

Effects of Season and Superovulatory Treatment on Embryo Yields in Fine-Wool Merinos Maintained Under Field Conditions

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Contents

The aim of the study was to assess the effects of superovulatory treatment (multiple FSH-dose vs single-shot FSH treatment) and seasonality on embryo yields in fine-wool Merino ewes. Treatment based on multiple FSH-dose consisted of 200 mg of FSH (Folltropin[®]) administered in seven decreasing doses. Single-shot treatment consisted of a single dose of 70 mg of FSH + eCG. In ewes treated with multiple FSH doses, number of recovered embryos was higher (6.0 ± 0.5 vs 3.5 ± 1.0), while non-fertilization rate was lower (12.8 ± 3.9 vs 40.3 ± 9.5) during the breeding season when compared to the non-breeding season ($p < 0.05$); although similar values of recovered Grades 1–2 embryos were observed between seasons. During the breeding season, proportion of responding ewes (98.1 vs 57.1%), ovulation rate (13.9 ± 0.8 vs 3.2 ± 1.2), recovered structures (7.9 ± 0.6 vs 1.7 ± 0.7), total recovered embryos (6.0 ± 0.5 vs 1.2 ± 0.6) and good-quality embryos (5.1 ± 0.5 vs 0.9 ± 0.6) were higher for the multiple FSH-dose treatment than for the single-shot protocol. In a similar way, in the non-breeding season, ovulation rate (11.3 ± 1.8 vs 6.0 ± 1.1) and recovered structures (6.6 ± 1.2 vs 2.7 ± 0.6) were higher for the multiple FSH injections protocol than those for the single-shot treatment, resulting in higher recovered Grades 1–2 embryos (3.2 ± 0.9 vs 1.4 ± 0.5). Current results indicate that seasonal anestrus affected embryo yields when applying multiple FSH-dose superovulatory treatment in Merino ewes, by decreasing the number of recovered embryos although the number of recovered good-quality embryos was not affected. During both seasons, multiple FSH injections produced higher ovarian response and number of viable embryos than the single-shot treatment.

Introduction

The yields obtained by the use of multiple ovulation and embryo transfer (MOET) protocols in sheep are largely hampered by factors causing variability in the superovulatory response and poor embryo recoveries (Sharma et al. 1993). Sheep breed, season and dose and regime of hormone treatment have been recognized as some of the most important of these factors (Gherardi and Lindsay 1980; Mutiga and Baker 1982; Bondurant 1986; Torres et al. 1987).

Embryo production programmes in sheep, being a seasonal breeder species, are mainly conducted during the reproductive period from autumn to winter (Forcada et al. 2006). Extending the application of the techniques to the non-breeding season would allow ewes involved in genetic improvement programmes to undergo embryo recovery procedures throughout the year (Mitchell et al. 2002). However, conflicting results

have been reported in studies designed for determining the effect of season on MOET protocols. Studies of superovulatory treatments in ewes from high latitudes have reported seasonal differences in the rates of ovulation (Gherardi and Lindsay 1980) and fertilization (Mitchell et al. 2002), as well as in embryo quality (Mitchell et al. 2002). Such differences are smaller or non-existent in the Mediterranean and tropical areas (Forcada et al. 2006).

Seasonal effects may be modulated by breed-specific differences (Samartzi et al. 1995; Fukui et al. 1998; Bettencourt et al. 2008). Most of the studies regarding MOET yields have been performed in dairy and, to a lesser extent, in meat breeds. Thus, the first objective of current study was to evaluate the effects of season on the superovulatory response and embryo production in Merino ewes, bred in Patagonia (Argentina) for fine wool production.

Merino constitutes 70% of approximately 7 millions of sheep reared in Patagonia (Paz and Mueller 1994); this breed is well adapted to harsh environment and thus reared under extremely extensive conditions. Management of these animals for the application of MOET protocols needs to be intensive and can involve errors in dosage and injection timing; furthermore, an excessive handling can be stressful for animals and detrimental to reproductive performances (Simonetti et al. 2008). Thus, simplification of superovulatory protocols is clearly beneficial.

Several studies, performed during the breeding season in Merino (Ryan et al. 1984, 1991; Maxwell and Wilson 1990; Maxwell et al. 1990; Evans et al. 1994) and Corriedale sheep (Yamada et al. 1996; Simonetti et al. 2008), have shown similar superovulatory response in classical multiple FSH-dose protocols and treatments based on a single injection of FSH combined with a moderate dose of eCG. On the other hand, other authors report that the use of a 'one-shot' of FSH/eCG induces lower rates of embryo recovery (Sarda sheep: Dattena et al. 1994; Leoni et al. 2001), fertilization (Sarda and Rasa Aragonesa breeds: Ledda et al. 1992) and a lower embryo quality (Sarda sheep: Leoni et al. 2001). Thus, alongside to the first objective of evaluating the effects of season on the ovulatory response and embryo production in Merino ewes, the second objective of current experiment was to determine the efficiency, in breeding and non-breeding seasons, of adapting a 'single-shot FSH/eCG' regime previously developed in our group for *in vitro* embryo production (Gibbons et al. 2007).

Material and Methods

Animals and superovulatory treatments

The sheep used in this experiment were involved in the MOET programme of the Laboratorio de Reproduccion de Pequeños Rumiantes (Estacion Experimental of the Instituto Nacional de Tecnologia Agropecuaria, Bariloche, Argentina; latitude of 41°S). The region presents an annual rainfall of 300 mm and temperature ranging from 2.3 to 13.0°C. Total solar lighting duration is 15:09 and 9:12 h (summer and winter solstices, respectively). All the donor ewes, 4–6 years old and having moderate body condition, were kept outdoors under natural day length and were fed a live-weight maintenance ration.

First trial was performed in non-breeding season (October) and involved a total of 46 sheep; twenty ewes were treated with a multiple FSH-dose protocol, while the remaining 26 were treated with a single-shot FSH/eCG treatment. Second trial was performed in breeding season (April–May) and involved 63 animals; for convenience of the MOET programme, 54 sheep were treated with multiple FSH doses, whilst nine ewes were treated with the single-shot FSH/eCG protocol.

In all females, oestrous cycle was synchronized by the insertion of an intravaginal progestagen impregnated pessary (60 mg of medroxyprogesterone acetate, Progespon®; Syntex, Lab. Syntex, Buenos Aires, Argentina) at Day 0. Two conventionally accepted superovulatory treatment procedures were applied. Multiple FSH-dose treatment consisted of 200 mg of FSH (NIH-FSH-P1, Folltropin®; Bioniche, Belleville, ON, Canada), administered in seven decreasing doses (48 mg × 2, 24 mg × 2, 20 mg × 2 and 16 mg × 1) (Herrera-Camacho et al. 2008). FSH injections were im administered twice daily, starting on the morning of Day 12, 48 h before pessary removal, and finishing 24 h after progestagen withdrawal. A single dose of eCG (200 IU in breeding season and 300 IU in non-breeding season, Novormon 5000®; Syntex, Buenos Aires, Argentina) was administered coincidentally with progestagen removal at Day 14. Single-shot superovulatory treatment consisted of a single dose of 70 mg of FSH plus a single dose of 300 IU eCG, both in breeding and in non-breeding seasons, coincidentally with pessary removal at Day 14 (Gibbons et al. 2007).

In all groups, oestrous detection was performed twice daily, from 24 to 72 h after sponge withdrawal, with adult vasectomized rams. Twelve hours after oestrous detection, ewes were artificially inseminated with 100 million frozen-thawed spermatozoa, by the laparoscopic method described by Maxwell and Butler (1984).

Embryo recovery and viability assessment

At Day 7 after sponge withdrawal, the number of corpora lutea was determined by laparoscopy and embryos, in the stage of late morulae to early blastocysts, were surgically recovered by implementing the prepubic laparotomy technique and under general anaesthesia, using xylazine (8 mg im, Kensol®; König, Montevideo, Uruguay) and ketamine (150 mg im, Ketalar®; Parke Davis, Buenos Aires, Argentina). Local anaesthesia was also administered in the surgical area

(2 ml of lidocaine hydrochloride, Frankaina® 2%; FatroVonFrankel, Buenos Aires, Argentina). All embryos were collected with the aid of laparotomy, flushing each uterine horn, with 20 ml of commercial embryo recovery medium (Bovi112pro®; Minitube, Verona, WI, USA), pre-warmed to 38°C and supplemented with 10% foetal bovine serum (FBS®, 013/07; Internegocios, Buenos Aires, Argentina). The embryo flushing medium was directed from the uterine horn towards the utero-tubal junction, where a catheter was inserted. General antibiotic was administered in the form of oxytetracycline (1 ml/10 kg im) and local antibiotic (gentamicine) at the site of the abdominal incision. Ethical concerns were always taken into account – according to local animal welfare regulations and practices. The flushed embryos were classified based on the morphological criteria and using the guidelines of the International Embryo Transfer Society (1998).

Indexes of superovulatory response

The number and percentage of sheep exhibiting oestrous and superovulatory response (ewes that had, at least, three corpora lutea) were recorded. Thereafter, the following information was recorded for each ewe: number of corpora lutea, number of total recovered structures (embryos + oocytes + zonae pellucidae), number of structures and number of embryos of each grade. The total rate of recovery was obtained, for each animal, by dividing the total number of recovered structures by the number of corpora lutea. Fertilization rate was assessed by dividing the number of recovered embryos by the total number of recovered structures. The rate of recovery of embryos of Grades 1–2 was obtained by dividing the number of recovered Grades 1–2 embryos by the number of total recovered embryos. All rates were expressed as percentages.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance of main effects and interactions between treatment and season on ovarian responses, recovery rates and embryo quality. Oestrous response and proportion of responding ewes were analysed using the CATMOD procedure. Analysis of data was performed using SAS computer package 2003. Results were expressed as mean ± SEM, and statistical significance was accepted from $p < 0.05$.

Results

Significant effects of season on the percentage of ewes bearing oestrous after a multiple FSH-dose protocol or in the mean interval from sponge removal to the onset of oestrous (Table 1) were not found. However, oestrous distribution over time showed a higher dispersion during the non-breeding season. In the breeding season, 94.4% of the ewes exhibited oestrus within 36 h after progestagen withdrawal; most of them (75.9%) were detected in oestrus at 36 h after sponge removal. In non-breeding season, only approximately 65% of the ewes were in oestrus within 36 h after progestagen withdrawal

Table 1. Oestrous response in Merino ewes in breeding and non-breeding seasons with decreasing doses of FSH and a single dose of FSH-eCG

Superovulatory treatment	Season	n	Oestrous response ^a	Hour post-removal of sponges					Interval to onset of oestrus (h) ^b
				24	36	48	60	72	
Multiple FSH-dose treatment	Breeding	54	100.0 ^{ab}	18.5	75.9	5.6			34.4 ± 0.8 ^a
	Non-breeding	20	90.0 ^b	30.0	35.0	15.0	10.0		36.7 ± 2.7 ^a
Single-Shot treatment	Breeding	9	77.7 ^b	22.2	22.2	22.2	11.1	0	49.5 ± 1.5 ^b
	Non-breeding	26	61.4 ^{bc}		3.8	50.0	3.8	3.8	51.4 ± 4.5 ^b

Different letters within columns indicate differences of $p < 0.05$.

^aProportion of ewes that exhibited oestrus.

^bInterval from sponge removal to onset of oestrus.

($p < 0.05$ when compared to breeding season) and only 35% of them in oestrus were observed at 36 h ($p < 0.05$ when compared to breeding season). In a similar way, significant effects of season were not found when applying a single dose of FSH/eCG on the percentage of ewes displaying oestrous signs, mean interval from sponge removal to onset of oestrus or in the time distribution of oestrous appearance. Comparison considering treatment and season showed no differences in oestrous response or oestrous time distribution; however, interval from sponge removal to onset of oestrus was longer for the single-shot treatment than for the multiple FSH-dose treatment, in both seasons (Table 1).

No significant differences were found, for both superovulatory treatments, in the percentage of responding ewes nor in any of the superovulatory indexes considered between seasons (Table 2), except for, in the multiple FSH-dose protocol, the number of total recovered embryos (higher in breeding season, $p < 0.05$) and non-fertilization rate (lower in breeding season, $p < 0.05$).

However, during the breeding season, percentage of ewes responding to treatment and number of corpora lutea, recovered structures, total recovered embryos and recovered Grades 1–2 embryos was significantly higher in the multiple FSH-dose protocol compared to the

single-shot superovulatory treatment (Table 2). In a similar way, in the non-breeding season, ovulation rate and recovered structures were higher for the multiple FSH injections protocol when compared to the single-shot treatment, resulting in higher recovered Grades 1–2 embryos.

Finally, statistical significances for the interactions between season and treatment were not found.

Discussion

Current study indicates a seasonal effect on embryo yields, in terms of total recovered embryos and non-fertilization rate, in fine-wool Merino ewes treated with a multiple FSH-dose treatment, although the number of good-quality embryos recovered was not affected. Overall, such yields were higher when applying a multiple FSH-dose protocol instead of a single-shot FSH/eCG treatment in both seasons.

Simplification of the superovulatory protocols for allowing treatments based on the use of a single injection of FSH has been previously afforded. In sheep, Lopez-Sebastian et al. (1993) showed that FSH diluted in propylene glycol and administered in a single dose significantly increased the ovulation rate in comparison with controls. Dattena et al. (1994) reported that the use

Table 2. Ovulatory and oestrous response, embryo production and quality in Merino ewes in breeding and non-breeding seasons with decreasing doses of FSH and a single dose of FSH/eCG (Mean ± SEM)

	Multiple FSH-dose treatment ^a		Single-shot superovulatory treatment ^b	
	Breeding season	Non-breeding season	Breeding season	Non-breeding season
Animals ^c (n)	54	20	9	26
Oestrous response ^d (%)	100.0 ^{ab}	90.0 ^b	77.7 ^b	61.4 ^{bc}
Responding ewes ^e (%)	98.1 ^a	83.3 ^{ab}	57.1 ^b	62.5 ^b
Ovulation rate (x)	13.9 ± 0.8 ^a	11.3 ± 1.8 ^a	3.2 ± 1.2 ^b	6.0 ± 1.1 ^b
Recovered structures (x)	7.9 ± 0.6 ^a	6.6 ± 1.2 ^a	1.7 ± 0.7 ^b	2.7 ± 0.6 ^b
Structures recovery rate (%)	58.9 ± 3.2 ^a	61.9 ± 5.5 ^a	64.6 ± 13.1 ^a	47.6 ± 6.6 ^a
Total recovered embryos (x)	6.0 ± 0.5 ^a	3.5 ± 1.0 ^b	1.2 ± 0.6 ^b	1.6 ± 0.5 ^b
Embryo recovery rate (%)	47.7 ± 3.6 ^a	36.1 ± 7.7 ^a	48.3 ± 15.6 ^a	24.3 ± 5.0 ^a
Recovered Grades 1–2 embryos ^f (x)	5.1 ± 0.5 ^a	3.2 ± 0.9 ^a	0.9 ± 0.6 ^b	1.4 ± 0.5 ^b
Grades 1–2 embryo recovery rate (%)	84.5 ± 3.2 ^{ab}	90.1 ± 4.5 ^a	50.0 ± 19.2 ^b	87.6 ± 5.6 ^{ab}
Non-fertilization rate (%)	12.8 ± 3.9 ^a	40.3 ± 9.5 ^b	30.0 ± 15.4 ^{ab}	37.6 ± 8.0 ^b

Different letters within rows indicate differences of $p < 0.05$.

^aMultiple-FSH dose treatment consisted of 200 mg of NIH-FSH-P1 given in seven decreasing doses with 200 or 300 IU eCG for the breeding and non-breeding seasons, respectively.

^bSingle-shot superovulatory treatment consisted of 70 mg of NIH-FSH-P1 given as a single injection along with 300 IU eCG in both seasons.

^cAll ewes subjected to treatment.

^dProportion of ewes that exhibited oestrus.

^eProportion of ewes that showed oestrus and had ≥ 3 corpora lutea.

^fEmbryos classified as Grade 1 (excellent or good), Grade 2 (fair), Grade 3 (poor) and Grade 4 (dead or degenerated) (IETS et al. 1998).

of a single dose of FSH in polyvinylpyrrolidone (PVP) resulted in superovulatory yields similar to that obtained with multiple doses of FSH or the injection of a single-shot FSH + eCG in saline. More recently, Simonetti et al. (2008) found a lower percentage of responding ewes to superovulation and a lower ovulation rate when applying FSH + PVP; however, fertilization and viability rates were higher.

The lack of seasonal effect on the percentage of sheep becoming in oestrus found in the present study for both superovulatory treatments, in spite of the reduced cyclic activity reported during the non-breeding season in Merino ewes reared under extensive conditions in Patagonia (Cueto et al. 2000), is in agreement with previous studies in other sheep breeds reared at different latitudes (Chios ewes, Samartzi et al. 1995; Portuguese Black Merino, Bettencourt et al. 2008).

Similarly, no significant differences were found in the mean percentage of total ewes showing oestrus between superovulatory treatments in both seasons, also confirming data from previous reports during the breeding (Corriedale ewes: Yamada et al. 1996; Simonetti et al. 2008 and Merino ewes: Ryan et al. 1984; Maxwell et al. 1990) and non-breeding season (Sarda ewes: Dattena et al. 1994; Leoni et al. 2001). However, interval from sponge removal to onset of oestrus was longer for the FSH/eCG single dose than for multiple injections of FSH combined with eCG. These observations contrast with other authors who have reported a similar or shorter interval from completion of progestagen treatment to onset of oestrus when comparing a single-shot FSH/eCG treatment vs a multiple FSH-dose protocol during the breeding (Simonetti et al. 2008) or non-breeding season (Leoni et al. 2001), respectively. Differences in current results are consistent with prior data, showing a delay in the preovulatory LH surge when eCG was not included in superovulatory treatments (Simonetti et al. 2008), and should be considered in timed artificial insemination programs.

Current study evidences seasonal differences in the number of total recovered embryos and non-fertilization rate when treating female donors with several FSH doses in the breeding season; although it should be bear in mind that both variables are related, the number of recovered embryos is determined by the ovulation and the fertilization rates. Considering that frozen-thawed semen was used in this study, a possible explanation would be related to the follicle population at FSH treatment. Ovarian follicle population is heterogeneous; any follicle ≥ 2 mm in diameter at the time of luteolysis has the potential to ovulate (Driancourt 1991), but the oocytes contained within the follicles need more time to become gradually competent and undergo fertilization and further development (Mitchell et al. 2002). Thus, owing to the short half-life of FSH preparations, multiple FSH-dose protocols need to be administered as once or twice daily injections over a 3- to 4-day period to maintain adequate levels of FSH for promoting follicle development (Akbar et al. 1974; Fry et al. 1987).

In brief, use of exogenous FSH promotes follicle growth but oocytes contained within initially small follicles lag behind in their development (D'Occhio et al. 1999). Currently, it is known that only those growing

follicles larger than 3 mm in size are able to develop in a viable embryo (Veiga-Lopez et al. 2005). The non-breeding season coincides with a period of reduced ovarian activity in ewes and the percentage of small follicles within the total follicle population is greater than during the breeding season, even after FSH stimulation (Mitchell et al. 2002). Therefore, it is possible to hypothesize that, in the present study, an increased number of small follicles was induced to ovulate during the non-breeding compared to the breeding season, many of which contained immature oocytes at the time of ovulation (Mitchell et al. 2002), unable of reaching fertilization.

On the other hand, some authors have reported higher yields, owing to higher recovery rates, in non-breeding season (González-Bulnes et al. 2003; Bettencourt et al. 2008). However, these authors related such effects to a completely different reason: the absence of inhibitory effect from large dominant follicles at the onset of FSH treatment. Another possible explanation, highlighted by Fukui et al. (1998), is related to the fact that premature luteal regression may widely impair the rate of normal fertilized embryos in superovulated sheep; incidence of premature luteal regression has been described to be higher in breeding season than in non-breeding season (Jabbour et al. 1991; Ryan et al. 1991). The lack of seasonal effects on the other superovulatory indexes when applying the classic superovulatory treatment is in agreement with several previous reports (López-Sebastián et al. 1990; Mitchell et al. 2002; Chagas e Silva et al. 2003; Bettencourt et al. 2008).

In addition, in the present study, effects of season in the response of Merino sheep to a single-shot superovulatory protocol were not found, which is also in agreement with other authors (Fukui et al. 1998; Okada et al. 1999), although Fukui et al. (1998) reported breed-related differences in the ovulation rate (no effect in South-Down and Merino \times polled Dorset ewes, but a higher ovulation rate in breeding season in Suffolk sheep). Thereby, from current and previous data, we can hypothesize that discrepancies among reports would be indicative of a different breed-related sensitivity to seasonal effects (i.e.: in the sensitivity of the ovary to exogenous gonadotrophins) (Gherardi and Lindsay 1980); such sensitivity of the ovary to seasonal shifts would be low in Merino ewes. Our results clearly show that, overall, embryo yields were higher when treating donor ewes with a multiple FSH-dose protocol instead of a single-shot FSH/eCG treatment during both seasons.

In the breeding season, proportion of responding ewes, numbers of corpora lutea, total recovered structures, total recovered embryos and recovered Grades 1–2 embryos were significantly higher for the classic treatment compared to the single-shot treatment. Such findings contrast with previous reports in sheep and goats during the same season, in which no differences in ovarian response and embryo yields were found between superovulatory treatments (Ryan et al. 1984; Maxwell and Wilson 1990; Maxwell et al. 1990; Dattena et al. 1994; Evans et al. 1994; Yamada et al. 1996). Similarly, in the current study and during the non-breeding season, ovulation rate, recovered structures and recovered Grades 1–2 embryos were higher for the multiple

FSH-dose protocol than for the single-shot protocol; again, these results contrast with previous findings in Sarda ewes (Dattena et al. 1994).

In this study, poor ovulatory response in the one-shot group in both breeding and non-breeding seasons could be related to a short period for ovarian follicular recruitment (Scaramuzzi et al. 1993; Bartlewski et al. 1999), which is a common phenomenon in superovulatory treatments beginning 24 h before the end of progestagen treatment (Boland et al. 1985; D'Alessandro and Martemucci 2004).

However, recovery rate of good-quality embryos was not affected by the single-shot protocol when compared to the multiple FSH classic treatment during both seasons, as described previously by other authors (Dattena et al. 1994; Yamada et al. 1996; Simonetti et al. 2008), although Leoni et al. (2001) reported differences in embryo quality, in terms of mean number of trophoblastic cells, when comparing the classic vs the one-shot superovulatory treatment, suggesting that the single FSH-eCG injection produced lower quality embryos because of accelerated follicle development when administering high gonadotrophin doses, which can derived in incomplete mature oocytes (Armstrong 1983).

Thus, we can conclude that season had an effect on embryo yields when applying a multiple FSH-dose superovulatory treatment. Results indicate that, during

the breeding season, the number of total recovered embryos increased when compared to the non-breeding season although the number of recovered good-quality embryos was not affected. The use of a single FSH injection for superovulation produced lower ovulatory response and number of viable embryos in Merino sheep during both breeding and non-breeding seasons. Thus, currently, the multiple FSH injections still appear to be the most advantageous hormonal treatment for fine-wool Merino sheep in Patagonia.

Acknowledgement

This study was supported by funds from INTA (Project AESA 203930).

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

M. Cueto and A. Gibbons participated in the research design, acquisition, analysis and interpretation of data; and to draft and revise the paper. F. Pereyra-Bonnet and P. Silvestre participated in the acquisition of data and drafting the manuscript. A. Gonzalez-Bulnes participated in the interpretation of data and drafting and revising the manuscript.

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Submitted: 8 Oct 2010; Accepted: 18 Nov 2010

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