences between groups (P < 0.05).

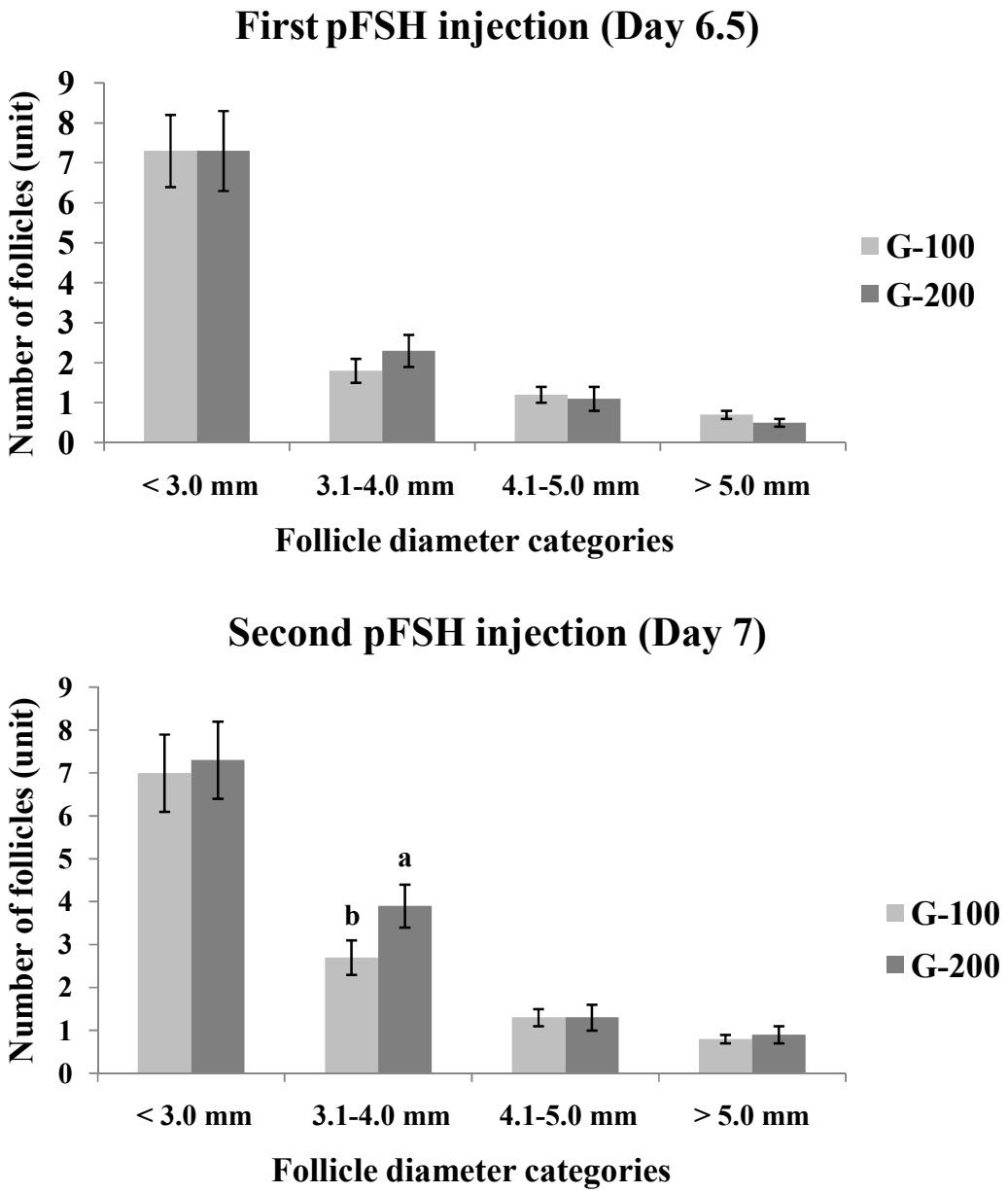

Fig. 2. Follicular counts (MEANS ± SEM) grouped by diameter categories (≤ 3.0 mm, 3.1– 4.0 mm, 4.1 –5.0 mm, > 5.0 mm) at the time of the first (Day 6.5) and second injection (Day 7) of pFSH treatment (six decreasing doses -25, 25, 15, 15, 10 and 10%, at every 12 h) with either 100 (G-100, light gray) or G-200 (G-200, dark gray) mg of pFSH during progesterone-based protocol for nine days to estrus synchronization in Lacaune. [ab] Denotes significant differences between groups ($P < 0.05$).

Transcervical penetration and uterine flushing were successfully performed in a total of 82% (32/39) of the ewes and did not differ ($P < 0.05$) between groups (Table 3). The transcervical penetration rate in ewes with CL count > 5 (95.4% - 21/22) was higher ($P < 0.05$) than in ewes with CL count ≤ 5 (40% - 6/15). Although the number of cervical rings did not differ ($P > 0.05$) between G-100 and G-200, the time of transcervical penetration was longer in the G-100 than in G-200. The total time of NSER procedure was similar ($P > 0.05$) between groups. There was no correlation between the number of CL with the time of cervical penetration ($r = -0.16$; $P > 0.05$). Both structures recovery rate and successful recovery rate were lower ($P < 0.05$) in G-100 group. The numbers of recovered structures, morulae, blastocysts, unfertilized oocytes, viable embryos, and freezable embryos were higher ($P < 0.05$) in G-200 than in G-100.

Table 3
End points (MEANS ± SEM or %) of transcervical penetration and embryo yield at non-surgical embryo recovery in Lacaune ewes treated with 100 (G-100) or 200 (G-200) mg of p-FSH* during progesterone-based treatment for nine days, in a crossover experimental design with two replicates.

	Groups		
Parameters	**G-100**	**G-200**	**P-value**
Transcervical penetration (%)	73.3 (11/15)	90.9 (20/22)	0.13
Cervical rings (n)	7.1 ± 0.3 (11)	7.6 ± 0.3 (20)	0.83
Time of transcervical penetration (min)	7.0 ± 2.0 (11)	3.9 ± 0.4 (20)	<0.01
Total time of procedure (min)	31.0 ± 2.2 (11)	26.3 ± 1.0 (20)	0.14
Corpora lutea in ewes collected (n)	4.6 ± 1.1 (11)	12.8 ± 1.1 (20)	<0.01
Successful recovery[c] (%)	63.6 (7/11)	100.0 (20/20)	0.01
Number of recovered structures	1.3 ± 0.5 (11)	8.7 ± 1.1 (20)	<0.01
Structures recovery[d] (%)	27.6 (14/51)	67.8 (173/255)	<0.01
Unfertilized oocytes (n)	0.2 ± 0.2 (11)	1.7 ± 0.8 (20)	<0.01
Morulae (n)	0.5 ± 0.4 (11)	4.0 ± 1.1 (20)	<0.01
Blastocysts (n)	0.5 ± 0.2 (11)	3.1 ± 0.8 (20)	<0.01
Viable embryos (n)	1.0 ± 0.5 (11)	6.9 ± 1.1 (20)	<0.01
Freezable embryos (n)	1.0 ± 0.5 (11)	6.5 ± 1.0 (20)	<0.01
Viable embryos[e] (%)	78.5% (11/14)	79.8 (138/173)	0.97

* Step-down – six decreasing doses (25, 25, 15, 15, 10 and 10 %), starting 60 h before device removal). ( ) Number of animals.
[ab] Denotes significant differences between groups ($P < 0.05$).
[c] Successful recovery rate: (number of ewes with at least one structure recovered / number of ewes in which the cervix was completely transposed for uterine flushing) x 100.
[d] Structures recovery rate: (Total number of recovered structures / total number of corpora lutea) x 100.
[e] Viable embryos rate: (Number of embryos Grade 1, 2 or 3 per ewe / number of recovered structures per ewe) x 100.

4. Discussion

At the beginning of the superstimulatory treatment there were no differences in ovarian conditions regarding follicular counts and presence of presumably dominant follicles and / or CL. It means that ewes were in similar ovarian conditions in both groups. However, 12 h after the beginning of treatment it was possible to observe significant difference in the count of 3.1-4.0 mm follicles, with higher superovulatory response and embryo yield observed in G-200 group. This fast-ovarian follicular change suggests that the reduced dose (100 mg) was less effective in prompt recruiting small antral follicles than the 200 mg of pFSH. Was it an initial important index for bad (100 mg of pFSH) or good (200 mg of pFSH) superovulatory response? In fact, it was reported that when a low FSH dose is administered a decrease in follicular recruitment may occur, reducing the ovulation rate and consequently the number of viable embryos (Boscos et al., 1997). Moreover, in a previous study a positive correlation was observed between the numbers of medium-size (4.0 mm in diameter) antral follicles, detected 12 h after the first pFSH injection, and the number of viable embryos (Bartlewski et al.,2008).

In G-200 group there was longer estrus duration compared to the G-100 group. It is likely that the highest dose of pFSH stimulated higher follicular recruitment and secretion of 17β-estradiol, which is the hormone responsible to promote estrous behavior. In support of that thought, it has been reported a positive correlation between serum 17β-estradiol concentrations at the time of the second pFSH injection and the number of luteal structures before collection (Bartlewski et al., 2008). In superovulated Dorper ewes, differences were detected on the estrous behavior following superovulatory treatment comparing 128 or 200 mg of pFSH (Loiola Filho et al., 2015). These authors observed that the ewes treated with higher pFSH dose (200 mg) had earlier estrus expression than those treated with lower dose (128 mg). Furthermore, the higher number of LAF could also be associated with this longer duration of estrus, since anovulatory structures can present in some cases a higher secretion of estrogens (Okada et al., 2000; Veiga-López, et al., 2006).

Although a higher number of LAF was found in the G-200 group that had higher total superovulatory response, there was a higher LAF rate in G-100 group. This demonstrates that the lower dose was insufficient to recruit high number of follicles and also did not allow the adequate follicular development and ovulation. Not only FSH doses but also other factors may be related to the occurrence of LAF (Rodriguez et al., 2019). According to these authors the variability in the release mode of LH secretion in superovulated sheep may be one of these

factors. In Santa Inês ewes the LH supplementation at the end of superovulatory treatment improved the frequency of ewes with high superovulatory responses (11 or more CL) and decreased the percentage of LAF relative to the total luteal structures (Oliveira et al., 2012). However, no effect was observed on the ovulation rate in the Manchega, Churra and Merino breeds treated with LH at the end of the superovulatory treatment (Picazo et al., 1996). Oliveira et al. (2012) suggested that the effects of changes in the FSH / LH ratio at the end of superovulatory treatment on the ovulation rate and embryo yield are controversial and appear to be related to intrinsic variability of breed. Therefore, under the experimental conditions of the present study, the application of GnRH as ovulation inductor seems to have been a favorable effect in Lacaune ewes treated with 200 mg of pFSH, because despite higher number of LAF accompanied by estrus duration longer, there was a lower LAF rate.

The greater superovulatory response in G-200 coincided with less difficulty in the cervix transposing. The structures recovery rate was higher in G-200 than in G-100 group (67.8 vs 27.6), and was similar to 65% reported by other authors performing NSER in superovulated animals (Barry et al., 1990; Mylne et al., 1992). With a higher superovulatory response, there is a greater chance of recovering at least one structure. In the current study, 100% of ewes had recovered at least one structure in G-200. In non-superovulated Lacaune ewes this rate ranged from 46 to 89% (Figueira et al., 2019). When cervical penetration rate was stratified by ewes that had CL count ≤ 5 or > 5 better results of structures recovery rate were observed in sheep with higher superovulatory response. Therefore, the data of present study corroborate with prior observations of our teamwork, suggesting that adequate luteal function seems very important for successfully NSER accompanied by the cervical dilation treatment with d-cloprostenol, benzoate estradiol and oxytocin (Fonseca et al., 2019a; Figueira et al., 2019a). Although the design of the present study does not allow elucidating the mechanisms involved, the low dose of FSH to ovarian superstimulatiion aimed at NSER may have limited embryo recovery efficiency. The blood progesterone concentration may not be directly related to the number of luteal structures present in ovaries after superovulatory treatment (Fuerst et al., 2009). Moreover, according to these authors, the supraphysiological progesterone concentration does not seem to be related to the embryo yield neither to embryo quality in ewes. Thus, it is possible that other hormones may play a major role, such as oxytocin. Further study is needed to determine how much the recovery efficiency of the NSER is affected by the number of ovulations and endogenous hormones in a specific breed.

The structures recovery rate in G-200 group (68%) was slightly lower than rates reported by laparotomy recovery in the same breed (77%, Torres et al., 1987) and in Santa

Ines breed (77%, Oliveira et al., 2014), but comparable to observed in Corriedale (Simonetti et al., 2008) and Dorper (Loiola Filho et al., 2015) breeds. The number of viable embryos obtained was comparable to world average of 6.3 embryos/flush (IETS, 2019). Therefore, the embryo yield obtained with protocol G-200 and NSER can be considered good. It was reported similar production of viable embryos (6.9 embryos) in Lacaune ewes submitted to long-term progestogen-based protocol (13.5 d) and superovulatory treatment with 16 mg Armour equivalent of pFSH plus 500 IU of eCG (Torres et al., 1987). These authors did not discriminate whether the animals were lactating or not and they have used surgical collection. The possibility of successive collections in a short period of time increases the potential for offspring generation with the use of NSER, with lower risks of sequel to future fertility of female when compared to surgical collections (Fonseca et al., 2019b). From the qualitative point of view, the viability of the embryos produced in this study was evaluated after they were submitted to cryopreservation by different techniques (slow freezing and vitrification). Reasonable survival and pregnancy rates after fixed-time transfer could be achieved with the slow freezing protocol, demonstrating that it is possible to conduct NSER-based MOET programs and embryo cryopreservation (Figueira et al., 2019b).

Finally, we demonstrated that the adjustment of duration of the progestogen protocols to mid-term (9-days) in synchronous estrus induced (Figueira et al, 2019) and superovulated Lacaune ewes (Figueira et al., 2018) plus to customization of the pFSH doses brings progressive increases in the structures recovery rates and embryo yield after NSER.

5. Conclusions

The dose of 100 mg pFSH appeared to be insufficient to reach a high superovulation response while 200 mg resulted in good superovulatory response and embryo yield after NSER. Furthermore, good structures recovery rate was observed only in the group with good superovulatory response. The association of G-200 protocol and NSER has potential to increase the generation of offspring in Lacaune ewes of high genetic merit in MOET programs.

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ARTICLE 6 - Pregnancy rate after fixed-time transfer of cryopreserved embryos collected by non-surgical route in Lacaune sheep

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ARTICLE 6 - Pregnancy rate after fixed-time transfer of cryopreserved embryos collected by non-surgical route in Lacaune sheep

Lucas Machado Figueira[1, 2], Nadja Gomes Alves[1], Ribrio Ivan Tavares Pereira Batista[2], Viviane Lopes Brair[2], Renato Ribeiro de Lima[1], Maria Emilia Franco Oliveira[3], Jeferson Ferreira da Fonseca[4], Joanna Maria Gonçalves Souza-Fabjan[2]

[1]Universidade Federal de Lavras, Minas Gerais, Brazil

[2]Faculdade de Veterinária, Universidade Federal Fluminense (UFF), Niterói, Brazil

[3]Universidade Estadual Paulista "Júlio de Mesquita Filho (UNESP), Jaboticabal, Brazil

[4]Embrapa Caprinos e Ovinos, Núcleo Regional Sudeste, Coronel Pacheco, Brazil

ABSTRACT

This study investigated the feasibility of applying fixed-time (cryopreserved) embryotransfer in ewes. Embryos (n = 106) were non-surgically recovered from superovulated donors (n = 39) on day 6–7 after oestrus. Straws containing one or two embryos (morulae and/or blastocysts) subjected to either slow freezing (SF, n = 62) or vitrification (VT, n = 44) were randomly used within fixed-time embryo transfer on Day 8.5. Recipient ewes were nulliparous (n = 58) bearing corpora lutea after synchronous oestrous induction protocol. The pregnancy rate was higher (p = .03) in SF (39.4%) than VT (16.9%) and survival rate tended (p = .08) to be higher in SF than in VT (25.8% vs. 15.9%). Lambing rates were similar (p = .13) between SF (20.9%) and VT (15.9%). Embryos recovered by non-surgical route after cervical dilation treatment and later cryopreserved by either slow freezing or vitrification produced reasonable pregnancy rates after FTET.

KEYWORDS: embryo cryopreservation, multiple ovulation and embryo transfer, ovine

1. INTRODUCTION

Non-surgical embryo recovery (NSER) by cervical route has been proposed to overcome the limitation of successive laparotomy procedures beyond animal welfare concerns. However, hormonal treatments for cervical dilation are necessary to produce efficient NSER. Our team's use of d-cloprostenol, estradiol benzoate and oxytocin has led to the achievement of promising cervical transposition and uterine flushing results in Brazilian native and Lacaune breeds (Fonseca et al., 2019). Previous studies demonstrated that some of the hormones used for cervical dilation (PGE2 and estradiol) did not inhibit embryo development or metabolism (McKelvey et al., 1997) and had no effect on foetal viability and growth after the fresh embryos were transferred (Mylne, Dingwall, King, McKelvey, & Robinson, 1999). However, the viability of these embryos following different cryopreservation techniques has yet to be evaluated. Thus, this study aimed to assess the survival capacity of non-surgically recovered and cryopreserved (vitrification or slow freezing) ovine embryos after fixed-time transfer.

2. MATERIALS AND METHODS

2.1. Experimental conditions

This research was approved by the Animal Care Committee of Embrapa Dairy Cattle (#2512100516/2016 – ATTACHMENT A). The experiment was performed during the breeding season on a commercial farm in Soledade de Minas (latitude 22°3′ S). Animals were kept in collective pens and received a diet of two meals with mineral salt and water *ad libitum*.

2.2. Non-surgical embryo recovery and cryopreservation

Lacaune donors (n = 39; 68.3 ± 6.7 kg of body weight, 3.5 ± 0.2 of body condition score and 130.4 ± 5.4 days in milk) were superovulated and mated. Embryos were recovered by transcervical method (Fonseca et al., 2019) 6–7 days after oestrus. Cervical dilation treatment was based on 1 mg estradiol benzoate (Sincrodiol®, OuroFino) and 37.5 µg d-cloprostenol (Prolise®, Tecnopec) i.m. at 16 hr and 50 IU oxytocin (Ocitocina forte®, UCB, Brazil) i.v. 20 min before NSER. Embryo

morphology was classified according to the IETS. Grade I and II embryos randomly underwent either slow freezing (SF; Fonseca et al., 2018) or vitrification (VT; Gibbons, Bruno-Galarraga, Fernandez, Gonzalez-Bulnes, & Cueto, 2019). The percentage of morulae and blastocysts transferred did not differ ($p < .05$, chi-square test) in SF (48.4%–30/62 and 51.6%–32/62) and VT (36.4%–16/44 and 63.6–28/44) groups, respectively.

2.3. Preparation and evaluation of recipients

Recipients received intravaginal sponges for 6 days (60 mg medroxyprogesterone acetate, Progespon®, Zoetis), and 200 IU eCG (Folligon 5000 IU®, Intervet) i.m. and 37.5 μg d-cloprostenol (Sincrocio®, OuroFino) i.m. was administered 24 hr before sponge removal (Day 0). Transrectal ultrasonography (M5VET®, Mindray– 8.0 MHz) was performed 24 hr before embryo transfer to detect the number and size of the corpora lutea (CL).

2.4. Fixed-time embryo transfer

Embryo transfer was performed using the semi-laparoscopic technique on Day 8.5 to the horn *ipsilateral* to the CL. After 24 hr fasting, recipients that presented at least 1 Cl (92%; 58/63) received one or two cryopreserved embryos (morulae and/or blastocysts)(Fonseca et al., 2018). Pregnancy rate (number of pregnant recipients/number of recipients receiving embryos) and embryo survival rate (number of embryos with heartbeat/number of embryos transferred) were recorded by ultrasonography on Day 31. Lambing rates (number of lambs born/number of embryos transferred) were also assessed.

2.5. Statistical analyses

Data of pregnancy, survival and lambing rates were analysed using SAS® program. The PROC GLIMMIX with binomial distribution and logit link function was used. The model for pregnancy rate included cryopreservation technique, type of transferred straw (1: one morula, 2: one blastocyst, 3: two morulae 4: two blastocyst and 5: morula and blastocyst) and number of CL as fixed effects. Values of $p < .05$ were

considered significant and $p < .10$ a tendency. Data are presented as percentage and mean ± *SEM*.

3. RESULTS

The number of CL per recipient was 1.3 ± 0.1 in SF and 1.4 ± 0.1 in VT, and the number of embryos transferred was 1.9 ± 0.1 in SF and 1.8 ± 0.1 in VT. Regardless of the cryopreservation technique employed, the embryos that were recovered by non-surgical method after cervical dilation treatment established and sustained pregnancy (Table 1). Lambing rates were similar ($p > .05$) between treatments; however, transfer of embryos from the VT treatment had lower ($p < .05$) pregnancy and tended ($p = .08$) to have lower survival rate.

TABLE 1
Pregnancy, survival and lambing rates after transfer of embryos recovered by transcervical route and cryopreserved by either slow freezing or vitrification.

Cryopreservation technique	Pregnancy (%)	Survival (%)	Lambing (%)
Slow Freezing	39.4 (13/33)	25.8 (16/62)	20.9 (13/62)
Vitrification	16.0 (4/25)	15.9 (7/44)	15.9 (7/44)
Average/total	29.3 (17/58)	21.7 (23/106)	18.8 (20/106)
p-value	.03	.08	.13

Note: Fixed-time embryo transfer was performed on Day 8.5 after sponge removal; Pregnancy: number of pregnant ewes/number of recipient ewes receiving embryos; Survival: number of embryos with heartbeat/number of transferred embryos; Lambing: number of lambs born/number of transferred embryos.

4. DISCUSSION

For the first time, we demonstrated that ovine embryos recovered by NSER (after exogenous hormonal cocktail) and subjected to cryopreservation can establish reasonable rates of pregnancy. The overall survival efficiency after cryopreservation was 21.7%, and VT group had lower pregnancy rate and tended to have lower survival rate than SF group. The survival rate of in vivo-derived cryopreserved embryos by vitrification technique described in the existing literature is 32.0%–36.0% (Folch, Olivera Muzante, & Aguilar Gomez, 2000), 41.2%–50.0% (Gibbons, Cueto, & Pereyra-Bonnet, 2011) or 60.1%–75.1% (Dattena et al., 2004). In the present study, the VT embryo survival rate was 15.9%, which is similar to the 22.6% rate that was recently reported for embryos recovered by laparotomy (Gibbons et al., 2019). However, our lambing rates (18.8%) were lower than the 62.9% rate observed by

Dattena, Ptak, Loi, and Cappai (2000) and the 80.0% rate recorded by Naitana et al., (1995). One potential explanation for this discrepancy is that these authors only transferred blastocysts that re-expanded after vitrification/rewarming.

Similar pregnancy rates were obtained in the current study on SF embryos (39.4%) as those achieved in a previous study in which embryos were surgically recovered (34.8%; Green, Santos, Sicherle, Landim-Alvarenga, & Bicudo, 2009). Establishment of pregnancy in ruminants depends on optimal interaction between the developing embryo-conceptus and the maternal uterine environment (Randi et al., 2016). Early-stage embryo-transfer studies in sheep (Naitana et al., 1995) and cattle (Rowson, Lawson, Moor, & Baker, 1972) have demonstrated the need for a close synchrony (±24 hr) between the donor and recipient, relative to oestrous onset. Pregnancy rates following the transfer of Day 8 bovine embryos to asynchronous (Day 5) or synchronous (Day 8) recipients were 4.8% and 61.1%, respectively, highlighting the low tolerance for an out-of-sync uterine environment (Geisert et al., 1991). The current study employed the FTET technique; this frequently does not take into account oestrous behaviour and ovulation, resulting in lower pregnancy rates in comparison with when oestrous behaviour and ovulation are assessed. This negative effect of asynchrony between recipient and embryo transfer appears to predominantly impact the VT embryos.

In conclusion, embryos recovered by non-surgical method after cervical dilation treatment and later cryopreserved are able to sustain a pregnancy after FTET, regardless of the cryopreservation technique used.

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ATTACHMENT A

Embrapa Gado de Leite

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que o Projeto intitulado "Estudo de pontos estratégicos para a elevação da eficiência da superovulação, colheita, criopreservação e transferência de embriões em ovinos", protocolado sob o CEUA nº 2512100516, sob a responsabilidade de **Jeferson Ferreira da Fonseca** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Embrapa Gado de Leite (CEUA/EGL) na reunião de 30/06/2016.

We certify that the proposal "Study of strategic points for increasing efficiency of superovulation, embryo recovery, cryopreservation and transfer in sheep", utilizing 30 Ovines (30 females), protocol number CEUA 2512100516, under the responsibility of **Jeferson Ferreira da Fonseca** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Embrapa Gado de Leite Corporate (CEUA/EGL) in the meeting of 06/30/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2016 a 03/2020 Área: Núcleo Produção E Bem Estar Animal

Procedência:	Campo Experimental José Henrique Bruschi						
Espécie:	Ovinos	sexo:	Fêmeas	idade:	02 a 06 anos	N:	30
Linhagem:	Santa Inês			Peso:	30 a 50 kg		

Resumo: Este projeto reflete a ação de um grupo de pesquisadores dedicados ao estudo dos fenômenos relacionados ao controle e manipulação da reprodução em caprinos e ovinos e à transferência de tecnologias associadas. Representa uma sequência de estudos finalizados ou em execução que tem abordado a superovulação, colheita, criopreservação e transferência de embriões em pequenos ruminantes apoiados por FAPESP, FAPERJ, FAPEMIG e Embrapa. Estima-se que 100% dos embriões ovinos produzidos in vivo no mundo sejam recuperados por procedimentos cirúrgicos. Tais procedimentos incorrem em riscos a vida do animal (anestesia e cirurgia), jejum prolongado, sequelas cirúrgicas (aderências), entre outros fatores que limitam a expansão de uso repetido de doadoras. Já há proibição em alguns países com relação a este método e, a exemplo do que ocorreu com bovinos, estima-se que os procedimentos não cirúrgicos substituam cada vez mais os cirúrgicos. Esta sequência de projetos citados propõe uma mudança para recuperação não cirúrgica de embriões. Uma vez identificada a dinâmica folicular associada a protocolos de superovulação na raça Santa Inês (FAPESP - 2008/05503-2), um novo desafio é o estudo de diferentes doses superovulatórias. Isto agora será possível por meio de procedimentos não cirúrgicos (ultrassonografia em modo Doppler e Colheita não cirúrgica de embriões). Diante da definição da melhor dose superovulatória, o desafio seguinte é a investigação do método e protocolo de acasalamento mais viável para fêmeas doadoras de embriões. Os resultados do projeto 03.12.01.031.00.02 tornaram viável a recuperação transcervical de embriões na raça santa Inês. Seguindo-se as recomendações baseadas nos resultados deste projeto, pode-se chegar a até 80% de sucesso de lavado não cirúrgico, fazendo que em apenas 20% dos animais fosse necessária a coleta cirúrgica. Ao final deste projeto, um piloto com quatro ovelhas da raça Morada Nova obteve 100% de sucesso de lavado uterino pela via transcervical. Esta possibilidade foi apontada como uma das consequências ou perspectivas do projeto. No novo formato proposto, além melhora do protocolo de relaxamento cervical em ovelhas Santa Inês, pretende-se estender abrangência para as raças Somalis, Morada Nova, Pantaneira e Lacaune. Face à expectativa de quantidade considerável de embriões a ser produzida, criou-se a possibilidade de trabalhar com protocolos de criopreservação. Como última escala de trabalho proposta, embriões criopreservados ou não poderiam ser transferidos para receptoras. Assim, o estudo de características das receptoras que podem interferir na taxa de gestação destes embriões é também necessário. Um piloto apontou para a possibilidade de incrementar o número de corpos lúteos e a progesterona plasmática de ovelhas nulíparas. Este novo projeto propõe a amplificação deste estudo. Os resultados darão suporte de conhecimento a respeito da eficiência e viabilidade da técnica de coleta embrionária não-cirúrgica transcervical em ovelhas de várias raças de interesse nacional. Conjuntamente, os resultados podem consolidar o Brasil como a maior referência mundial sobre produção in vivo de embriões ovinos recuperados por via não cirúrgica, abrindo perspectivas para aplicação em outras raças ovinas que não aquelas abordadas na proposta.

Local do experimento: Setor de Caprinos e Ovinos - Campo Experimental José Henrique Bruschi

Rua Eugênio do Nascimento, 610, Dom Bosco, Juiz de Fora, Minas Gerais, CEP 36038-330 - Sede da Embrapa Gado de Leite - tel: 55 (32) 3311-7496 / fax: 55 (32) 3311-7401
Horário de atendimento: 2ª a 6ª das 8:00h ás 12:00h : e-mail: cnpgl.ceua@embrapa.br
CEUA N 2512100516