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### **Fertility in the ewe following cervical insemination with fresh or frozen thawed semen at a natural or synchronized oestrus**

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Fertility in the ewe following cervical insemination with fresh or frozen thawed semen at a natural or synchronized oestrus

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#### **Abstract**

Artificial insemination (AI) in sheep is currently limited by the poor fertility obtained following non-surgical intracervical insemination of frozen thawed semen. An exception to this general finding is the non-return rate of around 58% reported for large scale on-farm AI in Norway. The objective of the present study was to determine if similar results could be obtained under Irish conditions. Comparisons were made between semen collected, and frozen, from rams in Norway (NOR) and Ireland (IRL). The effects of synchronisation and inseminator were also examined. Parous ewes (n=297) of various breed types were inseminated to a natural (N) or synchronised (S) oestrus with either fresh (from Irish rams) or frozen-thawed (IRL and NOR) semen. Ewes were randomly assigned, within breed, to the following treatment groups: (i) Fresh-N: n=28, (ii) Fresh-S: n=30, (iii) IRL-N: n=62, (iv) IRL-S: n=50, (v) NOR-N: n=68, (vi) NOR-S: n=59. Within each group, ewes were inseminated by an experienced Norwegian or by an Irish inseminator. Pregnancy rate did not differ significantly between ewes inseminated to a natural

or synchronised oestrus nor between Norwegian and Irish frozen semen. The proportion of ewes pregnant after insemination with fresh semen was 0.82 and 0.70 (treatments i and ii) compared with 0.40, 0.52, 0.34 and 0.37 (treatments (iii)(vi)) for frozen semen ( $P < 0.001$ ). Corresponding litter sizes ( $\pm$ S.E.), adjusted for ovulation rate, were  $2.9 \pm 0.22$ ,  $3.3 \pm 0.23$ ,  $2.2 \pm 0.21$ ,  $1.7 \pm 0.21$ ,  $2.2 \pm 0.21$  and  $2.1 \pm 0.21$  (fresh versus frozen;  $P < 0.001$ ). There was an interaction between semen type (fresh or frozen) and oestrus type (N or S) for litter size due to an increased adverse effect of frozen semen on litter size in synchronised ewes ( $P < 0.05$ ). Pregnancy rate was significantly influenced by breed of ewe ( $P < 0.01$ ) and inseminator ( $P < 0.05$ ). These results suggest that ewe breed may be a critical determinant of the potential for the exploitation of cervical insemination of frozen-thawed semen in sheep breeding programmes.

Author Keywords: SHEEP; Fertility; Cervical insemination; Fresh or frozen thawed semen; Synchronisation

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### 1. Introduction

Artificial insemination (AI), when used in conjunction with accurate progeny testing schemes, can substantially increase the rate of genetic progress (Eppleston and Maxwell, 1993). Such benefits have been clearly demonstrated in the dairy industry (Inskeep and Peters, 1981) and these benefits would also apply to sheep (Clarke et al., 1986). While cervical AI with fresh semen yields acceptable conception rates, the short shelf life of fresh semen coupled with a natural limitation on the number of semen doses achievable per unit time restricts the widespread use of individual sires (Gordon, 1997). Therefore, to maximise the genetic benefits through the use of AI, frozen thawed semen is a prerequisite. Ideally this should be by simple

cervical insemination rather than by invasive procedures such as laparoscopy. Unfortunately, expectations of obtaining a fertility rate similar to that obtained with fresh semen are not, or only rarely, fulfilled with cervical AI of frozen thawed semen (Salamon and Maxwell, 1995). Low fertility rates following cervical insemination of frozen thawed semen are attributed to damage to spermatozoa during the freeze thaw process resulting in impaired sperm transport, viability and fertilization capacity and increased embryonic mortality (Lightfoot and Salamon, 1970 and Salamon and Maxwell, 1995). While Maxwell et al. (1999) have shown that the deleterious effects of the freeze thaw process can be overcome by re-suspension of thawed spermatozoa in a diluent containing ram seminal plasma this would present practical difficulties under field conditions. Furthermore, a mean conception rate of 58% has been reported for cervical AI with frozen thawed semen involving large numbers of ewes under field conditions (Olesen, 1993).

Ewes in Norway are inseminated to a natural oestrus, which would be impractical under Irish conditions because flocks are small and ewes are at pasture during the breeding season (Gordon, 1997) whereas they are housed in Norway.

Furthermore the breeds involved are quite different from those found in Ireland. Thus, the primary objectives of the present study were to evaluate the effects of semen source (Irish rams or Norwegian rams) and oestrus synchronisation on pregnancy rate following cervical insemination with frozen thawed semen. The effect of inseminator was also examined.

## 2. Materials and methods

### 2.1. Animals

The experiment was carried out during the breeding season using culled parous ewes (n=297) which were either purebred Finnish Landrace, crossbred ewes from Scottish Blackface dams (Hill-cross) or other crossbreds (Lowland-cross) involving a variety of lowland (mainly Galway, Suffolk and Belclare) breeds. All animals used were reproductively sound and were managed in accordance with the guidelines for the accommodation and care of animals under Article 5 of the European Community Directive, 86/609/EC.

### 2.2. Semen collection and processing

Semen was collected from five mature rams of proven fertility (three Suffolk, one Texel and one Finnish Landrace) using an artificial vagina fitted with a graduated test tube. On each collection day, 2 or 3 consecutive ejaculates (during a time period of approximately 30 min) were collected from each ram. Semen was processed and frozen according to Norwegian techniques, as described by Andersen-Berg and Aamdal (1991) and Olesen (1993). Briefly, each ejaculate was assessed for concentration and initial motility. Ejaculates without a minimum concentration of  $3 \times 10^9$  spermatozoa/ml and a minimum initial motility of 3 (scale 5) were discarded. Acceptable ejaculates from an individual ram were pooled and then diluted in a

two-step procedure with a skim-milk egg-yolk glycerol extender. After equilibration and adaptation for 2-3 h at 5 °C the semen was reconcentrated by centrifugation at 700g for 10 min and rediluted to a sperm concentration of 800 x 10<sup>6</sup> ml, verified by haemocytometry. The semen was then loaded into 0.25 ml Minitub straws (Minitub GmbH, Tiefenbach, Germany) and frozen in liquid nitrogen vapour in a programmable freezer (Planar Series II, Planar Products Ltd., Middlesex, UK). The temperature was reduced from 5 to 10 °C at a rate of 5 °C/min, and from 10 to 13 °C at a rate of 5 °C/min.

Straws were then plunged into liquid nitrogen (-196 °C) and stored at this temperature. Random straws from the daily collection of each ram were tested after freezing and if the motility was less than 45-50% then that semen batch was discarded. Prior to insemination/testing, straws were thawed at 70 °C for 8 s (Andersen-Berg and Aamdal, 1991).

The same five rams were used to provide fresh semen on the day of insemination.

Semen was diluted in a skim-milk egg-yolk extender to give an insemination dose of 200 x 10<sup>6</sup> spermatozoa (0.25 ml Minitub straws) and held at 15 °C until insemination (within 1 h of collection).

Frozen semen from three Dala and two Spael rams was imported from a commercial AI centre in Norway, supplied in 0.25 ml Minitub straws, and held at -196 °C until insemination.

### 2.3. Experimental design and AI procedure

The design involved six treatment groups corresponding to three semen types by two synchronisation treatments. The semen types were fresh semen from Irish rams (Fresh), frozen semen from Irish rams (IRL) and frozen semen from Norwegian rams (NOR). Ewes were inseminated to either a synchronised oestrus (S) or to the first natural oestrus (N) after a synchronised oestrus. Ewes were randomly assigned, within breed, to one of the treatment groups subject to the restriction that a maximum of 60 ewes were inseminated with fresh semen. The treatment combinations (number of ewes) were as follows: Fresh-N (28), Fresh-S (30), IRL-N (62), IRL-S (50), NOR-N (68) and NOR-S (59).

Synchronisation of oestrus was accomplished by progestagen administration over a 12-14 day period via intravaginal pessaries containing 40 mg of flugestone acetate (Chronogest, Intervet UK Ltd., Milton Road, Cambridge). Ewes inseminated to a natural oestrus were initially synchronised with progestagen pessaries and inseminated at the subsequent return oestrus, which was detected by crayon harnessed vasectomised rams joined 13 days after pessary removal. Ewes detected

in oestrus (crayon mark) in the morning (07:00 h) were inseminated the same evening (17:00 h), while ewes detected in oestrus in the afternoon (16:00 h) were inseminated the following morning (09:00 h). Ewes inseminated to a synchronised oestrus received an injection of 400 i.u. PMSG (Intervet) at pessary removal and were inseminated once, 57 h later. All ewes were restrained in a standing position and the cervix was located using a speculum fitted with a light source. The semen was deposited as far as possible into the cervix without using force. Within each treatment group, half of the ewes were inseminated by an Irish inseminator and half were inseminated by an inseminator with extensive field-AI experience in Norway.

Ewes were maintained at pasture throughout and were slaughtered 27-42 days post insemination. The reproductive tracts were collected at the abattoir and the pregnancy rate, ovulation rate (number of corpora lutea) and litter size determined.

#### 2.4. Statistical analyses

Data on pregnancy rate were analysed using the GENMOD procedure of SAS (SAS, 1996) with the logit link function. Effects included in the model were semen type (fresh, Irish frozen, Norwegian frozen), synchronisation treatment, inseminator and ewe breed. The initial model included interaction effects.

Models for subsequent analyses were reduced by eliminating non-significant interactions ( $P > 0.05$ ). Individual ram effects were examined using only the data for ewes inseminated with frozen-thawed semen. Data on ovulation rate and litter size were analysed using least squares procedures to fit a model with effects for ewe breed, semen type, synchronisation treatment and their interactions (GLM procedure of SAS).

### 3. Results

Pregnancy rate for all combinations of semen type and synchronisation treatment are presented in Table 1. There was no significant difference in pregnancy rate between ewes inseminated to a natural oestrus and those ewes inseminated at 57 h after progestagen withdrawal. Ewes inseminated with fresh semen had a significantly higher pregnancy rate than any group inseminated with frozen-thawed semen, regardless of oestrus type ( $P < 0.001$ ). When ewes were inseminated (with frozen-thawed semen) to a natural oestrus there was no evidence for any difference in pregnancy rate between those ( $n=67$ ) inseminated in the morning and those ( $n=63$ ) inseminated in the afternoon ( $P > 0.9$ ), despite an expected mean difference of about 4 h in the interval from onset of oestrus to insemination. In ewes that were inseminated with frozen-thawed semen, a higher proportion was pregnant when Irish frozen-thawed semen was used, but this difference was not statistically significant ( $P > 0.1$ ). Inseminator significantly ( $P < 0.05$ ) influenced pregnancy rate (36% ( $n=117$ ) versus 45% ( $n=122$ ) for frozen semen and 66% ( $n=29$ ) versus 86% ( $n=29$ ) for fresh semen). In addition, breed of ewe had a significant effect on pregnancy rate (Table 2,  $P < 0.01$ ). There was no evidence for any interaction between ewe breed and semen type.

Table 1. Pregnancy rate (%) for ewes inseminated with fresh or frozen-thawed semen at either a natural or synchronised oestrus

Table 2. Effect of ewe breed on pregnancy rate after insemination with fresh or frozen-thawed semen

Litter size, adjusted for ovulation rate, was significantly lower when ewes were inseminated with frozen-thawed semen compared with fresh semen ( $P < 0.001$ , Table 3). There was no significant difference in litter size between ewes inseminated with Irish frozen-thawed and Norwegian frozen-thawed semen. While there was no overall significant difference in pregnancy rate between ewes inseminated to a natural or synchronised oestrus, there was an interaction between semen type (fresh or frozen-thawed) and oestrus type for litter size. This reflected the

fact that the adverse effect of frozen-thawed semen on litter size was greater in synchronised ewes ( $P < 0.05$ ) than in ewes inseminated at a natural oestrus.

Table 3. Effect of semen type and synchronisation on mean ( $\hat{A} \pm \text{S.E.}$ ) litter size adjusted for ovulation rate

The data in Table 4 show the individual variation in pregnancy rate among rams following insemination with frozen-thawed semen. While fertility ranged from 17 to 50% for Norwegian rams and from 40 to 58% for Irish rams this variation did not reach statistical significance (Irish:  $P = 0.7$ , Norwegian:  $P = 0.08$ ).

Table 4. Effect of ram on pregnancy rate for frozen-thawed semen

#### 4. Discussion

The pregnancy rate obtained following cervical AI with fresh semen is similar to that previously reported under Irish conditions (Smith et al., 1977). It is also consistent with lambing rates of 65-75% reported by Evans and Maxwell in Australia (1987) and by Jonmundson in Iceland (1986). Pregnancy rate was significantly lower in ewes inseminated with frozen-thawed semen regardless of whether ewes were inseminated to a natural or synchronised oestrus. This is consistent with the findings of others (Fiser et al., 1987; Findlater et al., 1991 and Maxwell and Watson, 1996) and is not surprising as the fertilizable life of frozen-thawed spermatozoa is often half that of fresh spermatozoa (reviewed by Salamon and Maxwell, 1995) and the proportion of progressively motile cells is reduced by the processes of cooling, freezing and thawing (Haresign, 1990 and Maxwell and Watson, 1996), resulting in impaired ability to penetrate the cervix (Lopyrin and Loginova, 1958 and Maxwell and Watson, 1996).

The finding that pregnancy rate did not differ significantly between ewes inseminated with Irish frozen-thawed semen and ewes inseminated with Norwegian frozen-thawed semen implies that the high conception rate achieved in Norway is not due to some inherent quality

that makes semen from Norwegian rams less susceptible to the deleterious effects of freezing. However, the pregnancy rate achieved in this study was clearly lower than that achieved in Norway (Olesen, 1993) despite the fact that Norwegian semen processing and insemination techniques were employed. The lower pregnancy rate is also unlikely to be due to insemination technique as levels of fertility obtained with fresh semen were acceptable. In addition, the Irish inseminator achieved a higher pregnancy rate than the Norwegian inseminator who achieves consistently good results in Norway (Olav Grotte, personal communication). Therefore, the discrepancy is more likely to be due to differences between ewe breeds used in Ireland compared with Norwegian ewe breeds. It was not possible to test the fertility of Norwegian ewes with Irish frozen-thawed ram semen in this study because of Norwegian restrictions on semen imports. However, the high pregnancy rate achieved in Finnish Landrace ewes (also a Northern European breed) add weight to the hypothesis that ewe breed is a factor in the exceptionally good results obtained in Norway.

Differences in pregnancy rate may be due to factors such as timing of ovulation or the structure of the cervix, which in turn may be breed dependent. Asynchrony of AI and ovulation is probably the commonest cause of failure of AI (Jabbour and Evans, 1991). The effect of asynchrony is likely to be exacerbated when frozen-thawed semen is used. The time of ovulation varies with many factors, including type of synchronisation, breed and season (Evans, 1988) and also varies both within and between flocks (Walker et al., 1989). It is possible that the timing of cervical AI in this study (57 h post pessary removal) was optimal for the Finn ewes but either too early or too late for the other breeds.

Therefore, further studies are required to determine if breed differences in the timing of ovulation are a factor in the breed effects on conception rate found in the present study.

Alternatively, the differences in reproductive performance between different ewe breeds may be due to differences in the structure of the cervix. The anatomical structure of the ovine cervix effectively prevents intrauterine deposition of semen. Consequently, cervical AI with frozen semen results in low fertility (Dattena et al., 1992). Ritar and Salamon (1983) found differences between herds of goats in depth of penetration of the cervix and these differences are related to pregnancy rate. It may be that a similar variation exists between sheep genotypes. The anatomy of the cervix was not examined in this study, but when Merinos and British breeds were compared (Halbert et al., 1990) no anatomical differences were found.

No significant differences in fertility were found between ewes inseminated to a synchronised oestrus and ewes inseminated to a natural oestrus contrary to reports that synchronisation causes reduced fertility after cervical AI and after natural mating (Quinlivan and Robinson, 1969; Hawk and Conley, 1975; Allison and Kelly, 1978; Andersen et al., 1973 and Findlater, 1989).

The negative effects of progestagen treatment on fertility have been attributed to reduced sperm transport through the cervix (Killeen and Caffery, 1982 and Armstrong and Evans, 1984) although such effects were not noted by Allison and Robinson (1970) Langford et al. (1979) or by Smith et al. (1995). The absence of evidence for an effect in the present study is encouraging as hormonal control of the oestrous cycle is considered essential for widespread application of AI in the Irish sheep industry (Gordon, 1997).

The finding that litter size, when adjusted for ovulation rate, was significantly lower in ewes inseminated with frozen-thawed semen concurs with the results of Olesen (1993) and Langford et al. (1979) who reported increased embryonic mortality between day 18 and term when frozen-thawed semen was used.

However, Olavsson (1980) reported little difference in non-return rate after 25 days and lambing rate but Olavsson used unsynchronised ewes whilst Langford et al. (1979) used synchronised ewes. This apparent difference may reflect the present finding that there was an interaction between semen type (fresh or frozen-thawed) and oestrus type indicating that any detrimental effect of frozen-thawed semen on litter size was exacerbated by synchronisation.

There is considerable variation in the fertility of ewes following AI (reviewed by Evans, 1991) and at least part of this variation is thought to be due to differences in fertility between semen from different rams. While there was variation in fertility between individual Irish and Norwegian rams in this study, it was not statistically significant. This is not consistent with most other studies where considerable variation in fertility among rams has been reported following both cervical (Curnock et al., 1984; Olesen, 1993; Smith et al., 1995 and Windsor, 1997) and laparoscopic (Butler and Maxwell, 1988; Eppleston et al., 1991; Eppleston and Maxwell, 1995 and Smith et al., 1998) insemination. An in vitro system that could accurately predict field fertility would facilitate stricter selection of AI rams with regard to the quality of frozen-thawed semen and would provide a valuable tool for increasing conception rate. However, this may also have the (undesirable) effect of decreasing the selection differential for production traits.

There is considerable potential for AI to impact favourably on the genetic improvement of sheep. The realisation of this potential would be greatly facilitated by the availability of an effective AI technique based on cervical insemination of frozen-thawed semen. This will require further understanding of the role of factors such as the optimal time for insemination and the physiological or physical determinants of the barrier presented by the cervix.

If ewe breed is confirmed as a critical determinant of the success of cervical insemination with frozen-thawed semen then this will provide an important tool for identifying the factors that limit pregnancy rate from this procedure.

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