Surgery on cervical folds for transcervical intrauterine artificial insemination with frozen-thawed semen enhances pregnancy rates in the sheep

SURGERY ON CERVICAL FOLDS FOR TRANSCERVICAL INTRAUTERINE ARTIFICIAL INSEMINATION WITH FROZEN-THAWED SEMEN ENHANCES PREGNANCY RATES IN THE SHEEP.

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ABSTRACT

In sheep industry, genetic progress rate achieved by artificial insemination (AI) is limited by the convoluted anatomy of the cervix, which does not allow the passage of an insemination catheter for uterine semen deposition. The aim of this study was to test, in 98 pregnant at term Sarda ewes, the effects of: Experiment 1) total or partial ablation of cervical folds and Experiment 2) 4 or 2 incisions of cervical folds, on the passage of an insemination catheter, deposition of frozen-thawed semen and pregnancy rates. Surgical procedures were performed within 24h from parturition providing deep sedation and epidural anaesthesia. Duration of surgeries and post-operatory recovery were carefully monitored. For both experiments, 5 months since surgery, independently of the stage of oestrus cycle, cervical patency was tested through the transcervical passage of a palpation probe. Six months since surgery, in Experiment 1, ewes were naturally mated with fertile rams. In Experiment 2, ewes submitted to incisions of the cervical folds and a control group underwent synchronization of oestrus and transcervical AI with frozen-thawed semen. Thirty days later, for both experiments, pregnancy rates were assessed by ultrasonography and lambing rates were recorded. Five months after surgery, in Experiment 1, transcervical passage of a palpation probe to reach the uterine lumen was possible in all ewes submitted to total and partial ablation of folds. In Experiment 2, this was achievable in 90.5% ewes with 4 incisions of the folds and in 89.6% ewes with 2 incisions with no significant differences among groups (P=0.44). In Experiment 1, pregnancy rates in ewes mated to rams after total or partial ablation of the cervical folds was 100%. In Experiment 2, following transcervical AI, pregnancy rates were higher in groups submitted to 4

(63.7%) or 2 (41.4%) incisions of the cervical folds compared to the control group (8%; $P<0.05$). These data were confirmed at lambing with rates of 56.8% and 41.4% in ewes submitted to 4 or 2 incisions respectively, significantly higher than the control group (4%; $P<0.05$). Surgical ablation or incision of the cervical folds in post-partum ewes represent valid procedures for transcervical intrauterine deposition of semen for AI, obtaining satisfactory pregnancy rates. These procedures might be useful in programs of genetic selection and MOET.

Key words: cervical surgery, fertility, frozen-thawed semen, sheep, transcervical insemination, lambing.

1. Introduction

Programs of genetic improvement are the base for progress in farm animal breeding. In the sheep industry this could be easily accomplished by a method of artificial insemination (AI) that is reliable and economically sustainable. However, in this species it has a poor uptake, the main reason being the poor quality and short life of frozen-thawed semen caused by damage to the spermatozoa associated with cryopreservation and thawing [1]. The impaired ability of frozen-thawed spermatozoa to move through the female reproductive tract and to reach the site of fertilization is one of the major problems of AI in the sheep. This is summed up to the impossibility to deposit the semen directly in the uterine lumen, because of the convoluted anatomy of the sheep cervix. This structure is in fact characterized by a series of funnel-shaped folds that protrude caudally and are often misaligned, precluding the transcervical passage and intrauterine delivery of semen by using conventional AI catheters. The anatomy of the sheep cervix is also highly variable among individuals. Breed, age, parity and physiological state [2-4] might influence its shape and degree of relaxation explaining the variability in the success of transcervical AI. These limiting factors explain the reason why, in the ovine species, AI is mostly performed using fresh semen deposited in the external *os* of the cervix (cervical insemination)[5].

Many attempts, mainly mechanical and hormonal, have been made in the past to overcome this anatomical barrier. Some studies focused on the design of new insemination catheters [6-10], but their successful passage through the cervix and consequent deposition of semen in the uterus was strongly influenced by the above mentioned differences in the breed and age of the animals [3, 4].

Another approach has been the use of hormonal treatments to enhance the dilation of cervical canal, mimicking the pathway that involves the oxytocin-mediated synthesis of PGE_2 enhanced by gonadotropins and oestrogens. Prostaglandins E_2 act on both cervical extra-cellular matrix and smooth muscle layers leading respectively to re-arrangement of collagen bundles and relaxation

[11]. Among others, 17β-oestradiol [12, 13], oxytocin[13-15], FSH [4, 16] and PGE_2 analogues [4, 16-18] were tested. Other studies investigated the effects of myorelaxing substances [19] or cytokines [20]. All of these methods have been, at best, only partially successful and in some cases completely unsuccessful with respect to pregnancy rates.

To our knowledge, no attempt has been made to enhance cervical patency and transcervical passage of insemination catheters by a surgical approach. Therefore, the aim of this study was to test, in pluriparous Sarda ewes: Experiment 1) if total or partial surgical ablation of cervical folds would allow the passage of an insemination catheter through the cervix up to the uterine lumen and would affect pregnancy and lambing rates after natural mating; Experiment 2) if a less invasive surgical procedure based on 4 or 2 incisions of cervical folds could enhance cervical patency and allow uterine deposition of frozen-thawed semen with satisfactory pregnancy rates.

2. Materials and methods

2.1 Animal management and experimental plan

The study started during lambing season (October-November 2015-2016) in Sardinia, Italy, and all the procedures were carried out under the European regulations on the Care and Welfare of Animals in Research. The experiments were performed on a total number of 98 multiparous Sarda ewes aged between 3 and 4 years old, all pregnant at term, randomly assigned to one of the 4 different surgical procedures on cervical folds, carried out in 2 different experiments. The experimental plan is shown in Fig.1.

2.2 Surgical procedures

Surgery was performed within 24h from parturition taking care that expulsion of fetal membranes had occurred. All animals were initially submitted to mild sedation with acepromazine maleate (0.5mL/50Kg BW, IM, Prequillan, Fatro S.p.A., Italy) and, after careful trichotomy and disinfection of the sacrococcygeal area, epidural anaesthesia was achieved by injection of Lidocaine 2% (30mg/10kg BW, Esteve S.p.A., Italy). The ewes were then placed in a cradle in dorsal recumbency with the hindquarters slightly elevated (Trendelenburg position). The perineal area and vulva were carefully cleaned with an antiseptic solution of 10% povidone iodide and after setting up the surgical field, a lubricated speculum was gently inserted in the vagina in order to locate the external os of the cervix and its folds. The most caudal fold was then grasped with Duval forceps and the cervix was gently retracted up to the vulva (Fig.2a). With the aid of Duval forceps, the remaining folds, up to the most cranial one, were progressively grasped and retracted (Fig.2b), exteriorizing them completely (Fig.2c). All surgical procedures were performed under sterile conditions and their duration was recorded.

At this point, cervical folds were either completely (n.ewes=5) or partially removed (n.ewes = 20; Experiment 1) or incised in 4 sites (dorsally, ventrally and 2 laterally; n.ewes = 44) or in 2 sites (dorsally and ventrally; n.ewes = 29; Experiment 2).

2.2.1 Experiment 1: Total or partial ablation of cervical folds

Total ablation was performed excising each fold from the most cranial one at 2-3mm from the base with Metzenbaum scissors (Fig. 3a). The edges of the wound were immediately sutured with a Schmieden suture (monofilament polyglecaprone 25,USP 5/0, Vetsuture®, Paris, France) that was interrupted and restarted in 4 points (dorsal, ventral and laterals).

Partial ablation was performed excising from each fold 2 trapezoid-shape pieces of tissue dorsally and ventrally at 2-3 mm from the base of the fold (Fig. 3b). These portions were removed with electrocautery.

2.2.2 Experiment 2: 4 or 2 incisions of cervical folds

A schematic representation of the sites of incision of the cervical folds is given in Fig. 3. For every fold, after exteriorisation and distension of the tissue, either 4 (dorsal, ventral and 2 lateral) or 2 (dorsal and ventral) incision areas were delimited by 2 Dandy forceps (Fig.4a-5a) and cut by electrocautery (Fig.4b-5b).

2.2.3 Post operatory care

After surgery, a topical antibiotic treatment (Orbenin; cloxacillin suspension, Pfizer Italy Srl) was applied and the cervix was repositioned. All animals were kept under careful post operatory observation for 24h, and afterwards reintroduced in the flock. Milk production performances were monitored by the farmer during lactation.

2.3 Assessment of cervical patency after surgery

Five months since surgery, all females in both experiments were evaluated for patency of the cervical canal and easiness in transcervical passage of a probe up to the uterine lumen. In detail, ewes were restrained in a cradle in Trendelenburg recumbency and after cleaning the vulvar area and inserting a vaginal speculum, the cervix was gently retracted caudally up to the vulva with the aid of Bozeman forceps. A palpation probe (commonly used in laparoscopic procedures, 3.5mm in Ø, Richard Wolf, USA), was inserted through the cervical canal. The patency test was carried out without considering the stage of oestrus cycle of the ewes. The ability and the time taken in reaching the uterine lumen was recorded and the easiness in the passage of the probe was scored from I to IV(I= very easy; II= easy; III= moderately difficult; IV=difficult).

2.4 Natural mating in ewes with total or partial ablation of the cervical folds (Experiment 1)

In order to test if total or partial ablation of cervical folds affected oestrus behaviour, mating and, finally, pregnancy rates, one month after the assessment of cervical patency, all ewes surgically treated with either total (n=5) or partial (n= 20) ablation of cervical folds were synchronised using intravaginal progestagen sponges (Crono-gest 20mg, Intervet Italia S.r.l, Italy) for 14 days. On the day of sponge removal, 300 IU of PMSG (Folligon, Intervet Italia S.r.l, Italy) were injected IM. The ewes were then allowed to mate to 3 adult rams of proven fertility for 2 consecutive cycles.

2.5 Transcervical artificial insemination with frozen-thawed semen in ewes with 4 or 2 incisions of cervical folds (Experiment 2)

2.5.1 Semen preparation

Briefly, semen was collected by artificial vagina from 3 rams of proven fertility and only ejaculates with a score of mass motility ≥ 3 (scale of 0-5; 0= no motility, 5 = vigorous swirling waves of movements) and ≥ $3x10^9$spz/mL were further processed. Semen was pooled (to avoid individual variability) diluted in home-made Tris-EY (Egg Yolk) based extender with 6% glycerol to reach a concentration of $1.6x10^9$spz/mL ($400x10^6$spz/straw), cooled to 4°C and loaded into 0.25mL straws (IMV technologies, France). The straws were submitted to LN_2 vapors and then plunged and stored in LN_2 until the day of insemination. Straws were then thawed warming them at 37°C for 30 sec. An aliquot of thawed semen (5µL) was collected and assessed for motility parameters through CASA (computers assisted sperm analysis; Ivos, Hamilton Thorne, Biosciences).Total and progressive motility were 65 and 45% respectively.

2.5.2 Artificial insemination with frozen-thawed semen

Six months after surgery, in order to assess pregnancy rates, the ewes that underwent incisions of the cervical folds (4 incisions, n=44; 2 incisions, n=29), and a control group of 25 animals (no surgery) were synchronised using intravaginal progestagen sponges (Crono-gest 20mg, Intervet Italia S.r.l, Italy) for 14 days. On the day of sponge removal, 300 IU of PMSG (Folligon, Intervet Italia S.r.l, Italy) were injected IM. At 56-58h from sponge removal, transcervical artificial insemination was performed using frozen-thawed semen. Ewes were placed in dorsal recumbence in a cradle, the perineal and vulvar area were carefully cleaned with an antiseptic solution and a lubricated speculum was gently inserted in the vagina. The fold of the external os of the cervix was localised and gently extruded using Bozeman forceps up to the vulvar vestibulum (Fig.6). The insemination catheter (Cassou mini-pistolet for ovine-caprine; IMV technologies, France), loaded with thawed semen, was then inserted through the cervical canal and the semen was deposited, when possible, directly in the uterine lumen. The animals in which passing the cervix to reach the uterus was not possible were recorded and semen was deposited in the cervix as deep as possible.

2.6 Pregnancy detection

For both experiments, return to oestrus was checked by introducing teaser rams wearing harnesses with crayons in the experimental groups from 15 to 20 days after artificial insemination. Pregnancy rate (pregnant ewes/ inseminated ewes) was determined at 30 days after insemination by transrectal ultrasonography (MyLab One, Esaote, Italy). Lambing rate was also recorded.

2.7 Statistical analyses

Statistical analysis was performed using Stata 11.2/IC (StataCorp LP, USA). Continuous data regarding the duration of surgery, the time taken to pass the cervix with the palpation probe during the assessment of post-surgery cervical patency were not normally distributed and were analysed by non-parametric Kruskall-Wallis test followed by two-samples Wilcoxon rank-sum test for pairwise comparisons with Bonferroni's correction. Categorical data regarding the ability to reach the uterus with the probe, the easiness in passing through the cervical canal, pregnancy rates and lambing rates were analysed by χ^2-test. The significance level was defined for $P<0.05$.

3. Results

3.1 Surgical procedures

The mean duration of surgery was, for partial ablation (Exp.1) and 4 or 2 incisions of cervical folds (Exp.2), 28 ± 6 min and no difference among these procedures was observed (P>0.05). Total ablation of cervical folds took around 30 additional minutes due to suturing time.

After the 24h of post operatory observation, all animals submitted to surgery were in good health conditions and were reintroduced in the flock. Milk production was not affected by the surgical procedure.

3.2 Post surgical assessment of cervical patency

The results obtained from the post surgical assessment of cervical patency are summarised in Table 1.

3.2.1 Experiment 1

Four months after surgery, the passage of the probe through the cervical canal up to the uterine lumen was allowed in all ewes submitted to total (5/5, 100%) and partial (20/20, 100%) ablation of cervical folds.

3.2.2 Experiment 2

Reaching the uterus was achievable in 40/44 (90.9%) ewes that underwent 4 incisions of the folds surgery and in 26/29 (89.6%) ewes that underwent 2 incisions surgery. The differences among procedures were not statistically significant (P>0.05). In those subjects in which the uterine lumen was reachable, passing the cervical canal was easier and effortless in ewes submitted to ablation of the folds compared to those that underwent incision (P<0.01). In the group submitted to 2 incisions, the passage of the probe was easy but not effortless compared to the other groups (P<0.001).

In addition, the time spent in reaching the uterine lumen was significantly lower in ewes that underwent total (2.4 ± 0.5 sec) or partial (4.9 ± 3 sec) ablation of cervical folds compared to those that had 4 (21 ± 26 sec) or 2 (26.2 ± 21 sec) incisions (P<0.05); no significant difference was found between the latter 2 groups (P>0.05).

3.3 Pregnancy and lambing rates

3.3.1 Experiment 1

Ultrasound check at 30 days after natural mating in ewes submitted to total or partial ablation of cervical folds showed pregnancy rates of 100% in both groups at the first oestrus after synchronisation. No relevant problems were reported during pregnancy and lambing.

3.3.2 Experiment 2

The site of deposition of frozen-thawed semen during transcervical AI in the control and in 4 or 2 incisions groups is reported in Table 2. Semen deposition in the uterus was possible in none of the ewes of the control group. No return to oestrus at 15-20 days and the ultrasound scanning performed at 30 days after AI with frozen-thawed semen, revealed that pregnancy rates were significantly higher in the groups of ewes submitted to 4 (28/44; 63.7%) or 2 (12/29; 41.4%) incisions of the cervical folds compared to the control untreated group (2/25; 8%; P<0.001). Among the 8 ewes (4 in the 4 incisions group and 4 in the 2 incisions group) in which frozen-thawed semen was deposited in the cervix, only 1 ewe with 4 incisions of the cervical folds was pregnant and lambed regularly. Data on pregnancy rates were confirmed at lambing with rates of 56.8% and 41.4% in ewes submitted to 4 or 2 incisions respectively, significantly higher than the control group (1/25; 4%; P<0.001; Table 2). Moreover, lambing occurred without relevant problems.

4. Discussion

The desired practical and commercial diffusion of intrauterine AI will be achieved if and when it becomes possible to pass an insemination catheter through the cervix allowing uterine deposition of semen without causing trauma to the cervix. Eppleston et al. showed that, in the sheep, there was a linear relationship between fertility and depth of deposition of frozen-thawed semen and that, when inseminating into the uterus, the site of deposition did not affect fertility. This suggested that an effective transcervical method of insemination would lead to good fertility rates of around 80%, a similar figure to the one achieved using laparoscopic insemination [21]. The anatomy of the sheep cervix represents a major limiting factor for intrauterine trans-cervical AI in the ovine species. In fact, its lumen is highly convoluted due to the presence of 4-7 cervical folds [2] in 3 distinct sections: the caudal section being the entrance to the external os with a large fold [whose shape has been classified in several previous studies [2, 22]], the central section having the majority of the larger folds, and the cranial section which meets the uterine body at the internal os and in which folds are smaller and less well defined [22]. The cervical folds project caudally into the lumen and are generally out of alignment with the first [23].

In the present experiments, we proposed a surgical approach for ablation/incision of cervical folds that allowed the passage of a Cassou insemination catheter and the deposition of frozen semen directly in the uterus. The surgery was performed within the 24h post-partum following expulsion of foetal membranes because during this time the cervix can be easily manipulated reducing the risk of traumas that could compromise the future reproductive ability. In the pre-partum period, the cervix undergoes a series of modifications that results in relaxation of smooth muscle layers and in softening of the connective tissue. This multifactorial event is controlled by reproductive hormones and is characterised by increase in inflammatory cells, in the amount of extra-cellular water and in dispersion of collagen fibrils [24]. The remodelling of cervical tissue provided, in our experiment, the optimal conditions to perform the surgery, limiting the effects of this invasive procedure. In the post- partum ewes submitted to surgery, cervical folds were in fact hypertrophic, softened and could be easily exteriorised from the vulva and manipulated to perform ablation or incision. Moreover, since in the peri-partum period, nearby tissues and ligaments are relaxed, excessive stretching that could result in traumas and potential fibrosis was avoided. This was confirmed, in ewes submitted to the surgery, by the post-operatory full recovery and by the full ability to carry the pregnancy at term and lamb with no complications.

Moreover, it is worth considering the important role of the cervix as a barrier protecting the endometrium and the conceptus from pathogens and that surgical ablation or incisions of cervical folds do not compromise this function. In fact, no infections were reported in the follow-up of the surgery nor during successive pregnancies. Providing that all animal welfare criteria are met and that sterility conditions are maintained during surgery, we can therefore propose this approach as a safe procedure with no risk for the animal health.

Pregnancy and lambing rates achieved after 4 or 2 incisions of cervical folds using frozen-thawed semen were as high as 63% and 56% respectively. This result is similar to those achieved by laparoscopic AI. With the laparoscopic technique, pregnancy rates using frozen-thawed semen are satisfactory and range from 43% [25] to around 72% [26]. However, it is a surgical procedure that cannot be used repeatedly on the same animal for problems of post-manipulation adherences in the abdominal cavity and ethical issues on animal welfare [27]. Moreover it requires trained personnel and expensive and delicate instruments. Previously reported pregnancy rates obtained by transcervical AI, if fresh semen is used, are satisfactory, ranging around 50-60% [25, 28]. Using frozen-thawed semen, rates range from 30- 32% [25, 29] to less than 10% [30], far below those obtained in this study. The surgical procedures we proposed in the present study are “once in a lifetime” procedures, that are not repeated on the same subject and that allow transcervical intrauterine insemination for the entire reproductive career of the animal (unpublished data). These findings are supported by the observations of the condition of cervical folds after post-surgical lambing (Fig.7).

Pregnancy rates were satisfactory in all 4 groups submitted to surgery. However, total ablation of cervical folds requires longer execution times that are inadvisable under field conditions. Pregnancy rates in animals submitted to 4 incisions of cervical folds were numerically but not statistically higher compared to the group submitted to 2 incision (63.7% vs 41.4% respectively). However, we can speculate that, increasing the size of the groups this difference would result statistically significant and therefore the technique of 4 incisions would be preferable. For what concerns partial ablation, the execution times are similar to those of the incisions of cervical folds and the penetration times are very low. This suggests that the above technique, together with the 4 incisions technique, are advisable in in field transcervical AI programs. These two procedures could provide consistent benefit in MOET (multiple ovulation embryo transfer) programs in the ovine species (unpublished data). In conclusion, surgical ablation or incision of the cervical folds in post-partum ewes represent valid procedures for trans-cervical intrauterine deposition of semen for AI, obtaining satisfactory pregnancy rates. Although it is a surgical procedure, animals recovered soon and their productive and reproductive careers were not compromised. Therefore we propose these techniques as useful tools for successful spreading of superior genotypes in selected animals.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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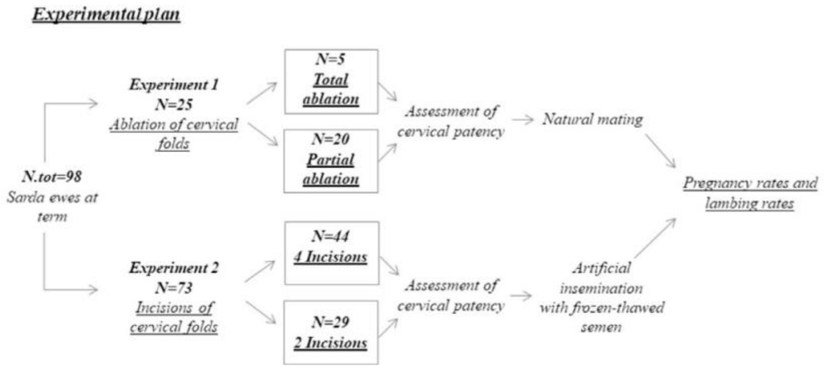

Fig. 1. Experimental design to test the effects of four different surgical procedures on cervical folds on fertility and lambing rates in pluriparous Sarda ewes.

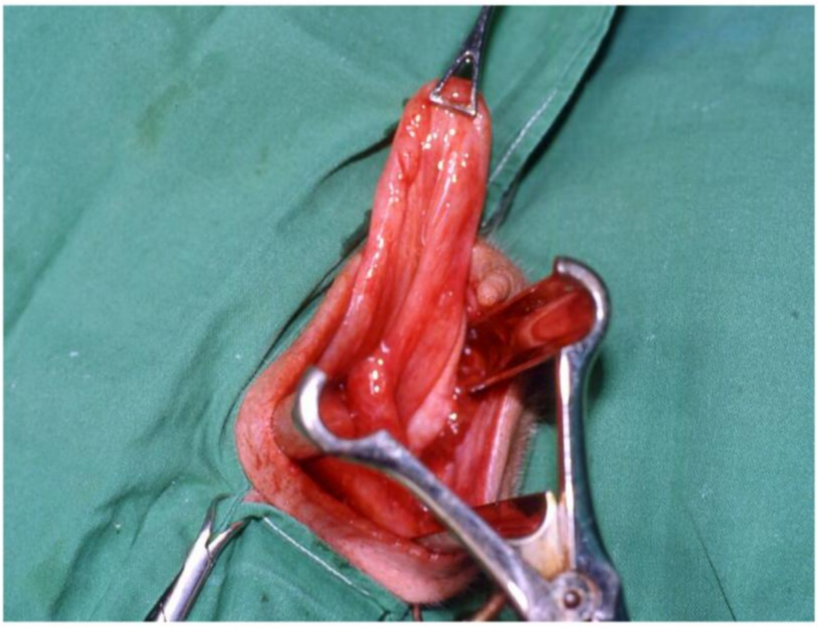

Fig. 2a. Procedure for the exteriorisation of cervical folds: a) the most caudal fold of the external os (arrow) was grasped with Duval forceps and the cervix was gently retracted up to the vulvar vestibulum.

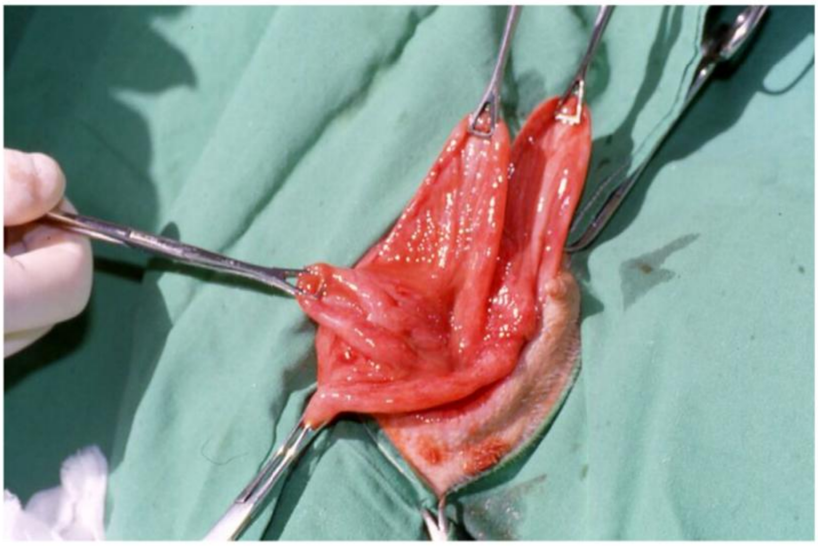

Fig. 2b. Procedure for the exteriorisation of cervical folds: b) progressively, with the aid of other Duval forceps.

Fig. 2c. Procedure for the exteriorisation of cervical folds: c) the cervical canal was completely exteriorised.

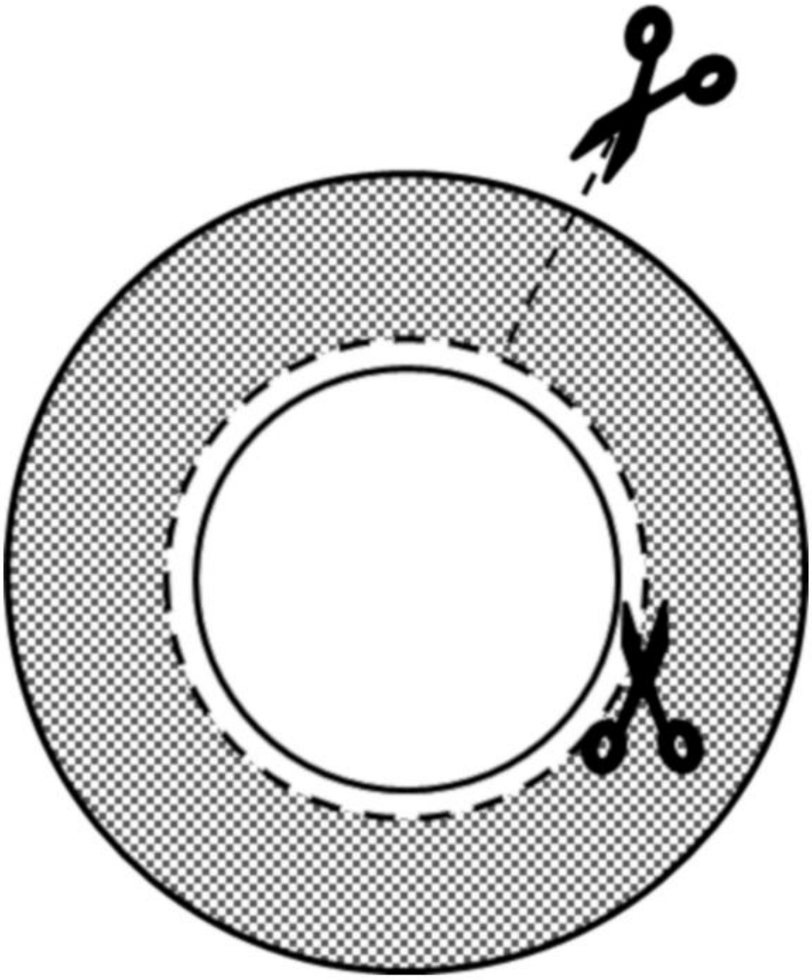

Fig. 3a. Schematic representation of total (a) and partial (b) ablation of cervical folds. The grey area represents the tissue removed from each cervical fold and the dotted lines represent the sites of incision.

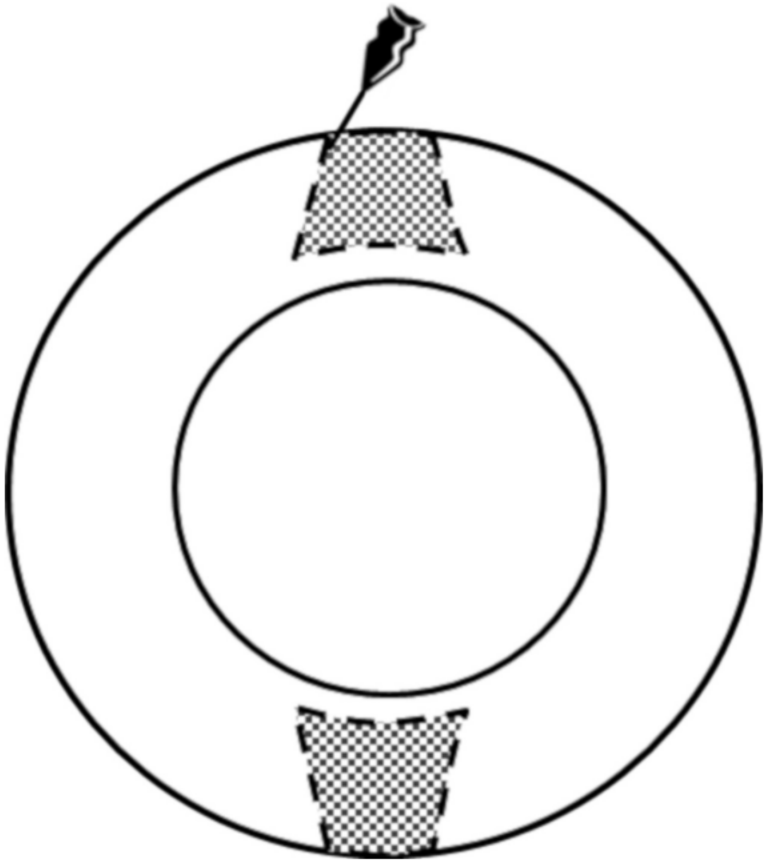

Fig. 3b. Schematic representation of total (a) and partial (b) ablation of cervical folds. The grey area represents the tissue removed from each cervical fold and the dotted lines represent the sites of incision.

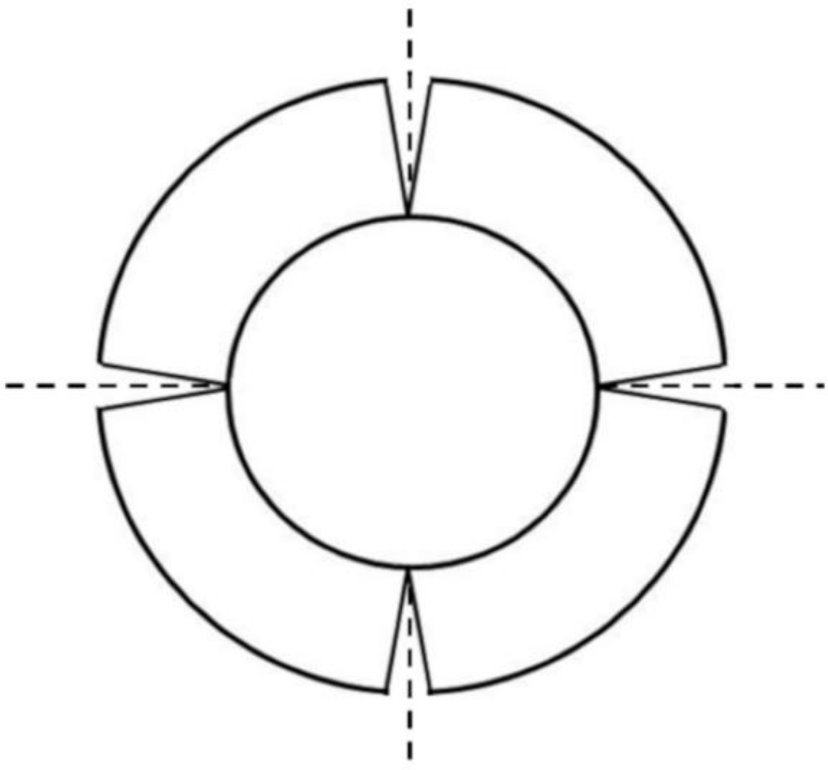

Fig. 4a. Schematic representation of the sites of incision of the cervical folds, indicated by dotted lines: a) 4 incisions (dorsal, ventral and 2 laterals).

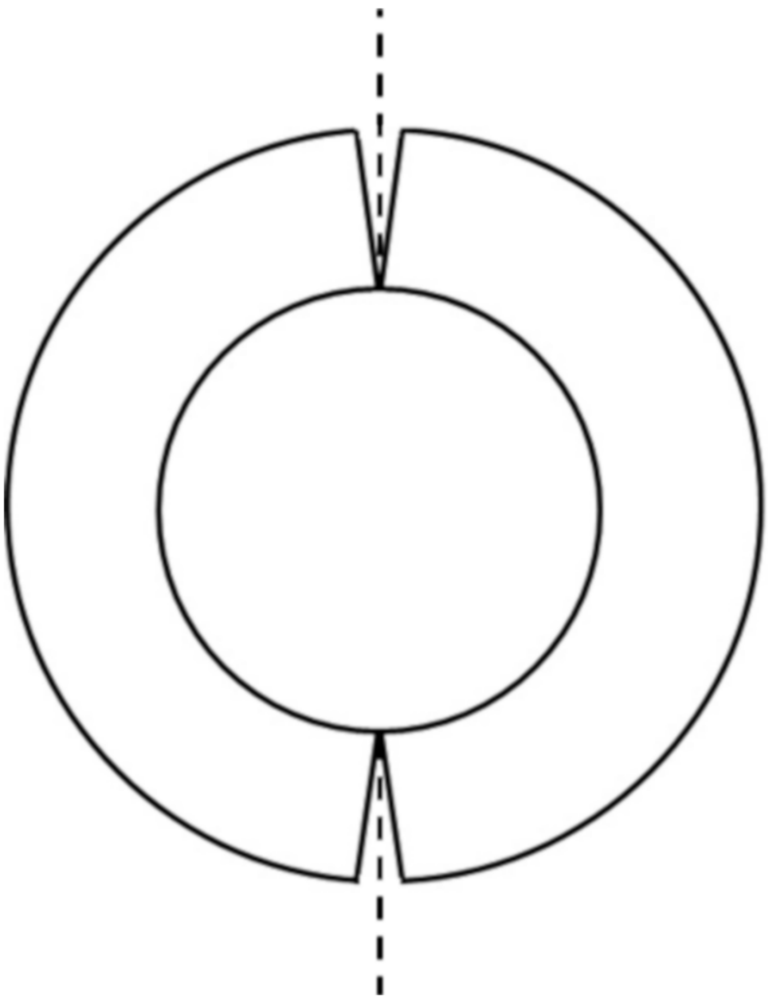

Fig. 4b. Schematic representation of the sites of incision of the cervical folds, indicated by dotted lines: b) 2 incisions (dorsal and ventral).

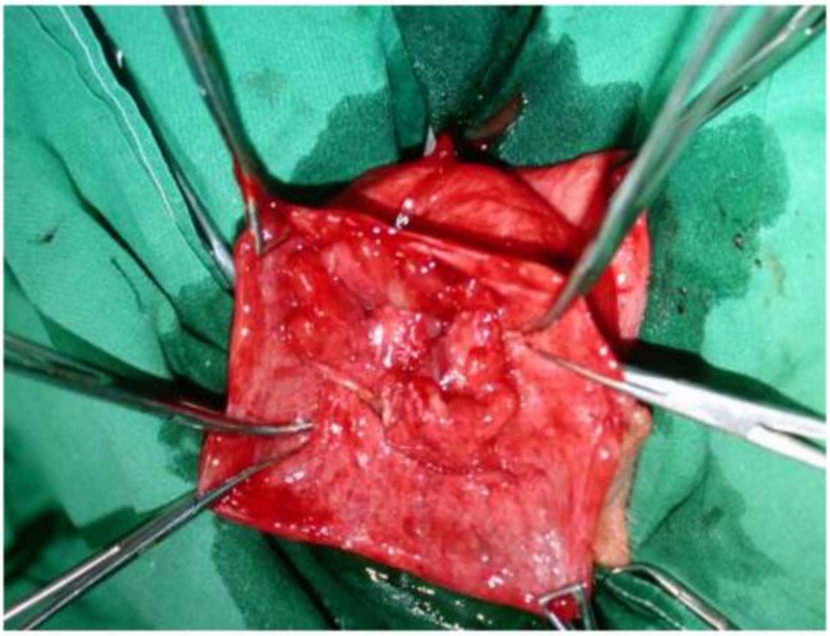

Fig. 5a. Incision of cervical folds: a) areas were delimited by Dandy forceps (arrows).

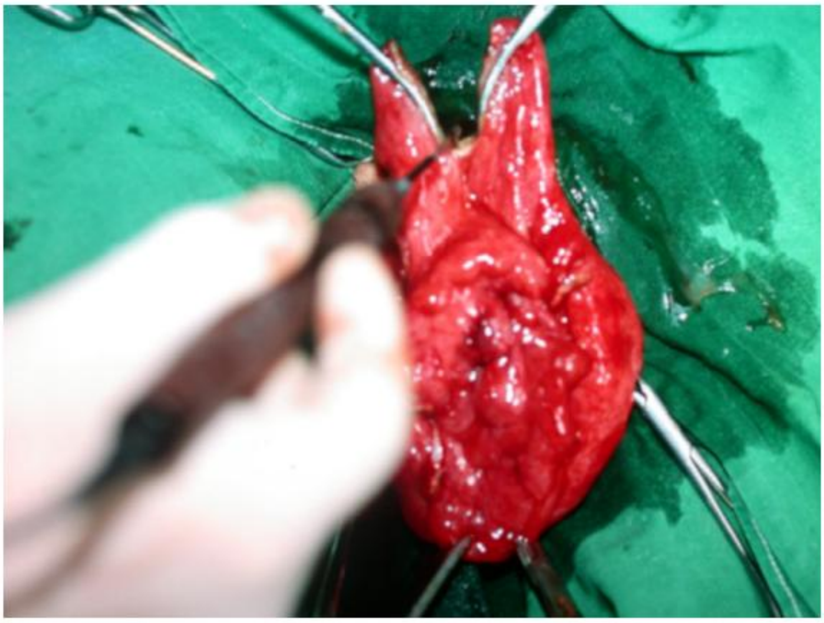

Fig. 5b. Incision of cervical folds: b) cut by electrocautery.

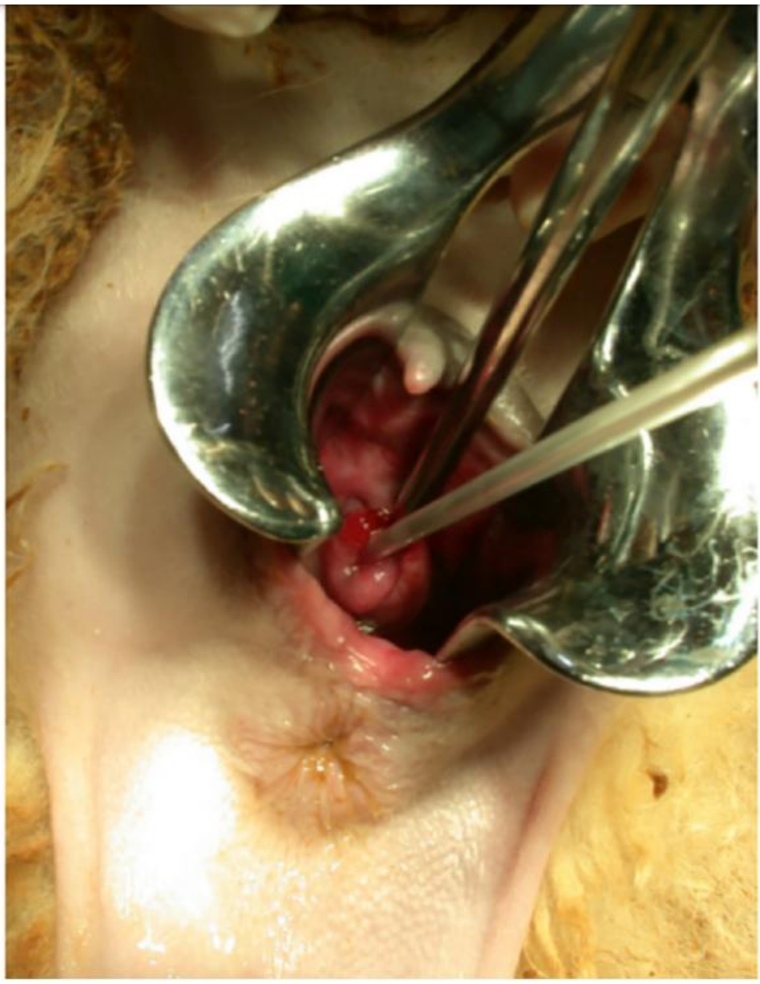

Fig. 6. Transcervical artificial insemination with frozen-thawed semen after incision of cervical folds: the fold of the external os of the cervix was gently extruded using Bozeman forceps up to the vulvar vestibulum and the insemination catheter was inserted in the cervical lumen and semen was deposited, when possible, directly in the uterus.

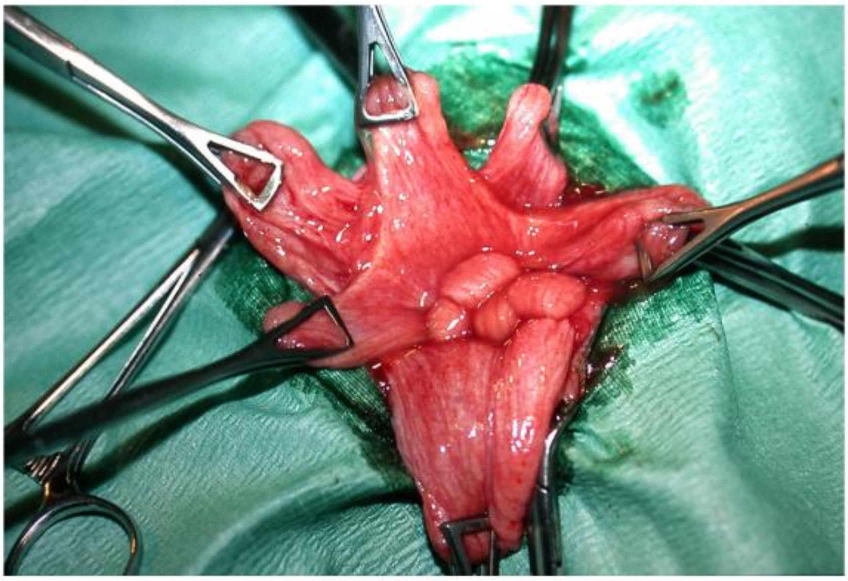

Fig. 7. Cervix of a ewe submitted to 4 incisions of cervical folds after post-surgical lambing.

Table 1
Cervical patency following 4 different surgical procedures on cervical folds.

	Surgical procedure on cervical folds	Ability to reach the uterus		Easiness in reaching the uterus (% score)				Time to reach the uterus (sec. ± SD)
			%	I	II	III	IV	
Exp. 1	Total ablation	5/5	100	**100**[a]	**0**[a]	0	0	**2.4 ± 0.5**[a]
	Partial ablation	20/20	100	**100**[a]	**0**[a]	0	0	**4.9 ± 3**[a]
Exp. 2	4 incisions	40/44	90.9	**65**[ab]	**25**[ab]	7.5	2.5	**21 ± 26**[b]
	2 incisions	26/29	89.6	**38.5**[b]	**46.1**[b]	15.4	0	**26.2±21**[b]

The easiness in passing through the cervical canal refers to ewes in which reaching the uterine lumen was possible. Scores: I) very easy; II) easy; III) moderately difficult; IV) difficult. The time taken to reach the uterus is expressed in mean ± SEM. Different superscripts indicate significant differences among procedures for $P < 0.05$.

Table 2
Site of deposition of frozen-thawed semen, pregnancy and lambing rates in control group and in ewes submitted to 2 or 4 incisions of the cervical folds.

	N° tot	Site of deposition of semen		Pregnant ewes	Pregnancy rates (%)	Ewes at lambing	Lambing rates(%)
		Uterus	Cervix				
Control	25	0	25	2/25	**8**[a]	1/25	**4**[a]
4 Incisions of cervical folds	44	40	4	28/44	**63.7**[b]	25/44	**56.8**[b]
2 Incisions of cervical folds	29	25	4	12/29	**41.4**[b]	12/29	**41.4**[b]

Different superscripts ([a, b]) indicate significant differences within column for $P < 0.05$.