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Repeatability of superovulatory response to successive FSH treatments in Merino sheep

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ABSTRACT

The efficiency of a multiple ovulation and embryo transfer program depends on the recovery of a large number of viable embryos per donor ewe at each successive superovulatory treatment. Embryo production, however, is characterized by high individual variability in the ovarian response to hormonal treatment. This study evaluated the repeatability of ovarian response and embryo recovery following three successive superovulatory treatments in 10 four-year-old Merino ewes over a short period of time. Oestrus was synchronized using an intravaginal progesterone pessary inserted on Day 0 and removed on Day 14. FSH was administered to induce superovulation in declining doses twice daily over 3 days as 2(18 + 14 + 8) = 80 mg total commencing on Day 12 after pessary insertion. A dose of 200 IU eCG was administered at pessary removal. Vasectomized rams were used for heat detection and laparoscopic AI was performed 12 h after oestrus detection. Surgical embryo recovery was performed at Day 8 after pessary removal, following which prostaglandin was administered and the next superovulatory procedures began 5 days later giving a 27-day interval between embryo recoveries. Ovulation rates (mean ± SEM) for the three successive superovulatory treatments were 13.8 ± 2.2, 12.1 ± 2.2 and 12.0 ± 2.2 (NS); and total embryos recovered were 8.3 ± 1.4, 6.3 ± 1.4 and 3.9 ± 1.4 ($P = 0.06$), respectively. Consequently, the embryo recovery rates declined from 66.1 ± 7.5% for the first recovery to 41.4 ± 7.5 and 34.5 ± 7.5% for the second and third recoveries ($P = 0.04$). The repeatability was $r = 0.84$ ($P < 0.05$) for ovulation rate, $r = 0.13$ (NS) for total number of embryos and $r = 0.09$ (NS) for number of grade 1 and 2 embryos. The study showed that an average of 16.3 ± 2.6 grades 1 and 2 embryos per donor ewe could be recovered over a period of 2.5 months. The high repeatability of ovarian response to successive superovulatory treatments would have to be associated with a high level of repeatability for the number of transferable embryos recovered for it to be used as a criterion for selecting donors on the basis of their response to the first hormonal treatment.

1. Introduction

Multiple ovulation together with embryo transfer is a powerful tool for genetic improvement in animal production by introducing or spreading selected breeds or

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genotypes with superior value. The yields obtained after treatment for multiple ovulation and embryo recovery (MOER) in donor females are mainly determined by the number of transferable embryos attained after MOER, by the feasibility for repeating hormonal treatment several times in the same female and, finally, by consistency in the number of total transferable embryos obtained in subsequent superovulatory treatments.

It is well known that the number of transferable embryos obtained after MOER treatment of donor females is dependent on the follicular growth at the beginning of the hormonal treatment, the ovulatory response to exogenous gonadotrophins and the viability of the embryos recovered (Gonzalez-Bulnes et al., 2004; Menchaca et al., 2010), having in mind that ovarian response to superovulatory treatment is highly influenced by great individual variability.

The feasibility for repeated embryo recoveries is determined by procedures applied for embryo retrieval. Embryos may be recovered from the genital tract of the donor either by surgery (Cordeiro et al., 2003; Tervit et al., 1991) minor surgery (laparoscopy: Bari et al., 2001; McKelvey et al., 1986) or non-surgical techniques (transcervical flushing: Gusmão et al., 2009). Surgical procedures allow recovery rates between 70 and 90% (Baril et al., 1995), but the performance of repeated embryo retrievals is compromised by scarrings and fibrous adherences in the reproductive tract (Torres and Sevellec, 1987). On the other hand, minor-surgery or non-surgical techniques yield lower recovery rates than those obtained by surgery, both using laparoscopy in ewes (50–60%, McKelvey et al., 1986) or transcervical flushing in goats (53% in Saanen goats, Lima-Verde et al., 2003; 60–80% in Boer goats, Holtz, 2005); however, it is assumed that efficiency of embryo recovery with these techniques does not decrease in successive interventions.

The consistency in embryo yields in successive MOER protocols is affected by a decrease in the total number of embryos collected. First, there is a significant reduction both in the number of ewes showing multiple ovulations (Al-Kamali et al., 1985; Fuki et al., 1985) and in the mean number of corpora lutea in ewes subjected to successive multiple ovulations (Boland and Gordon, 1982; Forcada et al., 2000). Second, decline in embryo recovery is influenced by the repetition of treatments and recoveries; mainly, when using surgical techniques (Torres and Sevellec, 1987). In addition, the possibility of obtaining high embryo yields in repeated MOER treatments is compromised, as mentioned previously, by the great individual variability in ovarian response to the same superovulatory protocol. Such high individual variability is one of the main factors compromising cost-effectiveness of genetic improvement embryo transfer programs, even when superovulatory treatments have been modified in the last years for excluding ovarian determinants (Menchaca et al., 2009, 2010).

In studies reported to date, the repeatability of ovarian response in subsequent hormonal treatments has been scarcely studied. In this regard, if a high repeatability of this variable is found, the identification of high-responding donor ewes to their first MOER treatment would be an easy and cost-effective method for selecting only those females that will give a good amount of transferable embryos in successive embryo recoveries. However, to the best of our group knowledge, there are no systematic studies supporting the last premise.

Thus, the objective of the present study was to evaluate the repeatability of the response to successive superovulation and embryo recovery in Merino ewes over a short period of time.

2. Materials and methods

Ethical concerns were taken into account by adhering to local animal welfare regulations and practices.

2.1. Animals and handling

The study was conducted at the Laboratory of Reproduction in Small Ruminants, Experimental Station of the Instituto Nacional de Tecnologia Agropecuaria, INTA, Bariloche, Argentina, at the latitude of 41° S. Total solar lighting duration is 15:09 and 9:12 h (summer and winter solstices, respectively). The experiment was performed during the breeding season (April–May). Ten donors Merino sheep, 4 years old and in good body condition (>3 out of 5) (scale: 0, emaciated; 5, obesity), maintained outdoors under natural day length and fed a maintenance ration of live weight, were used.

2.2. Successive MOER treatments

In all females, oestrous cycle was synchronized by the insertion of, at day 0 of the MOER treatment, an impregnated intravaginal progestagen pessary (60 mg of medroxyprogesterone acetate, Progespon®, Syntex, Buenos Aires, Argentina) for 14 days (Fig. 1). Superovulatory treatment consisted of 80 mg of FSH (NIH-FSH-P1, Folltropin®, Bioniche, Belleville, Canada), i.m. administered in six decreasing doses (18 mg × 2, 14 mg × 2, 8 mg × 2 Gibbons et al., 2010). FSH injections were applied twice daily, starting on the morning of Day 12, 48 h before pessary removal, and finishing 12 h after progestagen withdrawal. A single dose of eCG (200 IU, Novormon 5000®, Syntex, Buenos Aires, Argentina) was administered with progestagen removal at Day 14. Oestrus detection was performed twice daily (8 a.m. and 8 p.m.), from 24 to 72 h after sponge withdrawal, with adult vasectomized rams. Twelve hours after oestrus detection, ewes were artificially inseminated with 100 million frozen-thawed spermatozoa doses, from the same batch and ram, by the laparoscopic method described by Maxwell and Butler (1984). At Day 8 after sponge removal, embryos were surgically recovered under anaesthesia and a single i.m. injection of 125 μg cloprostenol (Estrumate®, Schering-Plough, Germany) was administered.

The subsequent MOER protocols were initiated 5 days after prostaglandin administration and performed in the same way as described for the first treatment, completing three successive embryo recoveries with a 27-day interval between them. The close repetition of surgical

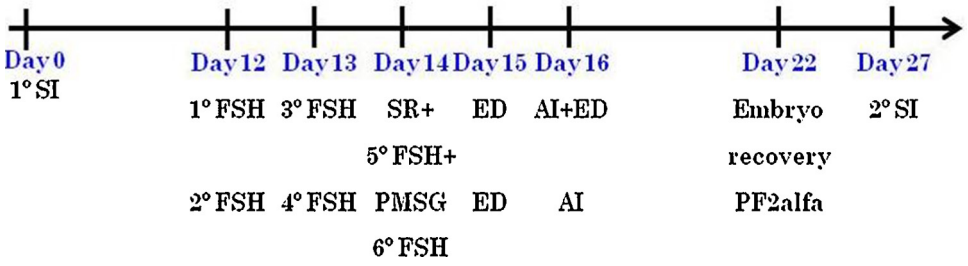

Fig. 1. Protocol for oestrus synchronization and hormonal stimulation for multiple ovulation in successive embryo recoveries in Merino sheep. SI: intravaginal sponge insertion. SR: intravaginal sponge removal. ED: oestrus detection. AI: artificial insemination.

interventions was chosen due to the short breeding season at 40° latitude.

2.3. Embryo recovery and viability assessment

At Day 8 after sponge withdrawal in each MOER protocol, the number of corpora lutea was determined by laparoscopy and immediately, embryos were recovered by surgical approach. Females, fasted 24 h prior, were anaesthetized with xylazine (2 mg/10 kg of Kensol® 2%, Konig, Buenos Aires, Argentina) and ketamine clorhidrate (25 mg/10 kg of Ketalar®, Parke-Davis, Buenos Aires, Argentina). Furthermore, local anaesthesia was applied in the surgical field (Lydocaine, Frankaina® 2%, Fatro-VonFrankel, Buenos Aires, Argentina). All embryos were collected with the aid of laparotomy, flushing each uterine horn with 20 ml of commercial embryo recovery medium (Vigro Complete Flush®, Bioniche, USA), pre-warmed to 38 °C and supplemented with 10% adult bovine serum (Internegocios, Buenos Aires, Argentina). The embryo recovery medium was injected by means of a sterile syringe with a blunt needle, inserted at the major curvature of the horn, and directed from the uterine horn towards the utero-tubal junction, where a catheter was attached with silk. After embryo recovery, uterine horns were externally washed with 40 ml of physiological solution in order to reduce possible fibrous adherences. Embryos were classified by morphological criteria (IETS, 1998), by using a stereomicroscope (Olympus SZ, Olympus Optical Co., Tokyo, Japan).

2.4. Indexes of superovulatory response

Firstly, number and percentage of sheep exhibiting oestrus and the interval from progestagen removal to onset of oestrus were evaluated. Second, number and percentage of ewes with superovulatory response were also determined. Ewes showing more than three corpora lutea were considered as superovulated (Azawi, 2011; Rexroad and Powell, 1991). Thereafter, the following information was recorded for each ewe: number of corpora lutea, number of oocytes, total number of embryos and number of embryos of each grade. The rate of total embryos recovered was obtained, for each animal, by dividing the total number of embryos recovered by the number of corpora lutea.

The proportion (%) of grades 1 and 2 embryos was calculated for each animal, by dividing the number of grades 1 and 2 embryos by the total number of embryos recovered. Fertilization rate was the total number of embryos recovered for each animal, expressed as a proportion (%) of the total number of oocytes plus embryos recovered. All rates were expressed as percentages.

2.5. Statistical analysis

Analysis of data was performed using SAS computer package (SAS, 2003). Test Fisher's Exact was used for assessing the proportion of sheep displaying oestrus and superovulatory response. Analysis of variance (ANOVA) was used to determine the effect of number of MOER protocols on the ovarian response, the rates of recovery and fertilization, and the quality of the embryos. Statistical analysis of results expressed as percentages (rates of total embryos recovered, grades 1–2 embryos recovered and fertilization rate) was performed after transformation of the values for each individual percentage to the arcsine square root. All the results were expressed as mean ± SEM and statistical significance was accepted from $P < 0.05$.

The repeatability was expressed as intra-class correlation coefficient and was calculated from estimation of variance components between and within ewes (Cardellino and Rovira, 1987). The variance components were estimated with the PROC VARCom Type I of SAS statistical program. The confidence interval (CI) of repeatability was estimated from the formula of CI for inter-class correlation (Snedecor and Cochran, 1980), with a probability of 95%. Statistical significance was accepted at 5% using the table for intra-class correlation coefficient for a bivariate population.

3. Results

Post surgical adhesions after embryo recovery were observed. At second surgical procedure, two females presented minor adhesions: one, between both uterine horns and the other, between one uterine horn and the omentum. At third surgical intervention, two donors developed multiple adhesions, hindering oviducts flushing, and a third ewe presented minor adhesions between one uterine horn and the ipsilateral oviduct.

There was a significant increase in the interval from progestagen withdrawal to the onset of oestrus in the second and third MOER treatments relative to the first superovulation ($P < 0.05$; Table 1). At the three successive MOER treatments, the percentage of ewes observed in oestrus were 100, 100 and 90%, respectively; the proportion of

Table 1
Effect of number of superovulatory treatment and embryo recovery on ovarian responses and embryo production in Merino ewes subjected to three successive hormonal treatments.

	Embryo recoveries			*P*
	First	Second	Third	
Ewe donors[a] (*n*)	10	10	9	
Sponge removal – onset of oestrus (h)	25.2 ± 1.6[c]	38.4 ± 1.6[d]	40.0 ± 1.7[d]	<0.0001
Corpora lutea (± SEM)	13.8 ± 2.2[c]	12.1 ± 2.2[c]	12.0 ± 2.2[c]	0.27
Total embryos recovered (± SEM)	8.3 ± 1.4[c]	6.3 ± 1.4[cd]	3.9 ± 1.4[d]	0.06
Total embryos recovered (%)	66.1 ± 7.5[c]	41.4 ± 7.5[d]	34.5 ± 7.9[d]	0.04
Grades 1–2 embryos recovered[b] (±SEM)	7.4 ± 1.3[c]	5.7 ± 1.3[cd]	3.2 ± 1.3[d]	0.06
Grades 1–2 embryos recovered (%)	84.8 ± 8.4[c]	89.5 ± 9.4[c]	67.4 ± 8.9[c]	0.24
Fertilization rate (%)	100 ± 5.6[c]	95.8 ± 6.3[c]	85.3 ± 6.0[c]	0.23

[a] Ewes that exhibited oestrus.
[b] Grades 1–2 (IETS, 1998).
Different letters within rows (c, d) indicate significance differences ($P < 0.05$).

Table 2
Repeatability and confidence intervals estimated for the number of corpora lutea and number of total and grades 1–2 embryos in Merino ewes subjected to three successive hormonal treatments.

Analysed variables	*R*[a]	S.E.[b]	Confidence interval	
			Φ_L	Φ_U
No. of corpora lutea	0.84	0.09	0.56	0.94
Total number of embryos	0.13	0.21	−0.25	0.51
No. of grades 1–2 embryos	0.09	0.21	−0.27	0.47

[a] Repeatability calculated as inter-class correlation.
[b] Standard error of the estimate of repeteability.

ewes showing a superovulatory response was 100, 80 and 80%, respectively; the mean number of corpora lutea was 13.8, 12.1 and 12.0 (SEM 2.2), respectively; and the fertilization rates were 100, 95.8 and 85.3%, respectively. None of these differences were statistically significant.

The rate of embryo recovery declined ($P = 0.04$; Table 1) and the proportion of grade 1 and 2 embryos fell between the first and third recoveries (NS). Consequently, the total number of embryos recovered was 8.3, 6.3 and 3.9 (SEM 1.4) for the first, second and third intervention ($P = 0.06$; Table 1), with a similar decline in the number of grades 1 and 2 embryos recovered ($P = 0.06$).

The total yield of embryos was 18.5 ± 2.8 and the total number of grades 1 plus 2 embryos was 16.3 ± 2.6 per Merino ewe over the three successive embryo recoveries.

The repeatability for ovulation rate in the three successive MOER treatments was $r = 0.84$ ($P < 0.05$), but repeatabilities for the total number of embryos and number of grades 1 plus 2 embryos were very low ($P > 0.05$) (Table 2).

4. Discussion

The results obtained in current study indicate, first, that MOER protocols can be applied on the same Merino ewes in a short time interval, allowing the retrieval of a high number of transferable embryos (around 18–19 embryos per donor ewe in a period of 2.5 months), in spite of a decreased recovery rate in successive recoveries. Second, the high repeatability of ovarian response in subsequent MOER treatments in sheep would indicate the possibility of selecting donor females with high ovarian response to first MOER treatment although, probably because of the decline in embryo recovery rates in successive interventions, repeatabilities for the number of total and transferable embryos were low. Third, in the current experiment, MOER protocol was equally effective for inducing oestrus and superovulatory response in the three successive embryo recoveries.

The percentage of females displaying oestrus behaviour was not affected by the number of previous superovulatory treatments, in agreement with other authors (Bari et al., 2001; Cordeiro et al., 2003; Torres and Sevellec, 1987), but the time-interval between progestagen withdrawal and oestrus onset increased in the second and third MOER treatments when compared with the first; same results were also found by Cordeiro et al. (2003). The variability in appearance of oestrus between successive protocols is especially important since, having in mind its incidence on the interval between the onset of oestrus and ovulation, it may affect the fertility obtained after applying fixed-time artificial insemination (FTAI) in genetic improvement embryo transfer programs. However, FTAI implementation in MOER protocols is not recommended, from our point-of-view, as intervals progestagen removal-oestrus-ovulation are dependent on endogenous (individual variability) and exogenous factors (type, protocol and dose of progestagens and gonadotrophins).

On the other hand, consistency in ovulation rate in successive hormonal treatments found in our work was in opposition to results obtained in other studies, in which, a progressive reduction in the number of superovulated ewes (Al-Kamali et al., 1985; Fuki et al., 1985) and rate of multiple ovulation (Boland and Gordon, 1982; Forcada et al., 2000; Torres and Sevellec, 1987) was observed. In our study, the use of low doses of pFSH, although yielded a lower rate of ovulation compared with traditional treatments of multiple ovulation (80 mg pFSH vs 200 mg pFSH, Gibbons et al., 2010), could be one of the reasons why ovulation rate did not decrease throughout successive MOER treatments. The interval between hormonal treatments has also been described as affecting ovarian response, since studies showing a decrease in the ovulatory response were based in periods from 50 to 75 days (Al-Kamali et al., 1985; Torres and Sevellec, 1987). In contrast, other authors found that ovulatory response remained constant in subsequent MOER treatments (Cordeiro et al., 2003; Kraemer, 1989), at longer intervals (60–90 days). Thus, existence of a certain

ovarian refractoriness to hormonal treatment might have adverse effects on subsequent ovulation when superovulation is repeated at short intervals; nevertheless in our study such refractoriness could have been avoided by using lower doses of gonadotrophins.

The results of current study indicate a significant decrease in embryo recovery rate over successive superovulatory treatments. Such finding may be related to various factors, among them the formation of fibrous adherences, in agreement with Torres and Sevellec (1987), who also used the surgical technique for collection of embryos. However, we have to note that other authors did not report a decrease in embryo recovery rates in successive interventions; either using surgery (Cordeiro et al., 2003; Tervit et al., 1991), semi-laparoscopic (Bari et al., 2000, 2001) or laparoscopy methods (McKelvey et al., 1986).

The decrease in the rates of embryo recovery in successive MOER protocols induced a decrease not only in the total number of embryos recovered but also in the number of transferable embryos. These observations are consistent with information provided by previous studies (Al-Kamali et al., 1985; Forcada et al., 2011; Torres and Sevellec, 1987), although Bari et al. (2001) and Cordeiro et al. (2003) found no significant differences in these variables between successive collections.

In any case, results obtained in current trial indicate the suitability of applying MOER protocols, repeated at short intervals, for the retrieval of a high number of transferable embryos.

The overall success of embryo production depends not only on the capability to obtain high rates of ovulation in response to multiple ovulation treatment, but also on the possibility to remove donor ewes with low ovarian response in subsequent treatments of superovulation (Bari et al., 2001); the effectiveness of this strategy depending on the repeatability of individual ovarian response to superovulation treatment when subjected to successive hormonal protocols. There is no specific data, to the best of our group knowledge, regarding the repeatability of superovulatory response in sheep at short time intervals; only previous information from Bari et al. (2001) who found a moderate repeatability in ovarian response ($r = 0.55$) when hormonal treatments were repeated at yearly intervals. In the same way, Saumande and Chupin (1977) in cattle and Cordeiro et al. (2003) in sheep reported that the same females that did not superovulate in response to first hormonal treatment, did not respond to second treatment. Thus the present study provides additional evidence for the existence of an "individual effect", either by genetic and/or nutritional factors, regardless of superovulatory treatment used.

However, in our study, repeatabilities for the total number of embryos and number of grades 1 and 2 embryos were low, similar to observations made by Bari et al. (2001) in sheep and Ireland et al. (2007) in cattle. Previous studies (Bari et al., 2000) indicated that the rate of ovulation is the most important determinant of embryo production, with a close relationship between these two parameters. Since the production of embryos depends on a number of additional factors (e.g., efficiency in fertilization rate, collection efficiency) (Bari et al., 2001), it is possible to assume that the influence of these factors on the number of total and transferable embryos was higher than on superovulatory response, thus explaining low values of repeatability for these two variables in the current study.

Nevertheless, when donor ewes were grouped in low and high ovulatory responders to FSH treatment (LR group, $n = 5$, ≤14 corpora lutea; HR group, $n = 5$, >14 corpora lutea), it was observed that the HR group (24.0 ± 3.2 and 21.6 ± 2.9, respectively) significantly doubled the total of recovered and transferable embryos per donor sheep compared with the LR group (13.0 ± 3.2 and 11.0 ± 2.9, respectively) (Bruno-Galarraga et al., 2011).

In conclusion, successive treatments of superovulation and surgical recovery in Merino sheep allowed the recovery of a high number of transferable embryos per donor ewe in a short period of time, although embryo recovery rate decreased in successive collections. The high repeatability of ovarian response to successive superovulatory treatments would have to be associated with a high level of repeatability for the number of transferable embryos recovered for it to be used as a criterion for selecting donors on the basis of their response to the first hormonal treatment.

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