End of Project Report

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AI FOR SHEEP USING FROZEN-THAWED SEMEN

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SUMMARY

International experience has been that cervical insemination of sheep with frozen-thawed semen usually yields unacceptably low pregnancy rates (10 to 30%). An exceptional case has been Norway where non-return rates in on-farm usage are around 60%. The objective of the work described in this report was to develop an AI procedure for Irish conditions, based initially on Norwegian protocols, using semen from individual rams. Such a procedure would greatly facilitate and enhance genetic improvement programmes for sheep.

The work undertaken had two separate aspects:-

(i) studies on semen, including processing and freezing methods, laboratory evaluation of semen quality post thawing and the relationship of *in vitro* evaluation to fertilisation rate *in vivo*

(ii) studies on pregnancy rate following AI in relation to issues such as ram breed effects, effects of synchronisation, operator differences and the role of ewe breed – inducing the timing of ovulation and various physical and physiological assessments of the cervix at AI.

The main results in relation to semen studies were that, while a range of differential staining procedures could be used to objectively evaluate semen with respect to proportion of live speramatozoa and the integrity of sperm cells after thawing, these results were not useful as indicators of fertilisation capacity in vivo. The *in vitro* fertilisation (IVF) of sheep oocytes recovered from abattoir material gave promising results as a method for evaluating the fertilisation capacity of frozen-thawed semen. The technique requires further validation.

A consistent feature of the studies on semen was the existence of significant variation among individual rams in the fertilisation capacity of their frozen-thawed semen - both in an IVF system and *in vivo*. The *in vivo* results clearly showed that, all other things being equal, the range in pregnancy rate among individual rams was from around 25% to around 65%. There was, however, no difference between breeds of ram, based on the fact that pregnancy rate was similar for ewes inseminated with semen from Norwegian rams (collected and frozen in Norway) and semen from Irish rams. It was concluded that the good pregnancy rates regularly achieved in Norway is not due to any inherently better freezability of semen from Norwegian breeds.

A major and highly consistent finding from the studies reported herein was major differences among ewe breeds with respect to pregnancy rate to AI with frozen-thawed semen, regardless of the source of that semen. Thus, the average pregnancy rate over the set of AI experiments was as follows:-

Finnish Landrace ewes	61%
Belclare ewes	44%
Belclare x Scottish Blackface ewes	44%
Texel ewes	31%
Suffolk ewes	12%

Follow-up studies of the possible reasons for this difference did not support the hypothesis that altering the timing of AI according to ewe breed would alter the results, nor were ewe breed differences attributable to cervical factors such as physical dimensions, mucus secretion or depth of penetration of the cervix AI.

One of the main conclusions from the project is that pregnancy rate can be raised by 15 percentage points if laboratory procedures, such as IVF, can

identify the rams that yield semen with a fertilising capacity, post freezing, in the top 25%. Thus, the expected pregnancy rate for Texel ewes would be raised to 46% while that for Belclare ewes would be over 60%.

It should be possible to achieve pregnancy rates over 50% for some breeds in the near future and this should make cervical AI of frozen-thawed semen a practicable technology in these cases.

Further work is required to identify the factors responsible for the large ewe breed effect on pregnancy rate and the perfect procedures for identifying the best “freezers” among panels of rams for use in AI programmes.

INTRODUCTION

Artificial insemination (AI) is probably the most important single technique devised to facilitate the genetic improvement of animals. The widespread use of AI in cattle has allowed accurate genetic evaluation and rapid dissemination of genetic merit on a national and international basis to the benefit of both breeder and consumer. It has also enabled the use of sophisticated data analysis procedures to identify animals of superior performance. The availability of an efficient sheep AI service would yield similar benefits and would greatly enhance the scope for pedigree and commercial breeders to respond positively and effectively to consumer demands. The widespread use of AI and the realisation of its full potential depend essentially on the use of frozen semen, and thus, on the availability of techniques that result in acceptable fertility. However, the very low level of fertility obtained when frozen-thawed semen is used for cervical insemination in sheep has stemmed widespread interest/uptake of AI by the sheep sector. The alternative, laparoscopic AI, is an effective method of insemination with frozen-thawed semen, but is costly and this limits its use. Welfare concerns may also limit the use of this procedure. It is therefore desirable and necessary to develop non-surgical procedures that could form the basis of making AI a practical reality for the sheep industry.

Advantages of AI

The use of AI, based on frozen-thawed semen, can greatly increase the number of offspring produced per sire per year because a ram has the potential to produce enough spermatozoa to inseminate thousands of ewes. Thus, genetically superior rams could be made accessible to all sectors of

the sheep industry, thereby rapidly improving the quality of output from the sector.

In Ireland, genetic improvement is hampered by the small size of pedigree flocks, which reduces the intensity of selection and leads to lack of consistency in breeding goals. Furthermore, available breeding value estimates are often only valid for within-flock selection. These constraints severely impair the rate of genetic improvement being achieved nationally (Hanrahan, 1997). Sire Reference Schemes are currently being adopted by some breeds to overcome these disadvantages and this also increases the precision of breeding value estimates through the application of BLUP procedures. Sire Reference Schemes are based on the use of a small set of rams to produce progeny in all flocks as a basis for providing genetic links among flocks. While these linkages can be produced by the transfer of ewes to a single location for mating and/or by rotating rams among flocks during the mating period these options are stressful and may affect fertility. There are also disease risks attendant upon intermixing of animals from different flocks. AI based on frozen semen would eliminate these problems and allow the widespread dissemination of valuable genetic material even to small flocks, thereby leading to effective genetic improvement of the national sheep population.

AI can also facilitate the introduction of new genetic material through international exchange of semen.

Disadvantages of AI

Fertility, encompassing both pregnancy rate and litter size, is adversely affected by AI, especially AI based on frozen-thawed semen. Despite

intensive laboratory studies, the freezing and thawing of ram semen still significantly reduce the viability of spermatozoa and make it difficult to achieve high fertilisation rates. The major obstacle to fertility in ewes cervically inseminated with frozen-thawed semen, is the establishment of a large enough population of viable spermatozoa in the cervix and impaired transport from the cervix to the site of fertilisation.

As with any technology, production costs are incurred when AI is used. Costs include collection and assessment of semen, processing, freezing and storage of semen, delivery of AI, labour, and drugs for synchronisation.

Semen collection and preparation

Semen can be collected by artificial vagina (AV) or by electrical stimulation. The AV method is preferable because it does not stress the animal, it is quick and simple and results in the collection of better quality semen. After collection the quality of semen is assessed for volume, motility, concentration and morphology on an individual ejaculate basis. Accepted ejaculates can then be inseminated raw (undiluted) as soon as possible after collection or stored in protective media on a short term basis. This is known as 'chilled' or 'liquid stored' semen where spermatozoa are held at 5 ^{o}C and inseminated within 24 hours. Semen can also be frozen for long term storage. Frozen semen may be stored in PVC straws (0.5 ml or 0.25 ml), Minitηb straws (0.25 ml) or as pellets. Pellet freezing of ram semen has produced better conception rates compared with PVC straws, but not significantly better than Minitηb straws. These provide a useful alternative to pellets allowing for individual dose identification (required for export), easier storage and are less time consuming at insemination.

Insemination techniques

Insemination of sheep may be vaginal, cervical, transcervical or intrauterine. The various methods differ in their complexity, cost and effectiveness.

Vaginal: This is the simplest form of insemination and involves depositing fresh semen in the anterior vagina without any attempt to locate the cervix. Reported success rates are highly variable and this method is unsuitable for use with frozen semen.

Cervical: This is a cheap and relatively easy method of insemination. The cervix is located, via a speculum fitted with a light source. The cervix of the ewe is convoluted in structure and does not dilate during oestrus. As a result it is generally only possible to deposit the semen in the first fold of the cervix. Conception rates with fresh or 'chilled' semen are good (65 to 75%) but unacceptably low (10 to 30%) if frozen-thawed semen is used. An exception is in Norway where mean conception rates of 60% have been reported (Olesen, 1993).

Transcervical: This method involves grasping the cervix and retracting it into the vagina with a pair of forceps to allow an inseminating instrument to be introduced into the cervical canal. Acceptable conception rates (57%) have been reported by Halbert *et al*., (1990) but not by others who have tried this method. This procedure involves a high degree of manipulation and any resultant injury could compromise the ewes ability to conceive naturally. As yet, no data are available on the efficacy of repeatedly using this technique.

Intrauterine: This involves a rapid laparoscopic location of the uterus and direct injection of semen into the uterine horns using a fine pipette. This circumvents the cervical barrier and radically improves fertilisation rates when using frozen-thawed semen; conception rates ranging from 50 to 80% have been reported (Maxwell and Hewitt, 1986). This method also has the advantage of only requiring a small number of spermatozoa, thereby allowing a more widespread dissemination of valuable genotypes. However laparoscopy has several disadvantages. It is an invasive procedure, requires veterinary expertise and is expensive in terms of equipment and labour. It is also possible that laparoscopic and transcervical AI may become unacceptable in the future based on welfare grounds.

OBJECTIVES OF PROJECT

The overall project object was to contribute to the development of an effective, non-surgical cervical insemination procedure for frozen-thawed semen from individual rams which can be employed at farm level. The availability of a cost effective AI procedure would facilitate and enhance genetic improvement programmes for sheep.

Whilst many workers report variable and generally poor pregnancy rates following cervical insemination with frozen-thawed semen, an exception is Norway where mean conception rates of 60% are reported at field level (Olesen, 1993; Donovan, 1997). Thus a central task was to determine if similar success could be achieved in Ireland by adopting the freezing and insemination procedures used in Norway. However, breeds used in Norway are quite different from those found in Ireland and insemination is to a natural oestrus. In Ireland this would be impractical and any serious thought of applying AI here would require set-time AI to a synchronised oestrus. It was necessary to determine if the good conception rates achieved in Norway are due to inherent Norwegian factors/practices and/or the breeds of sheep involved.

GENERAL METHODS

Semen collection

Rams were trained for semen collection using a simple mounting stand about 75 cm high and 3-4 m long (photo 1). An oestrus (teaser) ewe was restrained on the ramp and selected rams were trained to serve into an artificial vagina (AV) in the presence of an operator. The AV consists of an insulated outer casing and an inner liner. It is filled with warm water (45 to 55 ^{o}C) so that it simulates the thermal and mechanical stimulation from the ewe. Successful training depends on the skill of the operator and the age, sexual experience and temperament of the ram. Training usually began 2-3 weeks before collection as this allowed rams to become accustomed to their surroundings and handling operators and also allowed time for the assessment of semen quality and replacement of unsatisfactory males.

Semen processing

Following collection, each ejaculate was assessed for wave motion, volume and concentration. Only ejaculates with good wave motion (scoring >3, on a scale of 0 to 5) and containing more than 2.5 x 10^9 spermatozoa/ml were accepted. Generally two ejaculates per ram were collected on a daily basis, with an interval of 10 to 15 minutes between collections. If both ejaculates were acceptable, then they were pooled on a per ram basis.

The semen was then diluted in a two-step procedure with a skim milk-egg yolk-glycerol extender. After equilibration and adaptation for 3 h at 5 ^{o}C the semen was reconcentrated by centrifugation at 700 g for 10 minutes and rediluted to a sperm concentration of 800 x 10^6/ml before freezing in Minitub straws (0.25 ml) in liquid nitrogen vapour using a programmable freezer. The freezing protocol used was as follows: reduce the temperature from 5 ^{o}C to –10 ^{o}C at a rate of –5 ^{o}C/min, then from –10 ^{o}C to –130 ^{o}C at a rate of –50 ^{o}C/min and store in liquid nitrogen (-196 ^{o}C). After freezing a random straw is thawed from each ram and assessed for motility and viability using light microscopy (400 x). The straws for an individual ram in a particular freeze were retained for use **only if** the sample straw yielded a viability score ≥ 50% and progressive motility was judged to be satisfactory.

Insemination procedure

Straws containing $200x10^6$ spermatozoa were thawed at 70 ^oC for 8 s immediately prior to insemination. Ewes were cervically inseminated in a standing position (photo 2). The cervix was located, via a speculum with a light source (photo 3), and the semen deposited as far as possible into the cervix without using force by means of an insemination pipette with a bent tip (Minitub, Germany).

Freezing procedure

There is conflicting evidence in the literature on the effects of different freezing rates. The purpose of this study was to explore a number of variations in the rate at which semen was frozen and the temperature at which rapid freezing was initiated prior to plunging the straws into liquid nitrogen for permanent storage.

Methods: Ejaculates from individual rams (n = 15) were used. The semen was diluted and cooled to 5 ^{o}C and then packed in straws as described earlier. Then the temperature was reduced at one of three rates (-0.5 ^{o}C, -2.0 ^{o}C or –5.0 ^{o}C per minute) to one of two endpoints (-10 ^{o}C or –25 ^{o}C). Once the above endpoints were attained the straws were cooled to –130 ^{o}C at –50 ^{o}C/min and then plunged into liquid nitrogen (-196 ^{o}C) for long-term storage. Semen from 6 rams was used for each treatment and 3 straws per ram were used for post-thaw evaluation. Evaluation involved assessment of viability, mitochondrial activity and acrosome integrity using various differential stains (O'Neill, 1998)

Results and Conclusions: Freezing treatment had significant effects on viability of spermatozoa and on structural integrity. The viability results are summarised in Figure 1 and show that the 'Fast' freezing rate gave the best viability and that at this rate of freezing there was no difference between the –10 ^{o}C and –25 ^{o}C endpoints. The results on structural integrity showed only minor differences between the two endpoints (-25 ^{o}C was marginally

better) and also indicated that the fast rate of freezing was significantly better than slow.

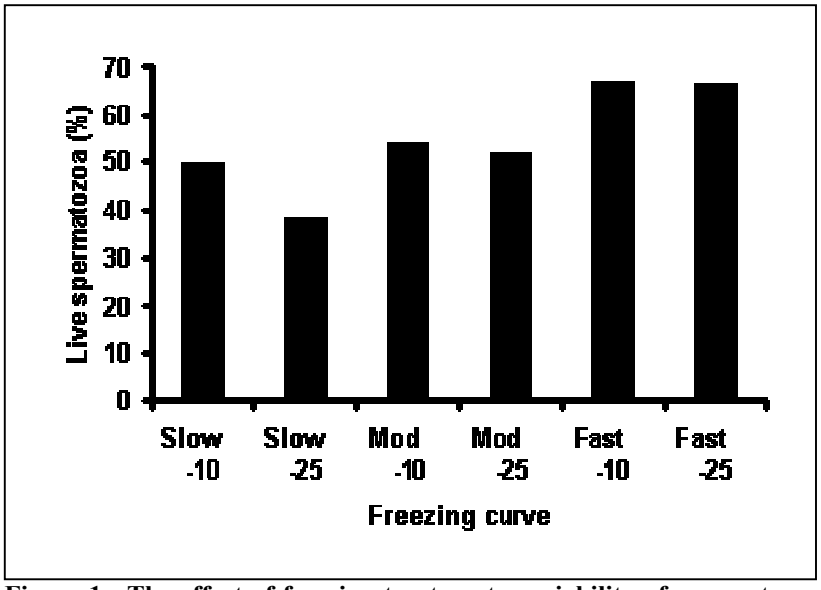


Figure 1. The effect of freezing treatment on viability of spermatozoa (Slow = -0.5 ^{o}C/min, Mod = -2.0 ^{o}C/min, Fast = -5.0 ^{o}C/min; -10 & -25 signify the temperature (^{o}C) at which freezing rate was increased to –50 ^{o}C/min)

There were highly significant differences among individual rams in the proportion of viable spermatozoa when thawed and assessed using differential staining. Similar findings were obtained in a subsequent study (Byrne, 1998) involving freezing rates of –0.5 ^{o}C/min and –5.0 ^{o}C/min.

Based on these results and information on the freezing curves used in Norway it was concluded that the fast freeze rate to –10 ^{o}C was suitable for routine freezing of semen.

Semen evaluation using differential staining

The freezing/thawing process induces a considerable number of structural, functional and biochemical changes in spermatozoa and quality control of frozen semen requires effective laboratory-based methods for the evaluation its fertilising potential. Various procedures have been proposed over the years for semen evaluation, including specific stains for assessing spermatozoa viability, mitochondrial function and acrosome integrity. The validity of these stains was examined in a series of studies.

Methods: Pooled semen from 3 rams was used. A combination of stains, one specific for live spermatozoa (SYBR-14) and one specific for dead spermatozoa (propidium iodide) were used to determine the proportion of live spermatozoa. Another stain (rhodamine) was used to identify spermatozoa with active mitochondria, while a fluorescent antibody was used to determine the degree of integrity of the acrosome membrane. The validation experiments involved using various procedures to quantitatively alter the quality of the semen being evaluated. Evaluations were done by fluorescent microscopy of frozen-thawed spermatozoa after staining. A minimum of 250 spermatozoa were assessed per sample.

In addition to the use of microscopy to count the differentially stained spermatozoa, flow cytometry was also used as an objective and potentially very precise procedure for assessing the results of staining. This equipment allowed evaluations to be based on 10,000 spermatozoa per sample.

Results and Conclusions: The results from specific staining to determine the proportion of dead spermatozoa and the proportion with mitochondrial activity yielded very high correlations ($\geq$ 0.95) between the expected and

estimated proportions and thus should provide reliable evaluations of these qualitative aspects of frozen-thawed semen. Similar results were obtained when flow cytometry was used and the variation in the assessments was lower. The ability of a fluorescent antibody to identify spermatozoa with an intact acrosome membrane was confirmed using both fluorescent microscopy and flow cytometry. However, interference by diluent particles with flow cytometer readings created a problem for the use of this technique given the nature of the diluents used for freezing.

It was concluded that the specific staining procedures employed can be effectively used to evaluate viability, functional and structural integrity of frozen-thawed spermatozoa. However, the ultimate utility of such laboratory procedures can only be judged in relation to actual conception rates *in vivo* following insemination.

Relationship between non-return rates and results from differential staining

The practical utility of differential staining for quality assessment can only be established by relating the results from staining with data on pregnancy rate. The animal resources to conduct such an insemination trial were not available. However it was possible to source frozen semen from a set of rams that had been used in large-scale on-farm AI in Norway.

Methods: Samples of frozen semen from 32 individual rams were obtained from Norway together with the official estimate for non-return rate following cervical AI. These AI results refer to on-farm usage in Norway. The validated procedures for determining proportion of viable spermatozoa and the acrosome status were used to assess the semen. The percentage mobility was determined using phase-contrast microscopy.

Results and Conclusions: The relationship between non-return rate and proportion of viable spermatozoa is shown in Figure 2. It is clear that the results from differential staining are not related to the success of cervical AI with frozen-thawed semen. Similar results were observed for the assessments of acrosome integrity and sperm motility.

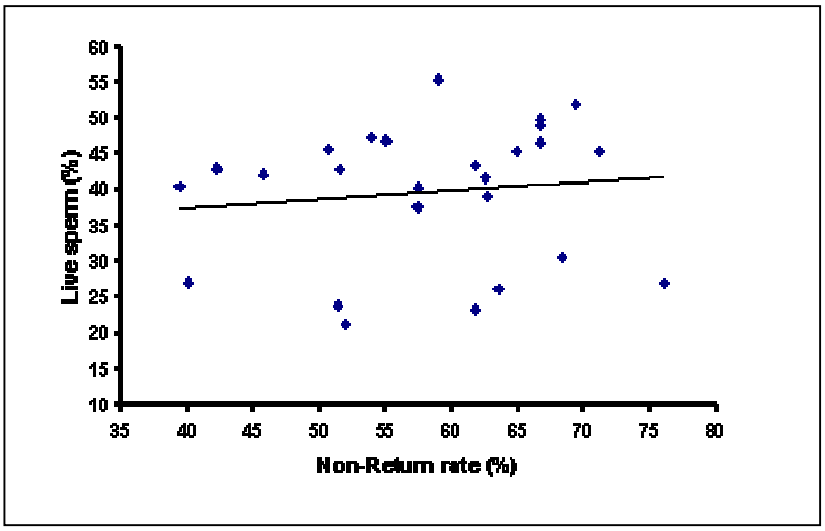


Figure 2. Relationship between sperm viability and non-return rate for rams used in AI in Norway

It was concluded from these results that while differential staining yielded consistent estimates of the structural and functional status of spermatozoa these methods were not useful in determining the capacity of semen to effect fertilisation of ewes following AI.

Use of IVF for semen evaluation

The inability of differential staining to yield useful information on the fertilising capacity of frozen-thawed semen led to the investigation of *in vitro* fertilisation (IVF) of sheep oocytes as a method for quality assessment.

Methods: The IVF procedure involved harvesting of oocytes from follicles on ovaries recovered from ewes immediately after slaughter in commercial abattoirs. The oocytes were matured in the laboratory and well-plates containing about 50 matured oocytes per well were inseminated with semen from individual rams (after appropriate conditioning). The proportion of oocytes that had been fertilised (per well and based on normal cleavage) was determined 48 h after insemination.

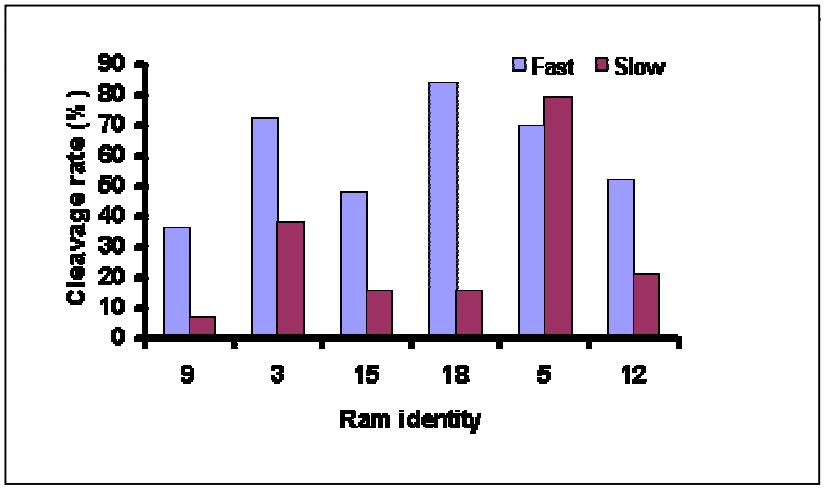
Semen from individual rams was frozen using either a fast (-5 ^{o}C/min) or a slow (-0.5 ^{o}C/min) rate of temperature reduction as outlined earlier and evaluated using the IVF procedure and by differential staining (viability and acrosome integrity).

Results and Conclusions: The main results are shown in Table 1. There was a highly significant ($P<0.01$) difference in fertilisation (cleavage) rate between the two freezing treatments. However, the differential staining failed to detect any significant treatment effect. These findings are consistent with the results from comparisons of non-return rate with results from differential staining.

Table 1: Effect of freezing treatment on semen quality

	Freezing rate	
	-0.5 °C	-5.0 °C
No. of oocytes	845	899
Cleaved oocytes (%)	26	57
Live sperm (%)	25	27
Sperm with intact acrosome (%)	52	49

Another feature of the results from this experiment was the presence of highly significant variation among individual rams (Figure 3) with respect to fertilisation rate. The range in fertilisation among individual rams was 36 to 84% for the fast freeze rate and 7 to 79% for the slow freeze rate. The results also showed that some rams gave a high fertilisation rate regardless of the freezing rate used. These findings are consistent with reports in the literature on variation among rams in conception rate following insemination of frozen-thawed semen.



It is concluded from these results that the IVF technique has potential as an effective means of assessing the fertilisation capacity of frozen-thawed semen and that it may enable the identification of individual rams whose semen is better able to survive the freeze-thaw process and yield high pregnancy rates. The results also support the earlier conclusion that differential staining methods are not a useful indicator of semen quality for AI.

***In-vivo* validation of IVF technique for semen evaluation**

The promising results from IVF evaluation of semen were tested by inseminating ewes with semen that was also evaluated by IVF.

Methods: Semen frozen using two freezing rates known to differentially affect IVF results was used to inseminate ewes. The study involved two parts.

Cervical or laparoscopic insemination of ewes followed by use of vasectomised rams to detect non-pregnant ewes and slaughter of ewes that did not return to service.

In vivo determination of fertilisation rate after laparoscopic AI of super-ovulated ewes, based on embryo recovery 5 days after AI.

The semen used was from 4 rams that had shown significant differences between fast and slow freezing rates when evaluated in the IVF system.

Results and Conclusions: The pregnancy rates following cervical and laparascopic insemination are shown in Table 2 which also contains the mean IVF results for the rams used.

Table 2: Effect of freezing procedure on pregnancy rate (%)

Freezing	IVF	Insemination method	
Rate	value (%)	Cervical	Laparoscopic
Fast	50	13	63
Slow	20	0	33

There was a significant ($P<0.01$) effect of freezing method on pregnancy rate and the difference was consistent with the IVF evaluation. The exceptionally low pregnancy rate to cervical AI was attributed to the fact that an error was made when determining the concentration of spermatozoa at the preparation of the straws. This error was only detected after the ewes had been inseminated. Thus, each ewe only received less than 50 x 10^6 spermatozoa instead of the intended 200 x 10^6 spermatozoa.

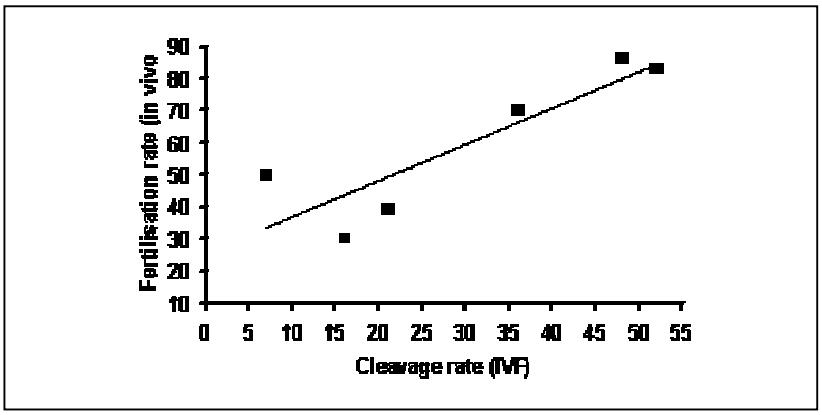


Figure 4. The relationship between in-vivo fertilisation rate and the fertilisation rate measured by IVF procedure.

The fertilisation rate portion of the study involved semen from 3 of the 4 rams and the results for each individual ram are plotted in Figure 4. The

results demonstrate a significant association between the *in vivo* fertilisation rate and the fertilisation rate observed using the IVF technique.

The results of this study support the earlier conclusion that an IVF procedure can be used to evaluate the quality of frozen-thawed semen for *in vivo* fertilisation. However, because of the very low pregnancy rate with cervical AI in this study further confirmation of the effectiveness of the IVF assay will be required.

Seminal plasma as an additive to freezing diluent

Studies in various species have shown that constituents of seminal plasma can enhance the viability and fertilising capacity of diluted or frozen semen. The effect of adding ram seminal plasma to the diluent used for freezing semen was studied as a possible means of increasing fertilisation rate.

Methods: Various concentrations (1% to 20%) of seminal plasma were incorporated in the diluents used for preparing semen for freezing and effects on viability and structural integrity of frozen-thawed spermatozoa were evaluated using flourescent stains. In a second study the evaluation was extended to include an IVF assay of fertilisation capacity.

Results and Conclusions: In the first study the results indicated that including 10 or 20% seminal plasma in the diluents increased the viability of spermatozoa by 10 to 15 percentage points over the standard diluent. There was also an indication that sperm membrane integrity was improved. However, in the second study, in which 20% seminal plasma was incorporated in the diluent, there was no significant effect on sperm viability or structural integrity of spermatozoa compared with the standard

diluent. Furthermore there was no evidence for any beneficial effect on fertilisation rate in the IVF assay.

It was concluded that adding seminal plasma to the freezing diluent was not likely to significantly improve conception rate to cervical AI. However, questions remain in relation to possible differences among individual rams in relation to effects of their seminal plasma (i.e. only seminal plasma from certain rams has a beneficial effect).

STUDIES ON PREGNANCY RATE

The effect of semen source, synchronisation and inseminator on conception rate

The principal objectives of this study were to test whether semen collected from rams of Norwegian breeds and frozen in Norway would yield a different pregnancy rate from semen collected from rams of breeds found in Ireland. An additional objective was to determine whether insemination to a natural oestrus – as is practice in Norway – was a significant factor in achieving good pregnancy rates following cervical AI of frozen-thawed semen.

Methods: Parous ewes (n=297) of various breeds (Finnish Landrace, Scottish Blackface cross, Belclare cross, Cambridge cross and Galway cross) were cervically inseminated with fresh or frozen thawed semen from individual rams, at either a natural or synchronised oestrus. The semen sources were (i) semen from Norwegian breeds frozen in Norway and imported for use, (ii) semen collected from local rams (Texel, Suffolk and Finn) and prepared using procedures already described. Synchronisation involved a 12-day pessary treatment (containing 40 mg FGA - chronogest) followed by 500 i.u. PMSG at pessary removal. This was compared with insemination to natural oestrus without PMSG. Inseminations were alternated between two inseminators, one of whom was an experienced Norwegian inseminator.

Results and Conclusions: Ewes inseminated with fresh semen had a significantly ($P<0.01$) higher pregnancy rate than any group inseminated with frozen-thawed semen (Table 3). However, there was no significant difference in pregnancy rate or litter size between Irish frozen-thawed and Norwegian frozen- thawed semen.

Table 3: Pregnancy rate (%) for ewes inseminated with fresh or frozen-thawed semen

Semen Type	Natural oestrus	Synchronised oestrus	Overall
Fresh	82	70	76
Frozen -Irish	40	52	46
-Norwegian	34	37	36

There was no significant difference in pregnancy rate between ewes inseminated to a natural or synchronised oestrus. There was an interaction between semen type (fresh or frozen) and oestrus type for litter size reflecting the fact that the adverse effect of frozen-thawed semen on litter size was greater in synchronised ewes (Table 4). The reduced pregnancy rate and litter size following insemination with frozen-thawed semen is consistent with the general pattern of results in the literature.

Table 4: Effect of synchronisation on litter size (±s.e.) adjusted for ovulation rate

Semen type	Natural oestrus	Synchronised oestrus
Fresh	2.7±0.23	3.2±0.23
Frozen -Irish	1.9±0.23	1.4±0.23
-Norwegian	2.0±0.24	1.9±0.24

An unexpected result emanating from this study was a highly significant effect of ewe breed on pregnancy rate, an effect that was independent of semen type.

The estimated pregnancy rates for the inseminators were significantly different ($P<0.05$) which was also reported for Norwegian conditions by Olesen, (1993)

Whilst the breeds involved in the initial study were mainly crossbred (with the exception of pure-bred Finnish Landrace), the results demonstrate the need to determine pregnancy rate for specific lowland breeds, as it is these breeds that are the target of breed improvement programmes.

Effect of ewe breed on pregnancy rate

In the light of the significant effect of ewe breed on pregnancy rate further studies were designed to confirm and extend this observation. The following study involved a number of pure breeds as well as crossbred types.

Methods: Purebred [Suffolk (n=84), Texel (n=110), Finnish Landrace (n=43), Belclare (n=25)] and crossbred [Suffolk cross (n=56), Belclare x Scottish Blackface (n=45)] ewes were synchronised and inseminated with either fresh (n=29) or frozen-thawed semen (Irish or Norwegian; n=334) from individual rams. The use of fresh semen was included as a quality control check. Ewes were maintained at pasture throughout, and most were slaughtered at 37 to 40 days post insemination to determine pregnancy rate and litter size. A proportion of the purebred Texel (n=47) and Suffolk (n=41) ewes were maintained as part of a breeding flock and fertility was determined at lambing.

Results and Conclusions: Ewes inseminated with fresh semen had, as expected, a significantly ($P<0.01$) higher pregnancy rate than those

inseminated with frozen-thawed semen, which is consistent with the previous study and general reports in the literature. Pregnancy rate was significantly dependent on ewe breed and this effect was independent of semen type (fresh or frozen).

Analysis of the results for the frozen-thawed semen confirmed the significance of ewe breed ($P<0.001$) as a major factor determining the success of cervical AI with frozen-thawed semen. The pregnancy rate for the different breeds is shown in Table 5. Finnish Landrace ewes had the highest pregnancy rate and purebred Suffolk ewes had the lowest pregnancy rate which was close to that for Suffolk-cross ewes. Belclare ewes and Blackface-cross ewes had similar results which were significantly better than for Suffolks and Suffolk crosses. Texel ewes were intermediate.

There was no significant effect due to source of semen (Norwegian vs Irish) but differences among individual rams were statistically significant ($P<0.05$).

The results of this experiment confirmed the importance of ewe breed and highlighted the need to examine possible reasons for this effect.

Table 5: Pregnancy rate (%) for ewes inseminated with fresh or frozen-thawed semen

Breed	Fresh semen	Frozen-thawed semen
Finn Landrace	100	77
Suffolk	57	18
Texel	67	30
Belclare	-	44
S.Blackface cross	80	43
Suffolk cross	-	19

Breed differences in timing of ovulation

The reasons for the very large breed differences in pregnancy rate are not known - but differences between breeds in the timing of ovulation was a likely factor since breeds are known to differ significantly in the timing of events around estrus. It is also known that the timing of insemination of frozen-thawed semen is a critical determinant of fertility, due primarily to the significantly reduced lifespan of frozen-thawed semen in the female reproductive tract. However, there are few direct estimates of the timing of ovulation relative to the withdrawal of progestogen, and particularly in relation to breed differences. This is despite the fact that treatment with progestogen pessaries and PMSG is almost universally used to control the time of oestrus and ovulation prior to artificial insemination in sheep. Consequently a study was undertaken to define the timing of ovulation in different purebred ewes to determine if differences in the timing of AI relative to ovulation could account for breed effects on pregnancy rate.

Methods: Four breeds were used (Suffolk, Texel, Scottish Blackface and Finnish Landrace). Time of LH peak was determined by radioimmunoassay of blood samples taken at 3-h intervals between 20 and 53 h post pessary removal. Ovarian status was examined in all ewes, by laparoscopy at 5-h intervals starting at 42 h post pessary removal, to identify the time of ovulation.

Results and Conclusions: Data on the timing of LH peak and ovulation and on the interval between LH peak and ovulation are summarised in Table 6.

Table 6: Means for the time intervals (h) from pessary removal to LH peak and to onset of ovulation, and the interval (h) from LH peak to ovulation for each ewe breed.

Breed of ewe	Time to LH peak	Time to ovulation	Interval from LH peak to ovulation
S. Blackface	36.4	60.1[a]	23.3
Finn	34.0	56.1[b]	22.6
Suffolk	35.5	57.5[ab]	22.6
Texel	36.7	58.3[ab]	21.9

[ab] Means without a common superscript are significantly different ($P<0.05$)

The results showed that there was no effect of breed on either the interval from sponge removal to the LH surge or on the interval from LH surge to ovulation. Ewe breed affected the time from sponge removal to ovulation but the differences were only about 2 h when the Finnish Landrace is compared with the Suffolk or Texel and the differences among these breeds were not statistically significant. Since this comparison corresponds to the greatest differences observed in pregnancy rate in the previous study it is concluded that differences in the average time interval between sponge removal and ovulation cannot explain the breed effect on pregnancy rate. The distribution of this time interval is shown in Figure 5 for Suffolk, Texel and Blackface ewes. An unexpected result from this study was that there was much less variation among ewes in the timing of ovulation in the case of Finnish Landrace breed than in any of the other breeds. This may be a factor in the breed effect on pregnancy rate.

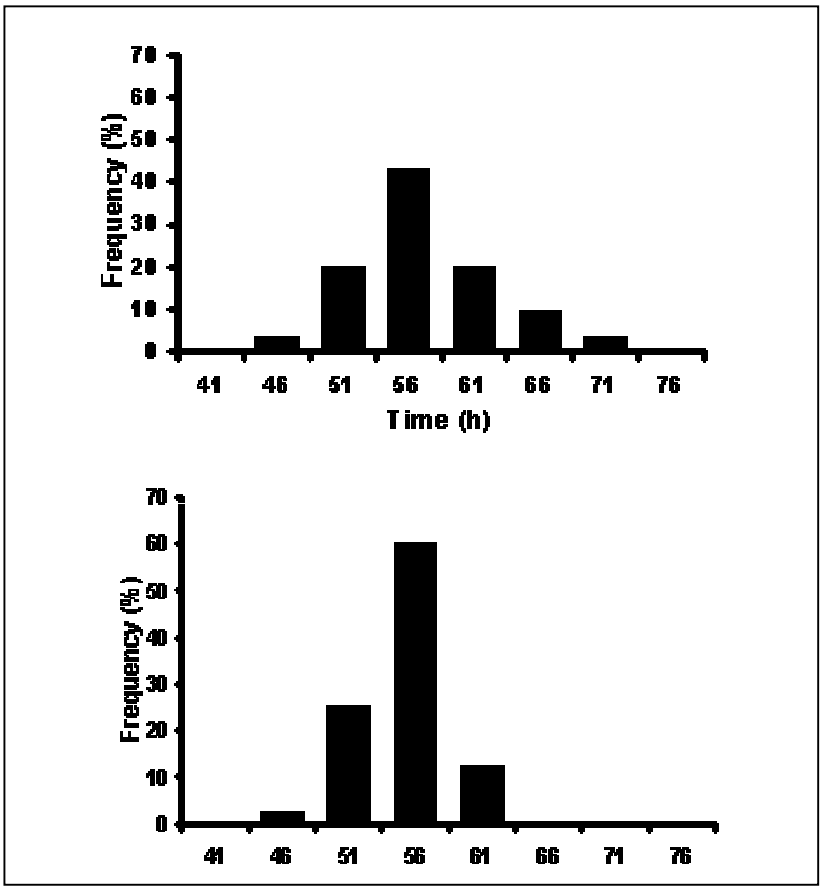


Figure 5: Distribution of time intervals from pessary removal to ovulation; upper panel - Finn ewes, lower panel - Suffolk & Texel ewes.

The major findings of this study were the absence of breed effects on the interval from LH surge to ovulation, the relatively small breed differences in the average interval from pessary removal to ovulation combined with the substantial difference between Finn and other breeds in the variability in the time to ovulation.

Effect of insemination time on conception rate

The lower pregnancy rates following cervical insemination of frozen-thawed semen in pure-bred lowland breeds, most especially the Suffolk, may be related to the within-breed spread of ovulation time. This problem is compounded by the severely reduced survival time of frozen-thawed ram semen in the female reproductive tract. Acceptable pregnancy rates may require a double insemination to overcome the problem of high variation in the timing of ovulation. The following experiment was designed to examine this hypothesis.

Methods: A single or double insemination with frozen-thawed semen was carried out on synchronised ewes of four breeds at different time points post sponge removal. The time of insemination relative to sponge removal was varied by 6 h and in some cases ewes were inseminated twice (6 h apart). The study involved purebred Suffolk (n = 56), Texel (n = 82), Finnish Landrace (n = 40) and Scottish Blackface (n = 42) ewes.

Results and Conclusions: The timing of AI did not have any effect on pregnancy rate and while ewes that were inseminated twice had a higher pregnancy rate this effect was not statistically significant (Table 7). The response to double AI is consistent with the gain of 8 percentage points reported by Olesen (1993). As in previous experiments the breed of ewe had a highly significant effect and this aspect of the results is presented in Table 8.

Table 7 : Effect of time of insemination on pregnancy rate

Insemination time[a]	Pregnancy rate[b] (%)
57 h	34
63 h	33
57 & 63 h	43

[a]After sponge removal; [b]Texel ewe basis

The semen for this experiment was obtained from 7 different rams and the difference between these in terms of pregnancy rate was significant ($P<0.05$).

Table 8: Ewe breed effects on pregnancy rate

Ewe breed	Pregnancy rate (%)
Suffolk	11
Texel	35
S. Blackface	29
Finnish Landrace	55

The results of this experiment lead to the conclusion that inappropriate timing of AI is not a likely explanation for the effect of ewe breed on pregnancy rate following AI. The failure of double insemination (at 6-h interval) to significantly increase pregnancy rate shows that the breed differences in the variability in ovulation rate timing was not an important factor. It seems reasonable to conclude that variation of the order of 6 h in the timing of AI will not greatly influence pregnancy rate. This conclusion, has favourable implications for the logistics of practical AI programmes at farm level.

The cervix and efficacy of AI

Absence of cervical penetration is cited as the major factor limiting the efficacy of cervical AI in sheep. Eppleston (1992) reported an increase of 7 to 12 % in fertility for every 1-cm increase in depth of insemination. Penetrability of the cervix may be associated with stage of oestrus or anatomy of the cervix. The cervix of the ewe is approximately 8 to 9 cm in length and contains about 5 funnel shaped rings. These rings have small

openings, are not concentrically aligned and do not dilate during oestrus. The anatomy of the ovine cervix therefore precludes the deposition of spermatozoa into the uterus via the cervix.

The quantity of mucus secretion is associated with stage of oestrus and is thought to be maximal in the early stages of oestrus (Eppleston, 1992). An assessment of this should provide an indication of the stage of estrus at AI and its effect on pregnancy rate.

In each AI experiment the depth of penetration was recorded as was the amount of cervical mucus. The ewes from the last experiment were used to determine whether breed differences in anatomical measurements of the cervix were reflected in differences in pregnancy rate.

Methods: A score was recorded for depth of penetration (0= none to 3 = deep) and amount of mucus secretion (0=dry to 3=copious) for all ewes inseminated in each study. Post mortem measurements were taken of the length and width of the cervix and number of rings for all ewes in the last experiment.

Results and Conclusions: No breed effects were detected in any of the AI experiments with respect to depth of penetration of the cervix at AI or in the amount of mucus secretion. Likewise there was no significant within-breed relationship between these traits and pregnancy rate. The observed differences in anatomical measurements (Table 9) are consistent with breed differences in mature body size but are not clearly associated with corresponding differences in pregnancy rate. These results do not support the notion that either anatomical differences or the state of the cervix (mucus, penetrability) at the time of AI are implicated in the breed differences in pregnancy rate.

Table 9: Cervical measurements (±s.e.) for different ewe breeds

Breed	Length (cm)	Width (cm)	No. of rings
S. Blackface	8.3 ± 0.40^{a}	1.0 ± 0.06^{ab}	5.2 ± 0.23^{ab}
Finnish Landrace	7.7 ± 0.43^{a}	0.8 ± 0.07^{a}	4.5 ± 0.24^{a}
Suffolk	10.2 ± 0.45^{b}	1.1 ± 0.07^{b}	5.4 ± 0.25^{b}
Texel	9.6 ± 0.33^{b}	1.2 ± 0.05^{b}	5.3 ± 0.19^{b}

[ab] Means without a common superscript are significantly different ($P<0.05$)

PRACTICAL IMPLICATIONS

The results from this project show that the potential for using cervical AI in breed improvement programmes depends on the ewe breed and the individual ram providing the frozen-thawed semen. The use of IVF to evaluate semen quality may allow effective screening of individual rams. Finally the effort required to apply IVF testing and then to establish a bank of semen from the best rams need to be considered. These issues are addressed in the following sections.

Ewe breed effect

The data from the three large-scale AI experiments were pooled to establish an overall picture of the breed effect and the results are presented in Table 10. These results are based on the use of rams without any selection for the freezability of their semen. The results show that the Finnish Landrace ewes were substantially better than any other breed and that the Suffolk gave the poorest results. It is notable that the average pregnancy for Finn ewes is essentially identical to the quoted figure for Norwegian ewe breeds (60%) and that these breeds are related to the north European race of sheep which includes Finnish Landrace.

Table 10: Overall summary of ewe breed effects on pregnancy rate

Ewe Breed	No. of ewes	No. of experiments	Pregnancy rate (%)
Finnish Landrace	127	3	61
Texel	183	2	31
Suffolk	133	2	12
Belclare	25	1	46
Scottish Blackface	42	1	29
Belclare x S. Blackface	121	2	44

The pregnancy rate for Belclare ewes is near to that which is likely to be acceptable to pedigree breeders for use in sire reference schemes. In particular, if rams can be effectively selected through IVF testing then cervical AI of frozen-thawed semen should be quite effective in such a breed.

Variation among individual rams

While the comparison of semen from Norway with semen frozen in Ireland did not reveal any significant difference in pregnancy rate, and therefore suggests that ram breed is not likely to be the limiting factor, it was evident in all the AI trials that there were considerable differences among rams of any given type. This observation is consistent with the IVF studies and results from the studies using differential staining. The absolute magnitude of this variation is relevant when evaluating possible increases in pregnancy rate if frozen semen could be effectively evaluated, for example by IVF, prior to use in AI programmes. Olesen (1993) reported a range of 24 percentage points among a set of 6 rams used in Norway in 1988.

Methods: Results from the three large-scale AI trials described earlier were used and the adjusted pregnancy rates for individual rams were obtained using appropriate statistical methods. The analyses were confined to those rams whose frozen semen was used to inseminate at least 20 ewes.

Results and Conclusions. Information was available for 20 rams and the results are plotted in Figure 6 as a frequency distribution. The range of values is quite wide. This significant variation among individual rams in the pregnancy rate following cervical AI with frozen-thawed semen highlights the need for a reliable method of identifying the best ‘freezers’ when

assembling a panel of rams for AI. The results of this project indicate that the IVF assay system is the only effective tool presently available short of conducting an actual AI trial. Based on the variation among rams, and allowing for sampling variation, it is calculated that if a panel of rams can be effectively screened and the best 25% identified this would raise pregnancy rate by 15% points regardless of ewe breed. Thus, in the case of Texel and Belclare ewes this would mean that the expected pregnancy rate to cervical AI would be:

Texel	**46%**
Belclare	**61%**

Thus, it is concluded that with the techniques and procedures developed in this project, cervical AI with frozen-thawed semen is practicable in breeding programmes for the Belclare and probably the Texel populations. Unfortunately, the expected success rate for Suffolks (28%) probably falls well short of what most breeders would be willing to accept.

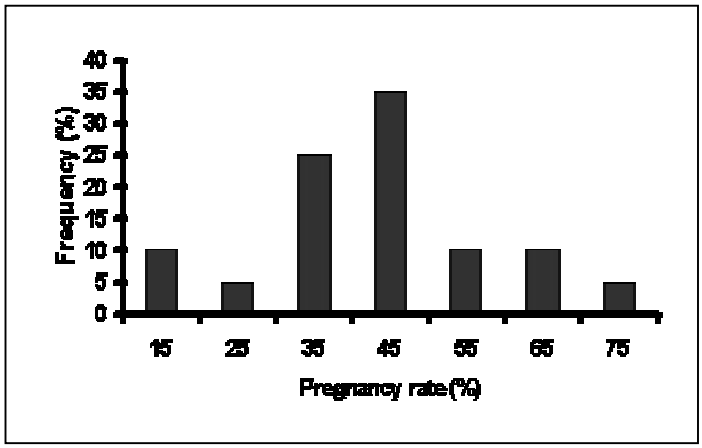


Figure 6: Differences among individual rams for pregnancy rate following cervical AI with frozen-thawed semen.

Establishing a semen bank

A central feature of an insemination programme, given that an acceptable and effective technique is available, is the logistics of semen collection. Important issues in this regard involve the performance of rams in relation to the quantity of semen produced, the proportion of ejaculates considered suitable for freezing and the freezability of the semen based on visual examination post thaw. The details of all routine collections of semen at Athenry for the project described in this report were analysed to estimate the essential parameters needed to make projections in relation to operation of an AI service.

The details are summarised in Table 11. These results should not be taken as direct evidence on breed differences as the various breeds were not always used at the same time. Rather the data provide a reasonable guide to what could be expected in practice. The data are consistent with previous studies with respect to sperm concentration but the volume per ejaculate is on the low side for all breeds. Thus, previous Irish studies of semen characteristics of rams yielded an ejaculate volume of 1.2 ml for Suffolk rams (Jennings, 1972) while Smith (1977) reported average volumes of 1.02 and 0.97 ml for Suffolk and Texel rams, respectively. The suggestion that Texel rams produce semen with a lower sperm concentration than Suffolk is consistent with the results reported by Smith in a direct comparison of these breeds. Smith also reported that about 20% of ejaculates had wave motion scores of 3 or less and therefore would be rejected for freezing. The lower ejaculate volume in the present project may in part be due to the fact that the rams used were not specifically managed for AI – being part of the pool of rams used for natural matings in various research flocks at Athenry.

With extra feed inputs over the pre-collection period as well as during the collection period higher volumes may have been produced.

Table 11: Characteristics of ejaculates from rams of five breeds

	Breed				
	Suffolk	Texel	Belclare	Cambridge	Finn
No. of rams	15	8	3	6	23
No. of ejaculates	187	124	43	78	210
Ejaculate volume (ml)	0.80	0.79	0.83	0.78	0.70
Sperm concentration ($x10^{-9}$/ml)	2.95	2.66	2.92	2.79	2.82
No. straws per ejaculate	11.4	10.5	11.6	9.9	9.6
Collections accepted after freezing (%)	72.2	57.9	73.2	67.8	83.6
Useable straws per ejaculate frozen	7.7	5.6	8.0	6.2	7.6

Based on the data from the earlier studies for semen volume (1 ml) and the results in Table 11 for the proportion of acceptable straws after freezing, it is calculated that, from 2 pooled ejaculates per ram per day and testing one straw per ram per freeze, an average of 20 acceptable straws would be obtained. This calculation assumes that ejaculates that are rejected initially based on wave motion are replaced by a useable ejaculate. Thus during a 5-day collection period each ram could be expected to yield about 100 acceptable straws for the semen bank. It is arguable that the number of collections during a week could be increased by 50% without a significant drop in sperm yield per ejaculate and, therefore, 150 straws could be banked per ram per week of collection. Thus, over a 3-week collection period a panel of rams could be expected to yield 300 to 450 useable straws each. This would be adequate for delivering the inseminations required for a sire

reference programme, based on 3 to 4 rams, to two or three of the major breeds in use in this country.

CONCLUSIONS

The use of differential fluorescent staining to evaluate the viability and structural integrity of frozen-thawed semen did not provide a useful indication of fertilisation rate *in vitro* or *in vivo*.

Significant variation was found among individual rams with respect to many aspects of semen quality post freezing and also with respect to pregnancy rate when frozen-thawed semen was used for cervical AI. The range in pregnancy rates observed was from around 15% to over 65%.

- The fertilisation capacity of frozen-thawed semen was evaluated using *in vitro* fertilisation (IVF) of sheep oocytes. The validation of this technique *in vivo* showed that it could provide an effective method for identification of rams whose frozen-thawed semen gave high pregnancy rates with cervical AI.
- The use of IVF testing to identify the best rams for AI could increase pregnancy rate by about 15 percentage points.
- No difference was found between pregnancy rates with frozen-thawed semen from Norwegian rams (collected and frozen in Norway) and semen from Irish breeds collected and frozen in Ireland. It was concluded that the high pregnancy rates reported for cervical AI in Norway are not due to any inherent difference in freezability of semen from Norwegian breeds compared with other breeds.
- The use of estrus synchronisation to facilitate set-time AI did not adversely affect pregnancy rate when compared with AI to a natural oestrus. Thus the poor pregnancy rates commonly obtained after cervical AI of frozen-thawed semen are not attributable to the use of synchronisation treatments.

- Based on the information collected on the number of useable straws per ejaculate it was calculated that between 100 and 150 useable straws could be stored per ram from a 1-week collection period with 2 ejaculates per ram per day.
- A short collection period for IVF testing followed by a 3-week collection period for rams that are chosen, based on the IVF test, would generate sufficient frozen semen to support sire reference schemes for two or three of the major breeds in use in this country.
- Ewe breed was consistently found to have a very large effect on pregnancy rate in the studies reported here. The range in values was from 12% for Suffolk ewes to 61% for Finnish Landrace ewes. Thus some inherent characteristics of the ewe are of fundamental importance.
- The differences between ewe breeds was not associated with differences in the timing of ovulation relative to sponge removal and hence using breed-specific set times for AI would not alter the breed effect on pregnancy rate. This conclusion was confirmed by testing the response to altering the timing of AI.
- Further work is required to identify the ewe-specific factors responsible for the large differences in pregnancy rate identified in this study. This should include a detailed study of factors affecting the population of spermatozoa that is established at the site of fertilisation following cervical AI. The target for this work is to achieve the pregnancy rate obtained in this project with Finnish Landrace ewes.
- The exploitation of IVF screening of rams to identify good 'freezers' and their use in AI is projected to yield pregnancy rates of over 75% for Finnish Landrace ewes, 65% for breeds like the Belclare and around 45% for the Texel breed. These values should make the

implementation of sire reference schemes based on cervical AI a practical proposition.

- The ability of the IVF procedure to reliably identify rams that yield semen with the highest pregnancy rates after freezing needs to be tested under field conditions and the required number of oocytes to be used for testing needs to be determined.
- Further work is necessary on possible refinements in instrumentation and in freezing protocols, including diluents and cryoprotectants.

REFERENCES AND PUBLICATIONS

Byrne G.P. (1998). Effects of freezing rate of ram semen on subsequent fertility *in vivo* and *in vitro*. M. Agr. Sc. thesis, National University of Ireland, Dublin.

Byrne, G.P., Lonergan, P., Wade, M., Duffy, P., Donovan, A., Hanrahan, J.P. and Boland, M.P. (1999). Use of IVF procedures for assessing the fertility of frozen-thawed ram semen. Proc. 25th Agricultural Research Forum, Dublin: 3-4 and Irish J. Agric. Food Res. 38 (1): 267

Byrne, G.P., Lonergan, P., Wade, M., Duffy, P., Donovan, A., Hanrahan, J.P. and Boland, M.P. (2000). Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro. Anim. Reprod. Sci. 62: 265-275

Donovan, A. (1997). Artificial insemination of ewes using frozen-thawed semen: A report on Norwegian procedures. Unpublished report, 15 pages

Donovan, A., O'Neill, D., Hanrahan, J.P., Duffy, P., Diskin, M., Crosby, F. and Boland, M.P. (1997). Artificial insemination in sheep using fresh and frozen semen. Proc. 23rd Agricultural Research Forum, Dublin: 309-310 and Irish J. Agric. Food Res. 36 (1): 128

Donovan, A., Hanrahan, J.P., Kummen, E, Duffy, P. and Boland, M.P. (1998). Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. Proc. 24th Agricultural Research Forum, Dublin: 43-44 and Irish J. Agric. Food Res. 37 (1): 108

Donovan, A., Hanrahan, J.P., Duffy, P., Byrne, G.P. and Boland, M.P. (1999). Cervical AI with fresh and frozen-thawed semen: effect of ewe breeed. Proc. 25th Agricultural Research Forum, Dublin: 1-2 and Irish J. Agric. Food Res. 38 (1): 267

Donovan, A. and Hanrahan, J.P. (1999). Artificial insemination of sheep – current status and possible developments. Irish Grassland and Animal Production Association Journal 33: 99-106

Donovan, A., Hanrahan, J.P., Duffy, P. and Boland, M.P. (1999). Cervical AI in sheep with fresh or frozen-thawed semen. Paper presented at EAAP 50th annual meeting, Zurich, 4 pages, and book of abstracts annual meeting of the European Assoc. for Anim. Prod. 5: 124

Donovan, A. Hanrahan, J.P., Duffy, P. and Boland, M.P. (2000). AI in sheep: breed differences in timing of ovulation. Proc. 26th Agricultural Research Forum, Dublin: 13-14 and Irish J. Agric. Food Res. 39 (3):

Eppleston, J. (1992). Studies on the fertility for frozen-thawed ram semen. Ph.D. thesis, University of New South Wales.

Halbert, G.W., Dobson, H., Walton, J.S., Sharpe, P. and Buckrell, B.C. (1990). Field evaluation of a technique for transcervical intra-uterine insemination of ewes. Theriogenology 33: 1231-1234.

Hanrahan, J.P. (1997). Exploiting genetic resources to enhance the competitiveness of lowland sheep systems. Irish Grassland and Animal Production Association Journal 31: 106-115.

Jennings, J.J. (1972). Some factors affecting the reproductive performance of rams. Ph. D. thesis, National University of Ireland, Dublin.

Maxwell, W.M.C. and Hewitt, L.J. (1986). A comparison of vaginal, cervical and intra-uterine insemination of sheep. J. Agric. Sci. Cambridge 106: 191-193

Olesen, I. (1993). Effects of cervical insemination with frozen semen on fertility and litter size of Norwegian sheep. Livestock Prod. Sci. 37: 169-184

O'Neill, D.J., Beckman, S.P.A., Roche, J.F., Hanrahan, J.P., Diskin, M. and Boland, M.P. (1997). The effect of different freezing curves on the viability, mitochondrial activity and acrosome integrity of ram spermatozoa. Proc. 23rd Agricultural Research Forum, Dublin: 335-336 and Irish J. Agric. Food Res. 36 (1): 132

O'Neill, D.J. (1998). Studies related to the cryopreservation of ram spermatozoa. M. Sc. (Agr) thesis, National University of Ireland, Dublin.

Smith, P.A. (1977). Studies in the artificial insemination of sheep. Ph. D. thesis, National University of Ireland, Dublin.