Embryo recovery from the oviduct in superovulated ewes: a method to improve MOET systems

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ABSTRACT: The efficiency of embryo recovery in a superovulatory treatment was studied by perfusing the oviduct or the uterine horn in 3.5 and 7 days after sponge withdrawal, respectively. Eighty-four and seventy ewes of Aragonesa breed were used as embryo donors and receptors, respectively. The donors were distributed in 3 replications. The oestrus was synchronized with the insertion of FGA intravaginal sponges (30 mg) for 13 days. Six intramuscular injections of 18 mg pFSH were applied in decreasing doses at 12 hour intervals starting 48 hours after sponge withdrawal. The recovery rate (RR) (83.2 vs. 75.8%), the viability rate (VR) (73.5 vs. 47.2%) and the number of viable embryos (VE) per donor ewe (5.9 ± 0.79 vs. 3.0 ± 0.37) were higher ($P < 0.01$) in ewes whose perfusion was done directly in oviducts. The interval from sponge withdrawal to oestrus had no influence on RR in any groups; however it had an effect on the VR in 3.5 and 7 days old embryos. Embryos from ewes showing the oestrus within 20 hours after sponge withdrawal had a higher viability. On the other hand, using morphologic criteria, 26.5% and 52.7% of 3.5 and 7 days old embryos, respectively, were discarded before transfer. The percentage of ewes in each group that became pregnant was similar in both groups. We conclude that in superovulated ewes the embryo recovery rate, viability and number of viable embryos are higher in embryos recovered from the oviduct compared to those recovered from the uterus, without decreasing the conception rate and the viability rate is higher in ewes that show the oestrus 20 hours before sponge withdrawal.

Keywords: embryo transfer; MOET; oviduct; uterus; superovulated sheep

The response to superovulatory treatment in small ruminants is variable and depends on many factors (Cognie et al., 2003; Gonzalez-Bulnes et al., 2004) while the most important factor is the hormonal treatment and the results depend on the type of hormone used (Driancourt and Fry, 1992), batch (Donaldson, 1990), hormone diluents (López-Sebastian et al., 1992; D'Alessandro et al., 2001), dose (D'Alessandro et al., 2001, 2005), if the treatment is fixed or decreasing (Gonzalez-Bulnes et al., 2000, 2002) and on the number of administrations (D'Alessandro et al., 2005). On the other hand, it is necessary to have an embryo recovery method that maximizes and guarantees the recovery rate in the embryo transfer programs in small ruminants. Principally there are two methods of embryo recovery according to embryo development: from the oviduct or the uterine horn. In both cases the uterine access can be surgical (McKelvey et al., 1986; Ramon et al., 1991) or non-surgical (Vallet et al., 1989). The cervical recovery of embryos was reported (Barry et al., 1990); however, the morphology of the sheep

cervix (Halbert et al., 1990; Kershaw et al., 2005) makes this method really difficult and implies the use of additional hormonal treatment for the cervical canal modifications in sheep.

The aim of this study was to find the best method of embryo recovery to obtain high quality and high embryo yield using novel techniques of embryo recovery and embryo transfer (Ramon et al., 1991) described by our group and to determine the influence of the oestrus onset in donor ewes on the embryo variables.

MATERIAL AND METHODS

Animals and treatment

One hundred and fifty-four adult and non-lactating ewes of Aragonesa breed were used as embryo donors (n = 84) or receptors (n = 70) in 3 replications of superovulation treatment and embryo transfers. The donors were distributed into balanced groups by live weight and corporal condition and the mean was 48.62 ± 0.58 kg and 2.92 ± 0.28, respectively.

The ewes' oestrus was synchronized with the insertion of intravaginal sponges of fluorogestone acetate (30 mg, Chronogest®, Intervet) for 13 days, the superovulatory treatment consisted of intramuscular application of 18 mg pFSH (Schering Corporation) in decreasing doses (4, 4, 3, 3, 2, 2 mg) every 12 hours, starting 48 hours before sponge withdrawal. In embryo receptor ewes the sponge withdrawal was done 12 hours before the donors, and these received 400 IU of equine chorionic gonadotrophin (eCG, Folligon®, Intervet). The oestrus detection was done every 4 hours starting in 12 hours after sponge withdrawal, using vasectomized rams fitted with a marker harness. The donor controlled mating (one copulation of each male) was allowed in 36 and 48 hours after sponge withdrawal. Later, mating ewes were divided into two groups for embryo recovery in 3.5 days or 7 days, and subgroups homogeneous to the oestrous time, the number of ewes in each group of oestrus onset is shown in Table 3.

Embryo recovery

The embryo recovery was performed semilaparoscopically in 3.5 days after sponge withdrawal in the oviduct (n = 37) or in 7 days from the uterine horn (n = 34), only in ewes that responded to superovulation treatment (three or more luteal bodies) to avoid unnecessary surgery in animals.

The embryos were obtained through prepubian laparatomy under general anaesthesia using 0.5 g of sodium pentothal intravenously and maintained through orotraqueal intubation and halothane administration.

The ovulation rate (OR) was determined by laparoscopy immediately before laparotomy according to standard procedures (Oldam and Lindsay, 1980). The same incision for laparoscope access was opened to 2.5 cm and the uterine horn was exteriorized using atraumatic forceps. For oviduct perfusion we used a modification for this study of Hunter et al. (1955), the fimbria was removed and the oviduct drawn through the fimbria using a blunt needle with additional holes in its side and fitted to 3.5 F silicone catheter (Tom Cat Catheter, Sherwood Medical) and held with the fingers. The next step was to perforate the uterine wall with a punch, one cm in caudal direction to the uterus tubal junction and to insert in this perforation another catheter fitted with a blunt needle and kept in place and occluding the uterine cavity at the same time with the fingers (Figure 1).

In the uterine perfusion (Ramon et al., 1991), the same abdominal access was used; the uterine horn was removed from the uterus-tubal junction

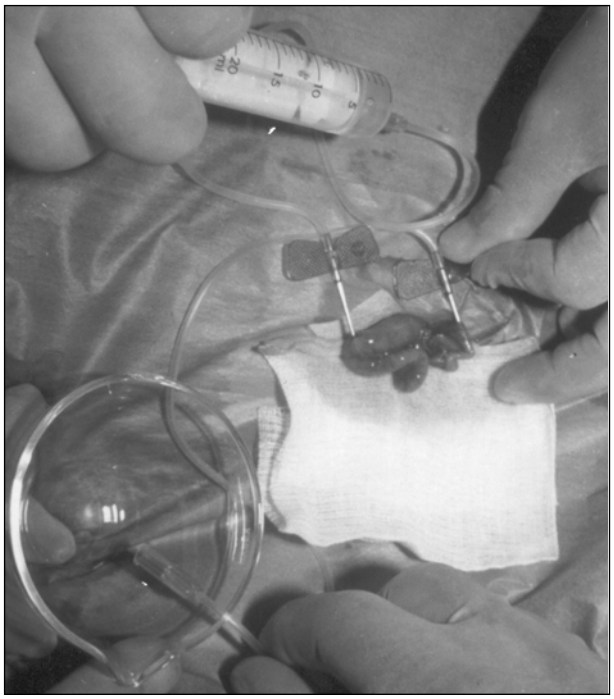

Figure 1. Embryo recovery from the oviduct

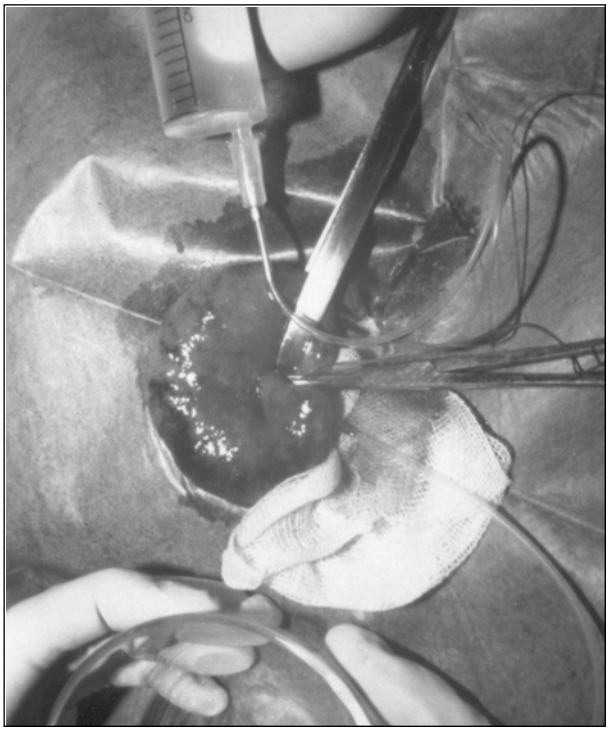

Figure 2. Embryo recovery from the uterus

to the horn bifurcation, and the uterine wall was perforated with a punch at 1 cm of the uterus-tubal junction in cervical direction and a needle and a catheter as previously described were inserted and held at this point with a silk suture rounding the needle and uterine horn and occluding the uterine lumen. At 3 cm in cranial direction of the uterine horn base the uterine wall was perforated and a needle was introduced and held in the position with an intestinal clamp in transversal position that occludes the uterine lumen (Figure 2).

The uterine flushing of the oviduct and uterine horn was performed using a non-rubber syringe attached to the perfusion catheter, using 10 ml of phosphate buffered saline (PBS) in the oviduct and 40 ml of PBS in the uterus while both the media were supplemented with 0.2% v/v bovine serum albumin (Sigma) and kept at 37°C. The flushing medium was recovered using a catheter attached to the cranial needle in flat bottom recipients previously sterilized and siliconized, using only the fingers to hold this catheter (Figures 1 and 2).

To avoid adherences in the abdominal wall, the oviduct and uterine horn were irrigated with abundant heparinized saline solution (1% v/v) and visible blood clots were removed. The oviduct and horn were reintroduced in the abdominal cavity and the other side of the uterine horn was flushed. With both uterine horns and oviducts flushed and inside the abdominal cavity, 250 ml of saline solution was introduced into the cavity and the abdominal incision was sutured using an eight-shape suture technique that holds the peritoneum, muscle and skin at the same time (Alexander, 1986), using polyamide monofilament 0 USP (Supramid®, B Braun). Asepsis of the abdominal cavity was performed and high spectrum antibiotics were administered.

The recovered embryos were considered viable if their development corresponded to the physiological age, without signs of cellular degeneration. The detected anomalies were: absence of symmetry, extruded blastomeres, increased granulation, vacuolated cells and zona pellucida damage, and they were classified according to Winterberger-Torres and Sevellec (1987). Seventy viable embryos were transferred in the 3.5 days group and fifty-four embryos in the 7 days group; two embryos of each group by receptor and allowing the maximum non-synchrony between receptors and donors of ±12 h.

Embryo transfer

For embryo transfer, receptor ewes were evaluated by laparoscopy for ovulation rate and previous local anaesthesia administration (Xylocaine, Sheerwood Medical). The uterine horn ipsilateral to the ovary with luteal body was exteriorized with endoscopic atraumatic forceps at 2 cm of the uterus-tubal junction. For oviduct transfers the catheter transporting the embryos was introduced 2.5 cm inside the oviduct and the medium containing embryos was deposited. For uterine transfer a small zone of the uterine horn was exposed and perforated with a punch and the catheter with embryos was introduced in cervical direction and embryos were disposed in the uterine lumen (Ramon et al., 1991). In both transfers (oviduct and uterus), two viable embryos in PBS were transferred using a catheter attached to a 1-ml syringe. Following the transfer the abdominal wall was sutured and antibiotics were administered.

Pregnancy diagnosis

The pregnancy diagnosis was performed in 17 days after mating using a radioimmunoassay for progesterone with a commercial kit (125 1-Progesterone Coatria, BioMerieux), the sensitivity was 0.05 ng/ml

and the intra and interassay variation coefficient for the reference sample was 15.3% and 18.6%, respectively. In 40 days ultrasound diagnosis was performed with a 5.0 Mhz probe (Sonolayer, Toshiba) according to Blasco and Folch (1989).

Statistical analysis

Discontinuous variables were compared using x^2 with Yates correction and Fisher exact test. The dichotomous variables were compared using multinomial sampling and x^2 (Brown, 1988) using a QBASIC program from the author and continuous variables were evaluated by Student's *t*-test.

RESULTS

Out of eighty-four donor ewes, five in the oviduct group and eight in the uterus group showed less than three luteal bodies and were not flushed. Only the ewes that responded to superovulatory treatment were included in the analysis; the three experimental replications did not show any statistical differences and were analyzed together. The results of each experimental replication are shown in Table 1.

No differences were found between embryo recovery groups in the length of oestrous cycle and ovulation rate, but recovery rate, viability rate and viable embryos per donor ewe were high when embryos were recovered from the oviduct ($P < 0.01$) and these results are shown in Table 2.

The time between sponge withdrawal and the onset of oestrous cycle has no influence on embryo recovery; however it affects percentages of viable embryos (Table 3).

The viability rate (Table 4) in the ewes that showed oestrous activity within the first 20 hours from sponge withdrawal compared to the ewes that showed the oestrus after 20 hours was highest in both groups (3.5 and 7 days embryos); however the difference was significant only in the 3.5 days group (81.6 vs. 60.5%; $P < 0.01$) and (55.8 vs. 42.4%; $P < 0.10$) in groups 3.5 and 7 days, respectively.

The fertility of receptors that received 3.5 days embryos in the oviduct was 64.7% and in 7 days embryos in the uterus it was 64.3%. The prolificity was 122 and 144 respectively, without any significant difference.

Table 1. Corpora lutea (CL), recovery of embryos (RE) and viable embryos (VE) in each replication by embryo group

Group	*n*	CL	RE	VE	VE/CL (%)
Oviduct					
Test 1	12	138	108	78	56.5
Test 2	18	149	129	96	64.4
Test 3	7	71	61	45	63.3
Total	37	358	298	219	61.1
Uterus					
Test 1	11	88	66	32	36.3
Test 2	15	130	101	46	35.4
Test 3	8	67	49	24	35.8
Total	34	285	216	102	35.8

Table 2. Results of embryo recovery from the oviduct and uterus in superovulated sheep

Group	*n*	Oestrus length[1]	OR[2]	RR[2]	VR[2]	VE/donor[2]
Oviduct	37	21.9 ± 0.76[a]	9.6 ± 0.79[a]	83.2[a]	73.5[a]	5.9 ± 0.79[a]
Uterus	34	22.9 ± 0.76[a]	8.3 ± 0.67[a]	75.8[b]	47.2[b]	3.0 ± 0.37[b]

[1]hours from sponge withdrawal

[2]mean ± SEM; different superscripts differ significantly; $P < 0.01$

Table 3. Corpora lutea (CL), recovery of embryos (RE) and viable embryos (VE) according to the time between sponge withdrawal and oestrus onset (SET)

Group	SET (h)	*n*	CL/ewe	RE/ewe	VE/ewe
Oviduct					
	12	–	–	–	–
	16	7	12.6	10.2	9.8
	20	14	9.9	8.0	5.8
	24	9	8.3	7.5	4.3
	28	4	8.2	7.5	4.5
	32	3	7.6	5.3	4.0
Total		37	9.7[a]	8.0[a]	5.9[b]
Uterus					
	12	2	6.5	5.0	2.0
	16	1	5.0	5.0	4.0
	20	10	8.8	6.2	3.5
	24	13	9.5	7.5	3.5
	28	7	7.1	5.7	1.7
	32	1	6.0	2.0	2.0
Total		34	8.4[a]	6.3[a]	3.0[b]

[a,b]different superscripts differ significantly; $P < 0.01$ (3.5 group); $P < 0.05$ (7 group)

DISCUSSION

The perfusion technique in the oviduct or uterus used in this study for the recovery of embryos of 3.5 and 7 days had a general recovery rate of 70%, higher than reported previously (Hunter et al., 1955). On the other hand, with the use of endoscopy for embryo recovery the surgical incision used is 2.5 cm in donor ewes and 1.5 cm in receptors the complete removal or manipulation of the ovaries is not necessary, with the added benefit of blood presence reduction.

The method of embryo recovery used in this study differs from a previously described semilaparoscopic method (Bari et al., 2000) in which a Foley catheter and a volume of 60 ml of the medium or a combination of the medium and air is used. Some small details incorporated in the procedure used in this study like the visible blood clot removal and irrigation with heparinised saline solution reduce postsurgical adherences and can increase the number of procedures in the same ewe (Ramon et al., 1991). In addition, the method of transfer is quick (4 mi-nutes per ewe) and assures the embryo position in the right place.

Table 4. Influence of the time between sponge withdrawal and oestrus onset on the number of corpora lutea (CL), ovulation rate (OR), recovered embryo number (RE), recovery rate (RR) and viable embryo number (VE) and viability rate (VR)

Group	SET (hours)	*n*	CL	(OR)	RE	(RR)	VE	(VR)
Oviduct	12–20	21	2 271	(10.8)	184	(81.0)	150	(81.6)[a]
	20–32	16	31	(8.1)	114	(87.0)	69	(60.5)[b]
Uterus	12–20	13	106	(8.1)	77	(72.6)	43	(55.8)[a]
	20–32	21	179	(8.5)	139	(77.6)	59	(42.4)[a]

[a,b]different superscripts differ significantly; $P < 0.01$

The number of ER was higher when the recovery was done from the oviduct. This can be influenced by the mechanical facility of flushing due to the part of the reproductive tract that was flushed being shorter and using a smaller volume than when flushing from the uterus. From this point of view, the technique of recovery is better. The time of sponge withdrawal has no influence in the RR contrary to the previous results of Torres and Cognie (1984), who observed that the RR of six-day embryos was affected positively by a shorter interval of the oestrus onset.

The morphologic classification of 3.5 days embryos is apparently less rigorous than that of 7 days embryos. However, the selection criteria from 7 days embryos are appropriate for eliminating each embryo that did not manifest any possible morphological defects in a more premature stage; in spite of this apparently more rigorous classification in 7 days embryos the VR was higher in the group of 3.5 days. A cause of this finding could be an adverse milieu in the embryo between 3.5 and 7 days of sponge withdrawal that provokes the presence of more degenerated embryos (Niemann et al., 1989). On the other hand, the interval of the oestrus onset clearly influences the VR, showing that embryos with more viability come from ewes that show the oestrus 20 hours before sponge withdrawal. In this respect, previous reports (Torres and Cognie, 1984) found that the number of VE/CL showed a tendency ($P < 0.1$) to affect in the same way.

The conception rate obtained was similar to other reports in 2–4 cell embryos (Vallet et al., 1989) and 5 days embryos (Brebion et al., 1989). In this study, no differences were found between both groups. This confirms that the evaluation method of 5 days embryos permits an evaluation of morphological changes during the embryo stay in the oviduct and its pass to the uterus (Wilmut et al., 1985; Gandolfi et al., 1991). However, the recovery of embryo from the oviduct improves the reliability of superovulatory treatment, making it nearly twice as reliable. The combination of embryo recovery from the oviduct and the combination of simple procedures like the application of growth hormone (Folch et al., 2001) or hCG (Khan et al., 2003) can improve embryo viability and increase sheep production through MOET programs.

In conclusion our results show that the oviduct level embryo recovery, in 2–8 cell stages, allows a high number of transferable embryos, without altering the embryo viability or conception rate and the viability rate is higher in ewes that show the oestrus 20 hours before sponge withdrawal. This technique of oviduct embryo recovery can improve sheep production using MOET schemes.

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