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Nonsurgical Embryo Recovery as a Feasible Tool for Supporting Embryo Biobanks of Locally Adapted Brazilian Sheep and Goats

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This study assessed the outcomes of nonsurgical embryo recovery (NSER) after superovulation (SOV) in five locally adapted Brazilian breeds of sheep and goats. The objective was to evaluate the feasibility and efficiency of using SOV combined with a less-invasive embryo collection technique for supplying the Brazilian animal gene bank with germplasm from specific genotypes of interest. Morada Nova ($n = 20$), Santa Inês ($n = 20$), and Somalis ($n = 20$) ewes received an intravaginal progesterone (330 mg) device for 9 days, while Canindé ($n = 15$) and Moxotó ($n = 15$) goats received an intravaginal medroxyprogesterone acetate (60 mg) device for 6 days. All females received 133 mg of porcine follicle-stimulating hormone (pFSH) administrated in six decreasing doses 12 hours apart, starting 60 hours before device removal, plus 37.5 μg of D-cloprostenol at the fifth and sixth pFSH dose. Donors in estrus were mated with fertile males. The corpora lutea (CL) number was assessed by ultrasonography 1 day before NSER. On day 6.5 or 7 after estrus, NSER was performed following hormonally induced cervical relaxation. A total of 97% of sheep and 90% of goats responded with estrus, and among those, 91% of sheep and 85% of goats presented a CL. In ewes, the numbers of CL were greater ($p < 0.05$) in the Santa Inês breed, while similar ($p > 0.05$) CL numbers were found among the goat breeds. All viable embryos were freezable (excellent and good quality) and the number per donor was 7.8 for sheep and 4.9 for goats. All parameters of NSER efficiency, embryo yield, and fertility post-NSER did not differ ($p > 0.05$) between breeds among each species. The SOV-NSER procedures applied for an embryo biobank supply of locally adapted Brazilian breeds of small ruminants were efficient regarding production of cryopreservable embryos, and preservation of donor fertility. Therefore, SOV followed by NSER is recommended for embryo biobank assembly in sheep and goats.

Keywords: caprine, ovine, transcervical, cryopreservation, genetic resources conservation, superovulation

Introduction

Since the beginning of the 16th century, Brazil has been introduced to many foreign domestic species for provision of food to colonizers, during expedition trips. Sheep and goats were examples of those animals. They readily adapted to the new environment and disseminated from the northeast coast to the inland toward the west and south. From that time, some desirable genotypes have been (naturally or man-created) selected and bred, originating the distinct locally adapted Brazilian breeds, which remained husbanded until recently. Those breeds survive and are relatively productive, even under tough environmental conditions of high temperatures and low water and food availability.

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The Brazilian northeast region has the highest concentration of those locally adapted small ruminants that include the sheep breeds Morada Nova, Santa Inês, and Brazilian Somalis and the goat breeds Canindé and Moxotó. Among those, only Santa Inês is of current economic interest, while the remaining breeds are raised under subsistence conditions. Those locally adapted breeds of low economic interest are under constant and progressive risk of extinction due to the reduction of their contingent. Thus, to preserve their adapted genotypes that might be of interest, they have been included in the Brazilian Conservation Program and Biobank of the Brazilian Agricultural Research Corporation–EMBRAPA.[1]

Despite the fact that the main priority of conservation initiatives is the preservation of species in their natural habitat, the relevance of gene banks for safeguarding endangered breeds is well recognized, especially considering a potential need of long-term conservation of biodiversity.[2] Embryos are excellent germplasm material for supporting the preservation of pure breeds, enabling a quicker and more efficient production of live animals compared to other sources of genomic material such as oocytes, sperm, or somatic cells.[3] The last report from Food and Agriculture Organization (FAO) of the United Nations, published $\sim$10 years ago, recommended collecting embryos from sheep and goats surgically, as nonsurgical embryo recovery (NSER) was of lower efficiency.[4] However, surgical approaches have many disadvantages such as relatively greater cost of equipment compared to nonsurgical collections. Risks during the trans- or postoperatory stages are of high concern: death, development of adhesions that disturb overall health, and posterior fertility. Limits to the number of embryo collections per animal[5] are critically undesirable in conservation approaches. Furthermore, challenges to animal welfare caused by the procedure itself or its sequelae should be carefully taken into consideration.[6]

The NSER procedure has the cervical penetration as its main limiting step, especially in sheep. In this species, the cervical anatomy differs among breeds.[7,8] Insufficient cervical dilation and anatomical characteristics, such as the high degree of misalignment of the cervical rings and vaginal narrowing (that prevent or hinder visualization, clipping, or retraction of the cervix), are the main caveats of NSER[6,9–11] and have discouraged the use of this technique for embryo recovery in small ruminants. In addition to the possibility of cervix transposition, the success of *in vivo* embryo production strictly depends on understanding follicular development and strategic gonadotropin administration. Studies conducted in sheep[9] and goats[12] revealed that administering the first follicle-stimulating hormone (FSH) dose at 60 hours before device removal yielded good viable embryos recovered by the transcervical route.

Brazil is nowadays in the frontline of producing embryos using NSER in sheep and goats. This latter achievement was attained especially because the major chokepoint of NSER, which is the transcervical access to the uterus, has been overcome.[13] During the last decade, successful NSER has been performed in small ruminant breeds of commercial interest: the ovine Creola,[5] Santa Inês,[14,15] Lacaune,[16,17] and Dorper,[18,19] as well as the caprine Toggenburg, Saanen and Alpine.[20–22] The adapted Morada Nova sheep has also undergone successful NSER.[10] However, data regarding the performance of the locally adapted breeds, Brazilian Somalis, Canindé and Moxotó, for embryo production and NSER are still lacking. Moreover, a simultaneous comparative study between different breeds of the ovine and caprine species has not yet been carried out. It was hypothesized that success in embryo production using superovulation (SOV) and NSER can also be achieved in locally adapted breeds that have not been subjected to a massive genetic selection. For that, their response to superovulatory protocols and the feasibility of the cervix transposition need to be tested.

The present study was designed to compare the efficiency of NSER in different Brazilian locally adapted breeds of sheep (Morada Nova, Santa Inês, and Somalis) and goats (Moxotó and Canindé) submitted, respectively, to 9- and 6-days progesterone-based estrous synchronization protocols and superovulatory treatment with 133 mg of FSH. It was expected that these superovulatory protocols could assure good ovarian stimulation and embryo production and, associated with NSER, could provide a sustainable conjunct of techniques able to support embryo biobank in locally adapted Brazilian sheep and goats.

Materials and Methods

Location

This study was carried out at the Embrapa Goats and Sheep experimental farm in Sobral–CE, in the northeastern region of Brazil. The research unit is located at 83 m altitude, 3°42′ S latitude, and 40°21′ W longitude. The climate is of the savanna type, known as AW according to the Koppen classification. The area has an average annual precipitation of 931.7 mm, an average relative humidity of 69.8%, and an average annual temperature of 28.1°C (22.3°C–34.1°C).

Animals and management

The procedures of this study were approved by the Ethics Committee for the Use of Animals of Embrapa Goats and Sheep (Protocol #006/2016) and were conducted under the principles of the Brazilian Society of Laboratory Animal Science. All animals were treated humanely, and the standards conformed to those of current ethical animal research practices. The donors (sheep, $n = 60$; and goats, $n = 30$) were kept on grazing semiarid systems during the day (0800–1600 hours) and retained in barns during the night, where they received chopped grass and a protein concentrate according to their nutritional demands, targeting a moderate adiposity (or body score condition).[23] Minerals and freshwater were offered *ad libitum*. The general characterization of the experimental animals is presented in Table 1.

Estrous synchronization protocol and superovulatory treatment

All females underwent the following procedure only once. On a random day of the estrous cycle, each ewe received an intravaginal silicon device containing 330 mg of progesterone (EAZI-BREED CIDR®; Zoetis, São Paulo, SP, Brazil) that remained for 9 days, while each doe received an intravaginal sponge containing 60 mg of medroxyprogesterone acetate (MAP, Progespon®; Syntex, Buenos Aires, Argentina) that remained for 6 days. All animals received a superovulatory treatment of 133 mg intramuscular (i.m.) FSH (porcine FSH, Folltropin®; Vetoquinol, Mairiporã, SP, Brazil), administered in six decreasing doses (25%-25%-15%-15%-

TABLE 1. PARAMETERS (MEAN ± STANDARD ERROR OF THE MEAN) RECORDED IN BRAZILIAN NATURALIZED SHEEP (MORADA NOVA, SANTA INÊS, AND SOMALIS BREEDS) AND GOATS (CANINDÉ AND MOXOTÓ) SUBMITTED TO ESTRUS SYNCHRONIZATION, SUPEROVULATORY TREATMENT, AND NONSURGICAL EMBRYO RECOVERY

	Sheep			*Goats*	
Reproductive parameters	*Morada Nova*	*Santa Inês*	*Somalis*	*Moxotó*	*Canindé*
Number of animals	20	20	20	15	15
Age, years	6.0 ± 0.2	5.2 ± 0.3	4.9 ± 0.5	6.0 ± 2.4	5.0 ± 0.6
Parity order[a] (1–8)	3.9 ± 0.2	3.7 ± 0.2	3.6 ± 0.5	3.0 ± 1.8	2.4 ± 0.4
Body weight, kg	33.7 ± 1.0	45.9 ± 1.0	29.0 ± 0.7	28.8 ± 4.9	30.2 ± 1.1
Body condition score[b]	2.7 ± 0.1	2.8 ± 0.5	3.0 ± 0.0	2.8 ± 0.2	2.7 ± 0.0
Postpartum period, days	149.6 ± 0.7	151.9 ± 0.4	146.7 ± 0.4	147.3 ± 2.1	145.7 ± 0.4

[a]Number of parturitions.
[b]Body condition score: 1, emaciated, 5 obese.

10%-10% of the total dose) at 12-hour intervals (0600 and 1800 hours). The superovulatory treatment started on the preestimated moment of ovarian follicle emergence, which was 60 hours before the intravaginal device removal.[9,12] Animals also received two 37.5 μg D–cloprostenol doses (Prolise®; ARSA S.R.L., Buenos Aires, Argentina) concomitantly to the fifth and sixth FSH doses. Estrus was observed after the removal of the progestogen implant (D0), for 30 minutes, in a 12-hour interval, until D4. As an antiluteolytic approach, 1.1 mg/kg body weight of flunixin-meglumine (Banamine®; MSD Saúde Animal, Cruzeiro, Brazil), was administrated i.m. daily from days 3 to 5 after estrus onset, totaling three doses. The schedules for estrus synchronization, SOV, and antiluteolytic treatments are illustrated in Figure 1.

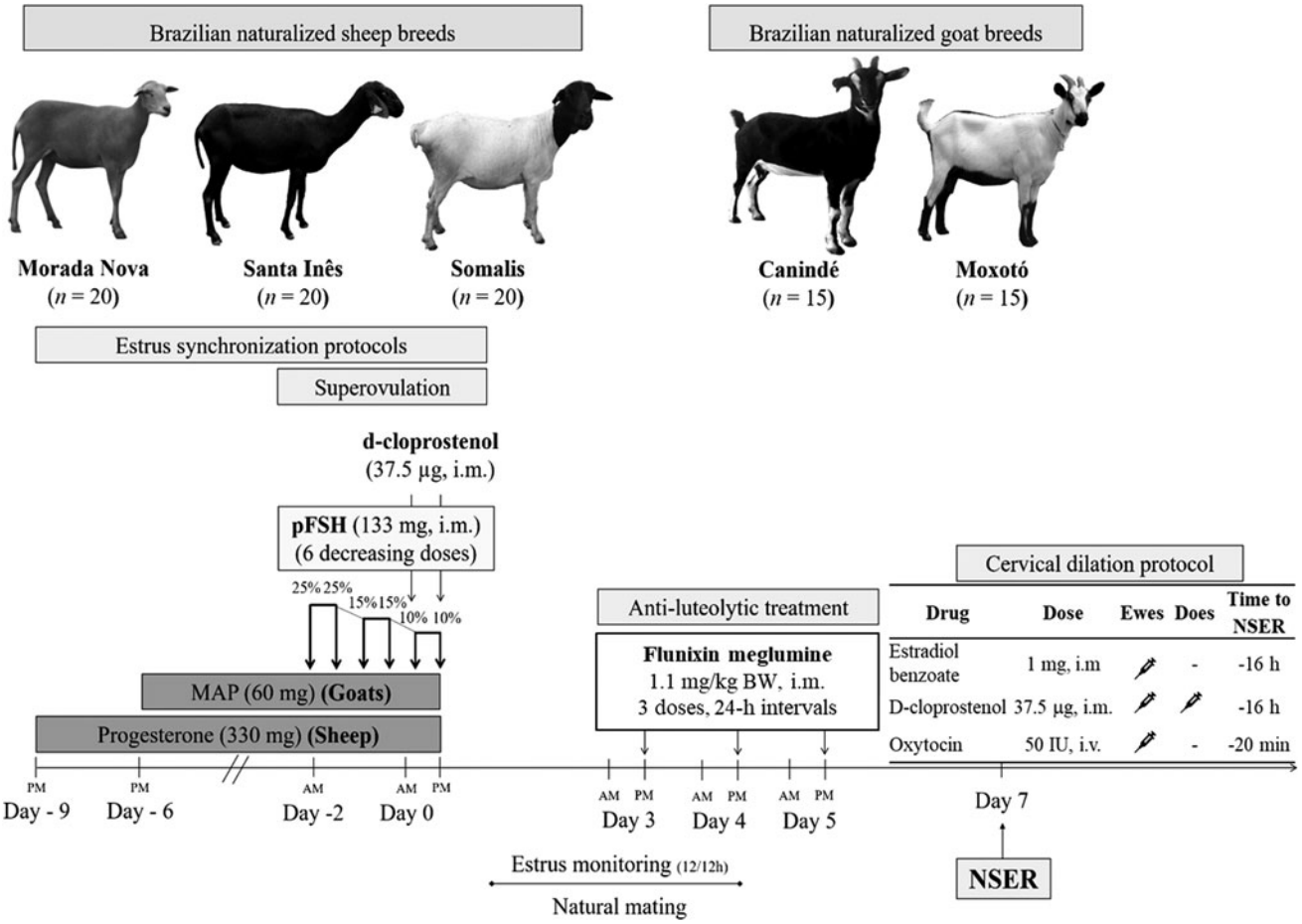

FIG. 1. Schematic representation of the experimental procedures designed to check the efficiency of NSER in different Brazilian locally adapted sheep (Morada Nova, Santa Inês, and Somalis breeds) and goats (Canindé and Moxotó breeds) for supplying Brazilian small ruminant embryo biobanking. All females were subjected to estrus synchronization protocol, superovulatory treatment, and antiluteolytic treatment, followed by cervical dilation protocols for each species. i.m., intramuscular; i.v., intravenous; MAP, medroxyprogesterone acetate (60 mg); NSER, nonsurgical embryo recovery; pFSH, porcine follicle-stimulating hormone.

Mating and superovulatory response evaluation

Upon estrus detection, donors were mated three times at 12-hour intervals. For that, fertile males were kept with donors in a 1:5 ratio (male:female). A preferential breeding schedule[24] was used as follows: in the first mating, females in the beginning of estrus were prioritized. In the second, females previously mated were mated again after the ones in the beginning of estrus, and in the third, the mating order was reversed again for prioritizing females from the first breeding order. This strategy aimed to roughly balance the quantity of sperm received by each donor. Evaluation of the ovaries for corpora lutea (CL) quantification was performed 12 hours before NSER, by B-mode transrectal ultrasonography, using the Z5 Vet® ultrasound equipment (Mindray, China) and a multifrequency transrectal transducer (at 8.5-MHz).

Cervical relaxation, transposing, and NSER

For cervical relaxation, ewes received, 16 hours before NSER, D-cloprostenol (37.5 μg, i.m) and estradiol benzoate (1 mg, i.m., Benzoato HC®; Hertape Calier, Janaúba, Brazil), as well as oxytocin (Ocitocina Forte®; UCB, Jaboticabal, Brazil) 20 minutes before NSER. Does received only D-cloprostenol, 16 hours before NSER (Fig. 1). The NSER procedure was performed on day 7 after intravaginal device removal (6.5–7.0 days after estrus onset). Neither drink nor food restriction was imposed. Before the procedure, all females received the same previously used sedation/analgesia/anesthesia protocol,[25] which consisted of administration of local and epidural lidocaine, as well as acepromazine (i.m) and dipyrone and n-butyl hyoscine bromide (i.m. and intravenous). Cervical immobilization, traction, and transposing were performed as described earlier[13,25] using two Pozzi forceps. A cervical map[25] of each animal was recorded during the Hegar dilator transposing attempt. The whole NSER procedure was performed as previously reported in dairy goats[22] and sheep,[16] and the time taken for cervix penetration and for flushing was recorded. Cervix penetration was attempted for up to 10 minutes. Fluid drained from the uterus was collected into specific filters (Minitubs Emsafe® embryo filter, Landshut, Germany), and recovered ova/embryos were placed in a holding medium (Dulbecco's phosphate-buffered saline plus 0.2% bovine serum albumin), identified using a stereomicroscope (magnification 40×), and classified according to the IETS manual.[26] After selection, freezable embryos were placed into a 1.5-molar Ethylene-Glycol solution for 10 minutes. Then, one embryo was aspirated into the central column of a 0.25 mL French straw, which had lateral columns filled with the same media, and were separated from the central one by air columns. Straws were heat sealed and introduced into a controlled rate freezing machine (Freeze Control CL 5500®; Cryologic, Victoria, Australia) at 20°C. A slow-cooling rate of 3°C/min from 20°C to −6°C was applied. After stabilization for 15 minutes (seeding after 5 minutes), the next cooling rate was 0.6°C/min from −6°C to −32°C when straws were plunged into liquid nitrogen.[27]

Post-NSER fertility

All animals that were submitted to SOV (including the ones that did not respond and were not flushed) received 37.5 μg D-cloprostenol 7 days after the scheduled NSER, for preventing undesirable pregnancy. Eight ewes and three does were culled following the annual substitution program of each herd and did not proceed to the next steps of the experiment. Ninety days after the scheduled NSER, the remaining females were kept with one fertile (previously subjected to breeding soundness evaluation) male, continuously for 30 days. Pregnancy diagnosis was performed 150 days after NSER by ultrasonography and parturition data were recorded. The pregnancy rate was calculated considering dams subjected to SOV (pregnant females after SOV), and considering only the females that were subjected to NSER (pregnant females after NSER).

Data analysis

The following parameters were recorded as outcome variables: estrus response (in %, females in estrus/treated females); estrus onset (in hours, from progestogen implant removal to estrus); estrus duration (in hours, from the first to the last estrus observation); percentage of females with CL 1 day before NSER (in %, females with CL/females in estrus); percentage of successfully flushed females (in %, females flushed/total number of females); number of CL per superovulated female; number of CL per flushed female; duration of the cervical penetration procedure by Hegar or stylet/catheter (in minutes, from Hegar dilator or stylet/catheter insertion to its removal); duration of the uterine flushing procedure (in minutes, from flushing catheter insertion to its removal); duration of NSER procedure (in minutes, from epidural anesthesia to catheter removal/cervical unclipping); fluid recovery efficiency (in %, volume of fluid retrieved/total fluid infused); total number of recovered structures (i.e., embryos and unfertilized eggs) per flushed female; number of unfertilized eggs per flushed female; number of viable embryos (number of recovered embryos with quality score 1–3[26]) per flushed female; number of freezable embryos (number of recovered embryos with quality score 1–2[26]) per flushed female; number of degenerated embryos (number of recovered embryos with quality scored of 4–5[26]) per flushed female; percentage of morulae (in %, number of morulae/number of viable embryos recovered); percentage of blastocyst (in %, number of blastocyst/number of viable embryos recovered); embryo viability rate (in %, embryos with quality score 1–3[26]/total structures recovered); percentage of females that had ova/embryo(s) recovered (in %, females that had at least one structure recovered/females flushed); percentage of females that had viable embryo(s) recovered (in %, females that had at least one viable embryo recovered/females flushed); percentage of females that had more than the average number of viable embryos recovered per flushed female; fertile females (in %, donors that lambed or kidded/total donors previously subjected to SOV or NSER) recorded in all females submitted to SOV and in all females submitted to NSER; and prolificacy (number of fetuses born/total donors that lambed or kidded).

Data regarding the reproductive parameters of different breeds among the same species were submitted to the Shapiro–Wilk test for normality analysis and compared using one-way ANOVA followed by the Tukey HSD test, if normal distribution was confirmed. The Kruskal–Wallis nonparametric test was applied to compare variables that did not have a normal distribution. Significant statistical difference was considered to be $p < 0.05$.

Results

Reproductive parameters of superovulated donors subjected to NSER followed by SOV and natural breeding are presented in Table 2 (sheep) and Table 3 (goats). The 9-day estrus synchronization protocol followed by SOV resulted in a later ($p < 0.05$) estrus onset and a greater ($p < 0.05$) number of CL in Santa Inês ewes compared with Morada Nova and Somalis ewes. Embryo flushing was feasible in every doe that responded to the SOV protocol. Five ewes that responded to SOV could not be penetrated by the catheter, and therefore, flushing was not performed on them. Among the flushed ewes, the number of viable embryos yielded per donor ranged from 1 to 12 in Morada Nova, from 0 to 42 in Santa Inês, and from 0 to 16 in Somalis. Among the does, the number of viable embryos yielded per flushed donor ranged from, respectively, 0 to 6 in Canindé and from 0 to 13 in Moxotó donors. All viable embryos were of excellent (grade 1) or good (grade 2) quality and at the morula (stage 4) or blastocyst (stage 5 or 6) developmental stages.[26] All dams that were determined to be pregnant at 150 days after scheduled NSER lambed or kidded, therefore, fertilization and parturition rates were the same. All parameters regarding NSER efficiency, embryo production, and fertility post-NSER did not differ ($p > 0.05$) among breeds within ewes and goats.

Discussion

One major challenge of the *in vivo* embryo production technique is the selection of the superovulatory protocol that should result in low variability and be of high efficiency. Both protocols used in this study for ovarian stimulation in sheep and goats were based on previous ovarian follicle dynamics studies, accompanied during synchronous estrus induction, in dairy sheep[9] and goats.[12] These studies confirmed

Table 2. Reproductive Parameters (% or Mean ± Standard Error of the Mean) Recorded in Brazilian Locally Adapted Sheep (Morada Nova, Santa Inês, and Somalis Breeds) Receiving Intravaginal Progesterone (330 mg) Device for 9 Days, 37.5 μg of Cloprostenol at 12 Hours Before Device Removal and Superovulatory Treatment (Superovulation) with 133 mg of Porcine Follicle-Stimulating Hormone Administered in Six Decreasing Doses (25%-25%-15%-15%-10%-10%) at 12-Hour Intervals Starting 60 Hours Before Device Removal and Nonsurgical Embryo Recovery 6 to 7 Days After Estrus Onset

Reproductive parameters	*Morada Nova*	*Santa Inês*	*Somalis*	*Total*
Estrus response, %	100.0 (20/20)	95.0 (19/20)	95.0 (19/20)	96.7 (58/60)
Estrus onset, hours	16.8 ± 2.2[b]	23.4 ± 1.1[a]	15.1 ± 1.2[b]	18.4 ± 2.5
Estrus duration, hours	21.6 ± 1.1	22.7 ± 1.3	24.0 ± 0.0	22.8 ± 0.7
Ewes with corpora lutea 1 day before NSER, %	80.0 (16/20)	100.0 (19/19)	94.7 (18/19)	91.4 (53/58)
Ewes successfully penetrated and flushed, %	93.7 (15/16)	89.5 (17/19)	94.4 (17/18)	92.4 (49/53)
Number of corpora lutea per ewe	8.5 ± 1.0[b]	14.3 ± 1.3[a]	9.9 ± 0.6[b]	10.7 ± 1.5
Number of corpora lutea per flushed ewe	8.7 ± 1.0 [136]	13.9 ± 1.4 [252]	9.9 ± 0.7 [178]	9.3 ± 0.5 [566]
Duration of the cervical penetration by Hegar, minutes[c]	1.8 ± 0.5	1.7 ± 0.4	2.0 ± 0.3	1.8 ± 0.1
Duration of the cervical penetration by stylet/catheter, minutes[c]	1.9 ± 0.6	1.6 ± 0.4	1.2 ± 0.4	1.6 ± 0.2
Duration of uterine flushing procedure, minutes	22.5 ± 0.8	26.9 ± 1.9	25.1 ± 1.4	24.8 ± 1.3
Duration of NSER procedure, minutes	26.9 ± 2.1	30.1 ± 2.2	28.6 ± 1.5	28.5 ± 1.6
Fluid recovery efficiency, %	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Total structures recovered per flushed ewe	9.0 ± 1.2 [135]	12.3 ± 3.0 [209]	5.9 ± 0.8 [101]	9.1 ± 1.8 [445]
Unfertilized eggs per flushed ewe	0.3 ± 0.3	1.5 ± 1.0	0.9 ± 0.7	0.9 ± 0.3
Viable (grades 1, 2 or 3[26]) embryos per flushed ewe	8.1 ± 1.3 [121]	10.2 ± 3.0 [174]	5.2 ± 0.9 [89]	7.8 ± 1.4 [384]
Freezable (grades 1 or 2[26]) embryos per flushed ewe	8.1 ± 1.3	10.2 ± 3.0	5.2 ± 0.9	7.8 ± 1.4
Degenerated embryos per flushed ewe	0.9 ± 0.4	2.0 ± 1.0	0.7 ± 0.3	1.2 ± 0.4
Morulae (stage 4[26]), %	86.8 [105/121]	82.8 [144/174]	67.4 [60/89]	82.0 [315/384]
Blastocyst (stages 5 or 6[26]), %	13.2 [16/121]	17.2 [30/174]	32.6 [29/89]	18.0 [69/384]
Recovery rate, %	99.3 [135/136]	82.9 [209/252]	56.7 [101/178]	78.6 [445/566]
Embryo viability rate, %	89.6 [121/135]	83.2 [174/209]	88.1 [89/101]	86.3 [384/445]
Ewes that had ova/embryos recovered, %	100.0 (15/15)	100.0 (17/17)	100.0 (17/17)	100.0 (49/49)
Ewes that had viable embryos recovered, %	93.3 (14/15)	88.2 (15/17)	94.1 (16/17)	91.8 (45/49)
Ewes that had more than the average number of viable embryos recovered, %	40.0 (6/15)	26.7 (4/15)	50.0 (8/16)	39.1 (18/46)
Pregnant females after SOV, %[d]	82.3 (14/17)	75.0 (12/16)	94.7 (18/19)	84.6 (44/52)
Pregnant females after NSER, %[d]	82.4 (10/12)	85.7 (12/14)	94.1 (16/17)	88.4 (38/43)
Prolificacy	1.9 ± 0.1	1.7 ± 0.0	1.0 ± 0.0	1.5 ± 0.1

() Number of animals; [] total number of corpora lutea or structures or their proportions.
[a,b]Within a row, the means with different letters differ significantly ($p < 0.05$) by the one-way ANOVA followed by the Tukey HSD test.
[c]Excluding ewes not successfully penetrated by the cervical catheter.
[d]Excluding culled animals.
NSER, nonsurgical embryo recovery; SOV, superovulation.

Table 3. Reproductive Parameters (% or Mean ± Standard Error of the Mean) Recorded in Brazilian Locally Adapted Goats (Canindé and Moxotó Breeds) Receiving Intravaginal Medroxyprogesterone Acetate (60 mg) Devices for 6 Days, 37.5 μg of Cloprostenol at 12 Hours Before Device Removal, Superovulatory Treatment (Superovulation) with 133 mg of Porcine Follicle-Stimulating Hormone Administered in Six Decreasing Doses (25%-25%-15%-15%-10%-10%) at 12-Hour Intervals Starting 60 Hours Before Device Removal, and Nonsurgical Embryo Recovery 6 to 7 Days After Estrus Onset

Parameter	*Canindé*	*Moxotó*	*Total*
Estrus response, %	93.3 (14/15)	86.7 (13/15)	90.0 (27/30)
Estrus onset, hours	30.9 ± 2.4	27.7 ± 1.6	29.3 ± 1.6
Estrus duration, hours	17.1 ± 1.7	15.7 ± 1.5	16.4 ± 0.7
Does with corpora lutea 1 day before NSER, %	78.6 (11/14)	92.3 (12/13)	85.2 (23/27)
Does successfully penetrated and flushed, %	100.0 (11/11)	100.0 (12/12)	100.0 (23/23)
Number of corpora lutea per doe	9.6 ± 1.4	13.9 ± 1.2	11.9 ± 2.1
Number of corpora lutea per flushed doe	9.6 ± 1.4 [106]	13.9 ± 1.2 [167]	11.9 ± 2.1 [273]
Duration of the cervical penetration by Hegar dilator, minutes	0.3 ± 0.2	0.2 ± 0.3	0.3 ± 0.3
Duration of the cervical penetration by stylet/catheter, minutes	0.5 ± 0.2	0.5 ± 0.1	0.5 ± 0.0
Duration of uterine flushing procedure, minutes	20.3 ± 0.8	19.1 ± 0.4	19.7 ± 0.6
Duration of NSER procedure, minutes	21.5 ± 0.7	19.8 ± 0.4	20.7 ± 0.8
Fluid recovery efficiency, %	100.0	100.0	100.0
Total structures recovered per flushed doe	3.4 ± 0.8 [37]	7.3 ± 0.9 [88]	5.4 ± 1.9 [125]
Unfertilized eggs per flushed doe	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Viable (grades 1, 2 or 3[26]) embryos per flushed doe	3.1 ± 0.8 [34]	6.5 ± 1.1 [78]	4.9 ± 1.7 [112]
Freezable (grades 1 or 2[26]) embryos per flushed doe	3.1 ± 0.8	6.5 ± 1.1	4.9 ± 1.7
Degenerated embryos per flushed doe	0.3 ± 0.2	0.8 ± 0.3	0.5 ± 0.2
Morulae (stage 4[26]), %	52.9 [18/34]	53.8 [42/78]	53.6 [60/112]
Blastocyst (stages 5 or 6[26]), %	47.1 [16/34]	46.2 [36/78]	46.4 [52/112]
Recovery rate, %	34.9 [37/106]	52.7 [88/167]	45.8 [125/273]
Embryo viability rate, %	91.9 [34/37]	88.6 [78/88]	89.6 [112/125]
Does that had ova/embryos recovered, %	72.7 (8/11)	100.0 (12/12)	86.9 (20/23)
Does that had viable embryos recovered, %	72.7 (8/11)	100.0 (12/12)	86.9 (20/23)
Does that had more than the average number of viable embryos recovered, %	50.0 (4/8)	33.3 (4/12)	40.0 (8/20)
Pregnant females after SOV, %[a]	91.7 (11/12)	73.3 (11/15)	81.5 (22/27)
Pregnant females after NSER, %[a]	100.0 (10/10)	83.3 (10/12)	90.9 (20/22)
Prolificacy	1.8 ± 0.0	1.6 ± 0.0	1.7 ± 0.0

() Number of animals; [] total number of corpora luteal/structures or their proportions. $p > 0.05$.
[a]Excluding culled animals.

the efficiency of administration of the first FSH dose 60 hours before the progestogen device removal. By using these protocols, we had previously observed a high CL count in dairy sheep (13.8)[16] and goats (4.9).[22] In the present study, these protocols provided on average, more than 10 CL per ewe or goat, with ~15% of variance. Curiously, in this study, 2 twin ewes of the Santa Inês breed yielded 42 and 32 viable embryos, and this is apparently the first report of a such high number of viable embryos yielded in sheep. These data support our hypothesis that the used superovulatoy protocols are efficient in various breeds of sheep and goats, including the locally adapted Brazilian breeds.

Similar superovulatory outcomes were shown among the goat breeds. The average of 4.9 viable embryos recovered in goats of this present study was similar to that reported previously in dairy goats.[22] The only difference between the two studies was that in this present study, MAP sponges were used, while in the aforementioned study,[22] P4 silicone devices were used. Those findings illustrate the consistency of the response, and following ovarian follicular turnover, elicited by this short-term estrus synchronization protocol in goats.[12] Among the sheep breeds of this present study, Santa Inês had a greater number of CL compared to Morada Nova and Somalis. In a previous study, it was shown that the emergence of the ovulatory follicular wave occurs ~6 days after the insertion of the progesterone device in superovulated Santa Inês ewes.[28] A greater superovulatory response has been observed in females when the FSH treatment is initiated close to the emergence of a follicular wave[29] as it appears to have occurred in the Santa Inês ewes in the present study. There is no report of follicular dynamics in Morada Nova and Somalis ewes, but the difference in the CL count might be the result of a distinct follicular population recruited in these females at the time of the first FSH dose. The later onset of estrus in Santa Inês ewes is another outcome of this present study that helps support our assumption that the follicular dynamics in Santa Inês might slightly differ from Morada Nova and Somalis breeds. The study of follicular dynamics in sheep and goats of different breeds may confirm this indication and support the recommendation of protocols that meet the particularities of ovarian activity in each breed. In the present study, despite the difference between sheep breeds for the number of CL per ewe, the embryo yield parameters were similar.

Superovulatory response, adequate breeding strategy, gamete quality, and uterine environment influence quantity and quality of embryos produced by a donor. The data presented here suggest that the superovulatory protocol combined with natural mating were efficient in supporting a suitable number of ovulations, as well as adequate fertilization. In addition, a high percentage of good or excellent (grade 1 and 2[26]) embryos yielded possibly resulted from healthy gametes and

a suitable uterine environment. Despite the aforementioned premises, for the full efficiency of the *in vivo* embryo production programs, embryos must be efficiently recovered. Our group has shown continuous enhancement for embryo recovery techniques, and recently, we demonstrated the potential of having successful NSER in up to 100% donors, as shown in dairy sheep[16] and goats.[22] In the present study, the efficiency of structure recovery was 56.7%–99.3% in sheep and 34.9%–52.7% in goat breeds. All viable embryos produced by all ewes and does were excellent or good, therefore, considered to be preservable by freezing. Taking into account the ultimate objective of producing embryos for cryobanking, this *in vivo* embryo production technique that included NSER resulted in the averages of 4.9 and 7.8 freezable[26] embryos per goat and sheep donor, respectively. Regardless of species or breeds, the whole success of SOV-NSER procedures was, in part, related to the strategic selection of donors, at 5 months or more postparturition. This strategy allowed the use of females that had been recently recognized as fertile. In addition, donors were not very far in time to a massive cervical opening event (i.e., parturition), which might have helped with cervical transposition. Finally, at 5 months postpartum, the females had their body reserves restored from pregnancy and lactation spoliation. All those conditions were considered important selection parameters for increasing NSER success in sheep.[30] and might have had contributed to the positive outcomes observed in this present study.

NSER has been shown to be safer, less invasive, and more aligned with ethical concerns than laparotomic embryo collection, as previously reported.[5,6] Importantly, a cervical relaxing protocol must precede NSER in sheep and goats. Combining estradiol benzoate, cloprostenol, and oxytocin in sheep or simply administrating cloprostenol in goats has been shown to be sufficient to promote cervix relaxation.[13] However, some previous concerns emerged regarding embryo quality and related consequences due to the temporarily nonphysiological hormonal environment promoted by cervical relaxing protocols. Despite a great increase in plasmatic estradiol concentration from the administration of estradiol benzoate and cloprostenol 16 hours before NSER, we have previously shown that no morphological changes were noted in embryos collected in sheep,[14] while *in vitro* survival, 48 hours after slow freezing was near 50%.[31] Goats subjected to NSER 16 hours after cloprostenol administration yielded embryos that result in good pregnancy rates upon transfer of fresh or slow-frozen embryos.[27] Nearly 40% of recipients became pregnant after receiving cryopreserved embryos recovered from Lacaune ewes subjected previously to an estradiol benzoate-cloprostenol-oxytocin protocol and NSER.[32] In addition, nonsurgical embryos recovered and transferred by the cervical route resulted in pregnancy rates reaching 57% upon transfer of embryos classified as excellent quality.[33] Given this aforementioned data, and the numbers of viable and freezable embryos recovered in the present study (similarly to the findings observed previously),[34] it can be inferred that cervical dilation protocols have no deleterious effects on the uterine environment and consequently on the quality of embryos yielded by sheep and goats.

Despite differences in SOV and cervical relaxation protocols, the same analgesic and anesthesia and the remaining steps of the NSER technique were used in both sheep and goat donors. Overall, the anesthetic and analgesic drugs used in this study are safe, not costly, and easily available in the Brazilian market. The routes of administration, including epidural, are easily performed in field conditions, not requiring laborious procedures such as those needed for drug preparation and laparotomy.[6] In the present study, animals did not need food and water deprivation, and they quickly returned to their routine management conditions.[10] These advantages of using the techniques of this present study are that they are aligned to the efforts suggested to sustain and promote animal welfare[35,36] and proved to be effective regardless of species or breeds subjected to NSER.

The reproductive parameters after SOV-NSER procedures and natural mating were also recorded. Results were similar to our previous findings in Dorper ewes submitted to estrus synchronization and NSER.[19] Interestingly, considering both the superovulatory regimen and the NSER procedure, in this current study, the fertility of all donors, independently from breeds or species, was very high, and no pregnancy loss was noted from ultrasonography detection (approximately from 30 to 60 days of pregnancy) to parturition. Prolificacy of the breeds studied here was similar to that previously reported in Santa Inês,[37] Morada Nova, and Brazilian Somalis[38] sheep and Canindé[39] and Moxotó[40] goats neither hormonally challenged nor subjected to NSER. The maintenance of fertility and prolificacy is a desirable outcome of NSER, especially when endangered species are the focus of the procedure, implicating a restricted or, perhaps, unique number of donors. Thus, NSER not only can be considered efficient for embryo recovery, but it also preserves the reproductive soundness of donors, allowing them to reproduce naturally and immediately after the procedure.

Cryopreserved embryos might be the main source for the preservation of pure breeds for long periods, as they support prompt flock/herd reconstitution, and can even be transferred to recipients of other breeds.[3] To reconstruct an endangered flock or herd, it was estimated that transferring 128 embryos might be sufficient to produce 25 females, taking into account embryo survival after cryopreservation (60%), as well as pregnancy (55%), birth (85%), survival (90%), rearing (98%), and sex rates (48%).[41] Using SOV and NSER techniques similar to those used in the present study, pregnancy rates of frozen/thawed excellent or good[26] embryos were 60% in goats[27] and 39% in sheep.[32] Hence, endangered flock reconstruction should consider specific data on overall efficiency for producing live animals, taking into account specific rates of embryo survival, pregnancy, birth, survival, and rearing, related to local conditions where embryo production and herd reconstruction occurs. The grouped results of this study strongly support using the sequential techniques applied for supporting programs of biopreservation and biobanking in sheep and goats. The data provided in this study might also serve as a reference for prediction of the number of donors and NSER procedures that might be necessary for assembling a biobank with a sufficient number of embryos. In addition, the techniques used in this study might be useful for establishing biobanks from other endangered breeds or species of sheep and goats around the world, such as Bighorn sheep (*Ovis canadensis*) and San Clemente Island goats, but this assumption deserves further investigation.

From SOV, animal preparation, embryo recovery to cryopreservation, the group of techniques used in the present

study has shown results that support both commercial and conservation purposes for *in vivo* embryo production and banking. It is particularly important to highlight that, among the five breeds involved in the study, four are under the risk of endangerment. To the best of our knowledge, this is the most successful NSER-based study involving a high number of sheep and goats, from different breeds, that reported sustainable and efficient embryo production and recovery. Postthaw embryo viability has been previously proven using the cryopreservation technique used in the present study in goats[27] and sheep.[32] All viable embryos recovered and cryopreserved in this study were inserted into the Brazilian animal gene bank managed by the Brazilian Agricultural Research Corporation (Embrapa). This bank currently has 649 embryos: 76.6% (497) recovered from NSER and 23.4% (156) from laparotomy.[42] Thus, the present study confirms the possibility of using NSER for supporting biobank embryo formation[10] and also supports changing the recommendation of FAO[4] that discourages NSER for this finality.

Altogether, the data presented here demonstrate the efficiency and consequent indication of NSER for supporting *in vivo* embryo production in goats and sheep, including those in need of conservation strategies. Importantly, the fertility of embryo donors was preserved after the procedures used to produce and retrieve embryos from them.

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