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Improving the efficiency of frozen-thawed semen inseminations in super-ovulated ewes

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Abstract

This project compared the effectiveness of two artificial insemination (AI) procedures using frozen-thawed ram semen in super-ovulated ewes of the Merino and Awassi breeds. In on-farm trials, half the ewes were inseminated twice (at 40h and 46h after progesterone withdrawal) and half were inseminated once (at 52h after progesterone withdrawal). Fertilisation outcomes were assessed at embryo collection 6 days after insemination. The delayed single AI increased the fertilisation rate by 14%, but decreased the proportion of embryos that were of transferable grade by 10%, compared with the standard double AI. Consequently, the overall efficiency of embryo production was similar in the two treatment groups. Also, the results were similar in Merino and Awassi ewes. Importantly, the delayed single AI used half as much frozen-thawed semen, required less labor, and reduced the procedural effects on the animals, compared with the standard double AI. The cost and welfare benefits of the delayed single AI procedure are undeniable, and should promote the use of frozen-thawed ram semen in advanced breeding programs of the sheep industry.

Executive summary

In current practice, the use of frozen-thawed ram semen in multiple ovulation and embryo transfer (MOET) programs usually involves two inseminations at around 40h and 46h after progesterone withdrawal. It was hypothesized that a single artificial insemination (AI) at a time closer to the expected time of ovulation (52h after progesterone withdrawal) would be effective in fertilising oocytes and producing embryos for transfer. The aim of this project was to compare the effectiveness of a standard double AI procedure with that of a delayed single AI procedure in Merino and Awassi super-ovulated ewes.

Three replicate on-farm trials were conducted from mid-April to mid-May in 2013. Two of the trials used Merino ewes (n=14 per trial) and the third used Awassi ewes (n=20). In each trial, the super-ovulated ewes were randomly allocated to two groups. One group received the standard double AI and the other group received the delayed single AI. Six days after AI, the uterine horns of the donor ewes were flushed to recover the embryos produced. Assessments at recovery included the proportion of ova fertilized, and the stages and grades of the embryos produced.

The fertilisation rate in ewes that received the delayed single AI was significantly greater than that in ewes that received the standard double AI (70% vs 56%). The proportion of embryos that were of transferable grade was significantly lower in ewes that received the delayed single AI than in ewes that received the standard double AI (84% vs 94%). Overall, the production of transferable grade embryos from total ova (59% vs 57%) and the mean number of transferable grade embryos per donor ewe (4.9 vs 4.7) did not differ significantly between the groups. There was no influence of breed on any of the outcomes measured.

These results show that the single delayed AI was as efficient as the standard double AI in producing embryos for transfer. Given that the delayed single AI used half as much frozen-thawed semen, required less labor, and reduced the procedural effects on the animals, compared with the standard double AI, the cost and welfare benefits of the delayed single AI procedure are undeniable. Larger scale trials are warranted to confirm the effectiveness of this simple AI modification for use with frozen-thawed ram semen in super-ovulated ewes.

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1. Background

Multiple ovulation and embryo transfer (MOET) is integral to genetic improvement schemes in the sheep industry (Ishwar and Memon 1996). Laparoscopic artificial insemination (AI), a key component of this technology, achieves acceptable fertilisation rates when either fresh or frozen-thawed semen is used (Evans and Maxwell 1987). However, due to the reduced fertilising lifespan of frozen-thawed semen, compared with that of fresh semen (Watson 1995), and the extended interval of ovulation in super-ovulated ewes, it is common practice to carry out a double insemination when frozen-thawed semen is used (Salamon and Maxwell 2000). Obviously, a double insemination requires twice as much frozen-thawed semen to be used and involves more labor, significantly increasing the cost of the procedure. A double insemination also places additional strain on the animal, and increases the potential for procedural-related problems to occur.

Early studies that established the use of frozen-thawed semen in super-ovulated ewes showed that depositing the semen at 6-10 hours prior to the expected time of ovulation was effective (Evans and Maxwell 1987). As ovulations in super-ovulated ewes occur around 54h after progesterone withdrawal, laparoscopic AI should be performed 44-48h after progesterone withdrawal when frozen-thawed semen is used (Evans and Maxwell 1987). Salamon and Maxwell (2000) recommended that a double insemination, with the first performed on the morning of detection of oestrus, would be expected to increase the lambing rate (Salamon and Maxwell 2000). Clearly, a double insemination increases the number of motile sperm present in the uterine horns, and extends the interval of their presence. Therefore, a double insemination procedure in which sperm are deposited at 40 and 46h after progesterone withdrawal has been adopted in commercial practice (D. Osborn, personal communication).

Freshly collected sperm must be exposed to the uterine environment for several hours in order to gain fertilising capacity. This final sperm maturation process, referred to as sperm capacitation, involves the removal of steroids and non-covalently bound epididymal and seminal glycoproteins from the acrosomal sperm head membrane. The resulting membrane destabilization increases permeability to Ca^{2+} and facilitates binding of the sperm to oocytes. It is well established that frozen-thawed sperm undergo capacitation-like changes due to the freezing-thawing process (Watson 1995). These capacitation-like changes affect the interaction of the sperm with the cells of the oviduct (Gillan *et al.* 2000) and decrease fertilisation rates following cervical AI (Salamon and Maxwell 1995). Watson (1995) proposed that capacitated sperm have a shorter fertilising lifespan than uncapacitated sperm and hence should be exposed to oocytes sooner to achieve fertilisation. Therefore it can be argued that insemination of frozen-thawed sperm closer to the expected time of ovulation may improve the rate of fertilisation.

2. Project objectives

The main objective of this on-farm MOET study was to determine whether a single dose of frozen-thawed semen, inseminated at 52h after progesterone withdrawal, produces transferrable grade embryos as effectively as a double dose, inseminated at 40 and 46h after progesterone withdrawal. In addition, Merino and Awassi ewes were used in the trials to determine the influence of breed on the fertilisation outcomes.

3. Methodology

The MOET trials were replicated on-farm in late autumn (mid-April to mid-May), at three different properties in New South Wales, Australia. Standard protocols (see Appendix I) were used to super-ovulate the donor ewes and synchronize the recipient ewes. A highly experienced veterinarian performed all the laparoscopic intrauterine inseminations, embryo collections, and embryo transfers.

Animals

A total of 28 Merino ewes (aged between 2 and 3 year old) and 20 Awassi ewes (aged between 1.5 and 4 years old) were programmed as embryo donors in three separate trials. In each of the two Merino trials, 14 donor ewes were randomly allocated to the two insemination groups (7 donor ewes per group per replicate). In the Awassi trial, 20 donor ewes were randomly allocated to the two insemination groups (10 donor ewes per group).

Super-ovulation of donors and synchronization of recipients

Super-ovulation of donors commenced with the introduction of Zoetis Eazi-Breed CIDRs (controlled intravaginal drug release) devices (on the morning of day 0, which marked the start of the treatment program). At the same time, intravaginal sponges impregnated with 30 mg fluorogestane were introduced to all recipient ewes. All donors had CIDRs replaced on day 7, and each donor received a single intramuscular treatment of Prostaglandin (Ovuprost) on that day. On day 11, 12, 13 and 14 of the treatment program, FSH (Folltropin) was administered twice daily to all the donor ewes in both treatment groups. On the morning of day 13, sponges were removed from recipients, followed by an injection of eCG (Pregnecol). Also on day 13, donors in the delayed single AI group (Donors B) had their CIDRs removed (at 12pm) 6 hours before donors in the standard double AI group (Donors A) had their CIDRs removed (at 6pm) as per Appendix I.

Semen collection

The ram semen used in this study was previously collected from 6 rams (semen from 2 rams was used for each trial). All sperm parameters in the ejaculates were within what is considered to be the normal range for ram semen (volume: 0.75-2 ml; sperm concentration: $\geq 3.2\times 10^9$ spermatozoa/ml; sperm motility: ≥70%; and frequencies of total morphological abnormalities: ≤10%).

Semen freezing and thawing

Briefly, the freshly collected semen was diluted with a Tris-based extender (Tryladyl, MiniTube) to a dilution ratio of 200 million sperm per ml at room temperature, and cooled to 4°C over a 90 min period. The cooled sperm was then loaded into 0.3 ml French straws which were placed on a rack and floated 4 cm above liquid nitrogen for approximately 10 min. The straws were then plunged into and stored in liquid nitrogen.

Thawing was carried out in a 37°C water bath immediately prior to the inseminations. The post-thaw motility of sperm was assessed using phase contrast microscopy. All frozen-thawed semen used for AI displayed greater than 40% progressively motile sperm.

Laparoscopic intrauterine insemination

Ewes were restrained in cradles, in dorsal recumbency. The lower abdominal area was scrubbed with antiseptic Chlorhexidine scrub (Chlorhex) and rinsed thoroughly. Dry gauze sponges were used to remove excess water and antiseptic. Two small stab incisions were made using a scalpel, one either side of the midline and 8 to 10 cm anterior to the mammary glands. The abdomen was insufflated with CO_2 gas via a cannula connected to a CO_2 gas cylinder. At the time of AI, the ovaries of each donor were briefly examined to determine whether ovulations had commenced. The presence of corpora lutea (CLs) indicated that ovulations had commenced, and the absence of CLs indicated they had not. For each donor ewe, semen was loaded into a Robinson pipette (0.2 ml containing a total of 25 million progressively motile sperm) and half (0.1 ml) was deposited into each uterine horn via a second cannula. The ewe was then removed from the cradle and, following a brief recovery period in a nearby pen, returned to pasture.

Standard double AI

In the standard double AI donor ewes (13 donor Merino ewes and 10 Awassi donor ewes), two separate laparoscopic inseminations were performed at 40 and 46 h (10am and 4pm on day 15) after CIDR removal (6pm on day 13).

Delayed single AI

In the delayed single AI donor ewes (15 donor Merino ewes and 10 Awassi donor ewes), only one laparoscopic insemination was performed at 52 h (4pm on day 15) after CIDR removal (12pm on day 13).

Embryo collection and transfer

Embryos were collected 6 days after AI. Ewes were anaesthetized with a gaseous mix of isoflurane, nitrous oxide and oxygen. Following externalisation of the uterine horns via an abdominal incision, a hole was made near the bifurcation of the uterine horns using a 19-G blunt needle, and a 10-G Foley catheter was introduced into the lumen of one of the uterine horns. After inflating the catheter cuff, the uterine contents were flushed with approximately 20 ml of flushing media (Minitube Vigro complete flush) and collected in an embryo searching dish. The flushing procedure

was repeated for the other uterine horn. The presence of corpora lutea (CLs) was confirmed at the time of flushing. Ewes that did not have any CLs present on their ovaries were not flushed and were excluded from the analysis.

Embryo assessment

Examination of the ova and embryos recovered was made by light microscopy. The fertilisation outcomes for each donor ewe included the fertilisation rate (number of embryos/total embryos and ova recovered), the transferrable embryo rate (number of transferrable grade embryos/total embryos), and the developmental stages of the embryos. The developmental and morphological criteria described in the IETS Manual (1998) were used to grade each embryo as being of good (grade 1), fair (grade 2) or poor (grade 3) quality (Manual 1998). The International Embryo Transfer Society scoring system is the standard reference for grading embryos that are exported/imported internationally. The stage category of each ovum/embryo was recorded: unfertilised ovum, degenerated/arrested embryo (2- to 8-cell), early (uncompacted) morula, late (compacted) morula, early blastocyst, blastocyst, expanding blastocyst, and hatching blastocyst. Only embryos of the early morula to hatching blastocyst stages were graded for quality. Grade 1 and 2 embryos were classed as transferrable grade embryos.

Statistical analyses

Statistical analyses were performed using the Genstat statistics software package (Version 16). Regression analysis was used to determine the effects of insemination treatment, breed and ovulation timing. A P value of less than 0.05 was considered to indicate a statistically significant difference.

4. Results

Embryo collections

Embryos were flushed and collected from a total of 44 donors (Awassi; n=18; Merino; n=26). Four of the donors were not flushed because a visual check of their ovaries immediately prior to commencing the flushing process revealed they had not responded (i.e. absence of CLs) to the super-ovulation treatment. The standard double AI group included 23 donors (Awassi: n=10; Merino: n=13), whilst the delayed single AI group included 21 donors (Awassi: n=8; Merino: n=13). The results of the embryo collections are summarised in Table 1.

In trial 1 (Merino ewes), all 7 donors of the standard double AI group were flushed with 6 producing embryos. Of 86 total structures recovered (ova and embryos), 47 were embryos (fertilisation rate: 54.7%), and 46 of these were of transferable grade (transferable rate: 97.9%). In comparison, 6 of the 7 donors of the delayed single AI group were flushed with 4 producing embryos. Of 36 total structures recovered, 32 were embryos (fertilisation rate: 88.9%), and 31 of these were of transferable grade (transferable rate: 96.9%).

In trial 2 (Merino ewes), all 6 donors of the standard double AI group were flushed and produced embryos. Of 64 total structures recovered, 38 were embryos (fertilisation rate: 59.4%), and 36 of these were of transferable grade (transferable rate: 94.7%). In comparison, 7 of the 8 donors of the delayed single AI group were flushed with 5 producing embryos. Of 92 total structures recovered, 60 were embryos (fertilisation rate: 65.2%), and 47 of these were of transferable grade (transferable rate: 78.3%).

In trial 3 (Awassi ewes), all 10 donors of the standard double AI group were flushed with 7 producing embryos. Of 58 total structures recovered, 31 were embryos (fertilisation rate: 53.4%), and 27 of these were of transferable grade (transferable rate: 87.1%). In comparison, 8 of the 10 donors of the delayed single AI group were flushed and produced embryos. Of 63 total structures recovered, 41 were embryos (fertilisation rate: 65.1%), and 34 of these were of transferable grade (transferable rate: 82.9%).

All together, the average numbers of total structures (ova and embryos), embryos and transferable grade embryos recovered per flushed donor ewe was 9.04, 5.04 and 4.74, respectively, for the standard double AI group, and 8.30, 5.78 and 4.87, respectively, for the delayed single AI group.

The distribution of ova/embryos collected from the two insemination groups is shown in Figure 1. The proportion of unfertilised ova was lower in the delayed single AI group than in the standard double AI group. However, the proportion of embryos that had degenerated or arrested development was greater in the delayed single AI group than in the standard double AI group. The delayed single AI group had a greater proportion of morula stage embryos and a lower proportion of blastocyst stage embryos compared with the standard double AI group.

Table 1. Summary of the embryo collection data for the three MOET on-farm trials.

Trial	Breed	Donors flushed	Ova/embryos collected	Total embryos	Fertilised (%)	Total transferable	Transferable (% fertilised)
Standard double AI							
1	Merino	7	86	47	54.7±5.4	46	97.9±2.1
2	Merino	6	64	38	59.4±6.1	36	94.7±3.6
3	Awassi	10	58	31	53.4±6.6	27	87.1±6.0
	TOTAL	**23**	**208**	**116**	**55.8±3.4[a]**	**109**	**94.0±2.2[a]**
Delayed single AI							
1	Merino	6	36	32	88.9±5.1	31	96.9±3.1
2	Merino	7	92	60	65.2±5.0	47	78.3±5.3
3	Awassi	8	63	41	65.1±6.0	34	82.9±5.3
	TOTAL	**21**	**191**	**133**	**69.6±3.3[b]**	**112**	**84.2±3.1[b]**

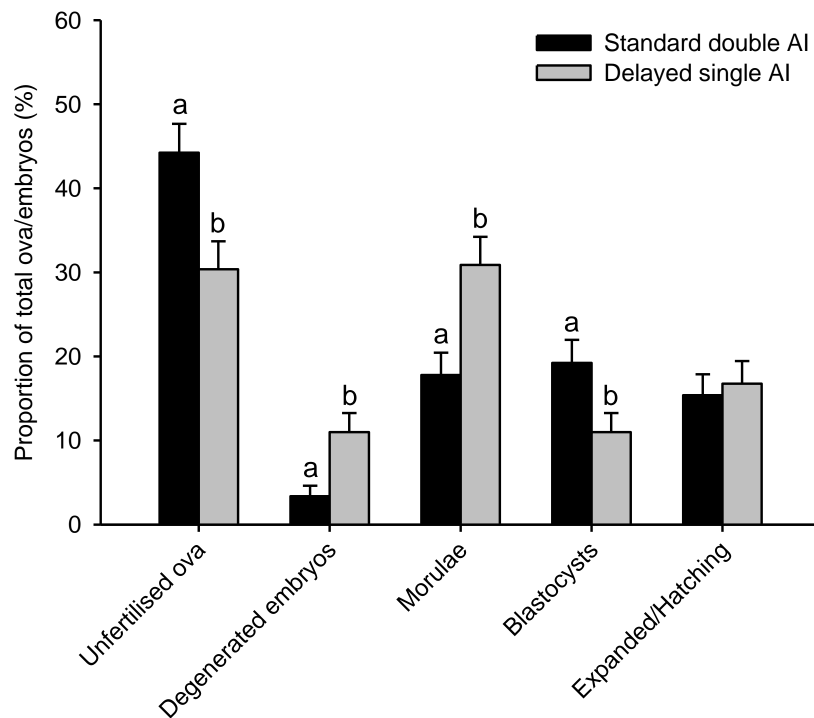

Figure 1. Distribution of ova/embryos collected on Day 6 after insemination. For each stage classification, bars labelled with different letters are significantly different ([ab]$P<0.05$).

Fertilisation rates

As shown in Figure 2, the fertilisation rate for the donor group given the delayed single AI was significantly higher than that for the donor group given the standard double AI (55.8±3.4% vs 69.6±3.3%).

Transferable embryo rates

As shown in Figure 3, the proportion of embryos that were of transferable grade was significantly lower for the delayed single AI group than that for the standard double AI group (84.2±3.1% vs 94.0±2.2%). However, when calculated as a proportion of the total structures recovered (unfertilised ova and embryos), the transferable embryo rates for the 2 groups did not differ (58.6±3.6% vs 57.1±3.6%).

Ovulation timing

Examination of the ovaries at the time of insemination revealed that ovulations had commenced well ahead of the expected 54 h post-CIDR removal in a significant proportion of the donor ewes in both groups. Nine of the 23 (39%) donor ewes of the standard double AI group had commenced ovulations by the 46 h insemination, and 15 of the 21 (71%) donor ewes of the delayed single AI group had commenced inseminations by the 52 h insemination. When the timing of ovulation was considered as a factor, there were no effects on the fertilisation rates or the transferable embryo rates within each donor group.

Pregnancy and lambing data

A total of 61 transferable grade embryos recovered from Awassi donor ewes were transferred to 41 Merino recipient ewes. The recipient ewes were scanned at 55 days of gestation and 26 (carrying a combined total of 37 embryos) were detected as pregnant (63% stick rate; which falls within the normal range of industry results). Subsequently, a total of 23 lambs were born. Unfortunately, a number of late pregnancy abortions occurred, which was attributed to the ingestion of plant toxins. Pregnancy and lambing data was not obtained for the transferable grade embryos recovered from Merino donor ewes because these embryos were frozen for transfer at a later date.

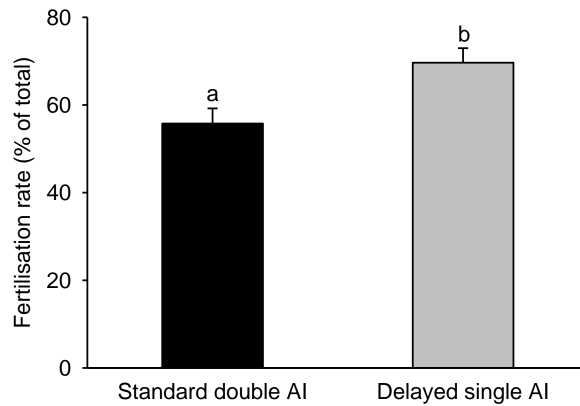

Figure 2. The effect of insemination procedure on fertilisation rates. Bars labelled with different letters are significantly different ($^{ab}P<0.05$).

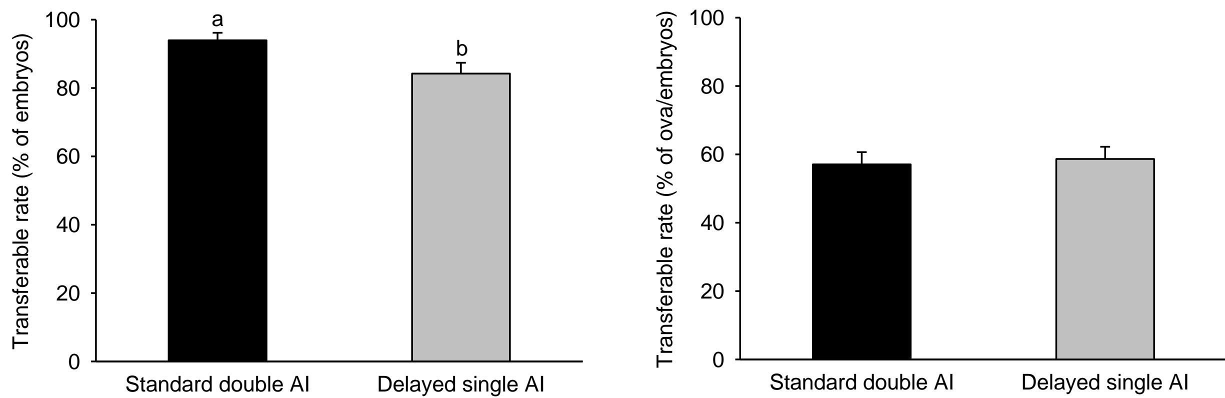

Figure 3. The effect of insemination procedure on transferable embryo rates. The left panel shows the values as proportions of the embryos produced, and the right panel shows the values as proportions of total ova/embryos recovered. Bars labelled with different letters are significantly different ($^{ab}P<0.05$).

5. Discussion

The results of this on-farm MOET study demonstrate that a single insemination of frozen-thawed semen at 52h after progesterone withdrawal produced embryos in super-ovulated ewes as effectively as a double insemination procedure, which involved separate inseminations at 40 and 46h after progesterone withdrawal. Surprisingly, the fertilisation rate was greater in donor ewes that received the delayed single AI than in those that received the standard double AI. Moreover, the difference in fertilisation rate between the insemination groups was consistent among the three trials performed, as well as among the two breeds examined.

Concomitantly, the proportion of embryos that were regarded as being of transferable grade was lower for the delayed single AI group than for the standard double AI group. This disparity may be due to the oocytes of the delayed single AI ewes being fertilised slightly later than those of the standard double AI group. If the oocytes were fertilised later, some of the oocytes may have aged marginally prior to fertilisation, reducing the quality of the resulting embryos. This proposal is supported by the finding that embryos of the delayed single AI group degenerated and arrested development to a greater degree. The greater proportion of morula stage embryos in the delayed single AI group provides further evidence that fertilisation occurred later in this group. However, despite the observed differences between the groups, the two insemination procedures yielded similar overall efficiencies of transferable grade embryo production.

Successful AI using frozen-thawed ram semen can be achieved by depositing the semen in the uterine horns 6-10 h prior to the expected time of ovulation (Evans and Maxwell 1987). Additionally, a double insemination, with the first insemination performed in the morning of detection of oestrus, has been proposed to increase lambing rates (Salamon and Maxwell 2000). In the present study, the inseminations of the standard double AI procedure were performed at times that are consistent with those recommended. The results clearly show that inseminating frozen-thawed semen only 2h prior to the expected time of ovulation was also effective. This suggests that frozen-thawed semen requires minimal exposure to the uterine environment in order to gain fertilising capacity, which may be quite limited once acquired. That the first insemination of the double insemination procedure appears to have been of little or no benefit supports this suggestion.

The ovulation timing results indicate that there was considerable variation between animals. Previous studies have shown that most ovulations in FSH-treated ewes occur between 54 and 60h after progesterone withdrawal (Walker *et al.* 1986, Evans and Maxwell 1987). In the present study, 39% of standard double AI ewes had commenced ovulations by the 46h insemination, and 71% of delayed single AI ewes had commenced ovulations by the 52h insemination. The observed lack of synchrony in the time at which ewes began to super-ovulate was similar for Merino and Awassi ewes and is consistent with the previous observations of Walker *et al.* (1986). The findings of Walker *et al.* (1986) indicate that premature ovulations contribute little (about 10%) to total ovulations and that a significant proportion of ewes (about 30%) ovulate over a prolonged period (at least 12h). Such asynchrony

and ovulation timing spread may explain the lack of an effect of ovulation timing on the fertilisation rate and transferable embryo rate within each group in the present study.

The numbers of transferable grade embryos recovered per donor ewe was approximately 25% lower than the industry average (D. Osborn, personal communication). A possible reason for this result is that the trials were performed towards the end of the breeding season. The ovulation rate in ewes is known to decline gradually over the course of the breeding season (Scaramuzzi and Radford 1983). A decreased responsiveness to gonadotrophins later in the breeding season, or other factors such as reduced body condition, may have contributed to the lower than average rates.

In conclusion, the delayed single AI procedure was as effective as the standard double AI procedure for the insemination of super-ovulated ewes with frozen-thawed semen. The delayed single AI procedure used half as much frozen-thawed semen, and required less labor, than the standard double AI procedure. Furthermore, the delayed single AI procedure reduced the strain of a double insemination on the animals. The findings indicate that the first of the double inseminations provided little or no benefit, and that a single insemination at 52h after progesterone withdrawal was sufficient to achieve effective fertilisation. Larger scale trials that examine lambing rates are warranted to confirm the effectiveness of the delayed single AI procedure when frozen-thawed semen is used. The cost and welfare benefits of such a simplified procedure are undeniable, and should promote the use of frozen-thawed ram semen in advanced breeding programs of the sheep industry.

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Appendix I: Protocol for super-ovulation of donors and synchronization of recipients

Day	Time	Animal	Activity	Drug	Dose
0	8 am	Donors Recipients	Insert CIDRs Insert sponges		
7	8 am	Donors	Change CIDRs and inject	Ovuprost	1.0 ml
11	8 am 6 pm	Donors Donors	Inject (Adult/Maiden) Inject (Adult/Maiden)	Folltropin Folltropin	2.5 ml/2.0 ml 2.0 ml/2.0 ml
12	8 am 6 pm	Donors Donors	Inject (Adult/Maiden) Inject (Adult/Maiden)	Folltropin Folltropin	2.0 ml/1.5 ml 2.0 ml/1.5 ml
13	6 am	Recipients	Remove Sponges & Inject	Pregnecol	2.0 ml
	8 am	Donors	Inject	Folltropin	1.5 ml
	12 pm	Donors B	Remove CIDRs and Inject	Folltropin	1.0 ml
			and Inject	Pregnecol	1.0 ml
	6 pm	Donors A	Remove CIDRs and inject	Folltropin	1.0 ml
			and inject	Pregnecol	1.0 ml
14	8 am	Donors	Inject	Folltropin	1.0 ml
	6 pm	Donors	Inject	Folltropin	0.5 ml
			Remove feed and water		
15	9 - 10 am	Donors	AI frozen-thawed semen		39 - 40h
	3 - 4 pm	Donors A	AI frozen-thawed semen		45 - 46h
	3 - 4 pm	Donors B	AI frozen-thawed semen		51 - 52h
20	1 pm	Donors	Remove feed and water		
	5 pm	Recipients	Remove feed and water		
21			Collect and transfer embryos		