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Fertility of frozen ram semen under field conditions

With special reference to influence of extenders and freezing procedures

Jorge Gil Laureiro

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Av fakultetsnämnden utsedd opponent: Prof. Michael McGowan, Dept. of Farm Animal Medicine and Surgery, Royal Veterinary College, University of London, UK.

Abstract

A freezing method for ram semen is currently applied in Scandinavia, with good fertility using cervical artificial insemination (AI). The method consists of a two-step extension procedure in a milk-egg yolk extender (7% glycerol), followed by centrifugation to concentrate the extended semen before packaging in 0.25 ml straws and freezing in a programmable freezer. However, this method is tedious and includes steps considered stressful for spermatozoa, such as centrifugation. Moreover, the extender used contains components of animal origin, a potential source of undesired contamination. If genetic improvement of current sheep flocks in developing countries is to be done using frozen-thawed (FT) semen for AI, it is imperative to use cheap, uncomplicated freezing procedures and extenders free from potential contamination. The FT-semen must be easily inseminated (using cervical sperm deposition) and acceptable fertility levels after AI should be reached under prevailing conditions of animal husbandry.

The overall aim of this thesis was, therefore, to simplify current procedures to freeze ram semen in mini straws (0.25 mL), including the evaluation of several extenders and freezing protocols using a split-sample design. The procedures should result in acceptable post-thaw sperm survival with potential fertilizing capacity when tested *in vitro*, as well as in reasonable fertility after single cervical AI of extensively managed Corriedale ewes during spontaneous oestrus in Uruguay. Thus, the studies comprised trials performed in Sweden (*in vitro*) and in Uruguay (*in vitro* and *in vivo*).

The *in vitro* studies included alternative extenders and elimination of centrifugation during handling. Semen frozen in a programmable freezing chamber (studies in Sweden) or frozen in an isotherm box, 4 cm above the surface of the liquid nitrogen (LN_2) for 10 min (studies in Uruguay), was analyzed for several sperm parameters post-thaw. These were subjective motility (SM), computer assisted sperm-motility analysis (CASA), membrane integrity (SYBR-14/PI), and capacitation status (CTC). The *in vivo* studies were done under commercial-like conditions in Uruguay during two consecutive breeding seasons (April 2000 and April 2001), where a total of 1,270 Corriedale ewes were cervically inseminated, once, during spontaneous estrus. Fertility after AI was recorded as

non-return rates at 21 days (NRR-21); non-return rates at 36 days (NRR-36); pregnancy rates (PR-US, ultrasonography at 50 days) and lambing rates (LR).

Replacement of the centrifugation by an adjusted semen extension, yielded post-thaw significantly higher numbers of spermatozoa with desirable characteristics per straw, compared to controls (centrifugation). The use of a clarified milk-based extender resulted in slightly better (NS) post-thaw sperm quality compared to the non-clarified milk extender, and significantly better if compared to a conventional TRIS-citrate-fructose extender (SM: 57.5% vs. 43.8%; linear motile spermatozoa [CASA]: 46.5% vs. 33.7%, uncapacitated spermatozoa: 49.7% vs. 34.4%). Increasing the concentration of egg yolk in the milk-extender above 5-10% did not have any beneficial effects on post-thaw sperm parameters. Furthermore, post-thaw sperm quality was, generally, better preserved when the second fraction with glycerol was added at 5°C rather than at 15°C. For further studies, a two-step (two-fractions) freezing protocol using a clarified milk extender with 5% egg yolk and 7% glycerol (added in the second fraction at 5°C) was used as control and compared in *vitro* to Bioexcell® (IMV, a commercial extender free from animal-derived components available for bull semen) containing either 3.2% or 6.4% glycerol. No significant differences in sperm characteristics post-thaw were seen between the milk-egg yolk extender (control) and Bioexcell® 6.4% glycerol. Both gave significantly higher ($P<0.001$) results than Bioexcell® 3.2% glycerol.

Fertility after AI was tested, in two field trials, by single cervical deposition of FT-semen (200 x 10^6 total spermatozoa/straw, frozen in an isotherm box) in ewes during spontaneous estrus. The two above mentioned procedures of adjusting sperm numbers prior to freezing, tested in 300 Corriedale ewes (April 2000) did not differ significantly (for either NRR-21 [30.8% vs. 29.7%], NRR-36 [28.5% vs. 27.8%] or LR [21.9% vs. 21.4%], respectively). This indicated that the simplified protocol without centrifugation not only resulted in higher numbers of viable spermatozoa, but also seemed to be a suitable procedure for freezing ram semen under field conditions. The fertility results after AI in 970 Corriedale ewes (April 2001) with FT semen extended in Bioexcell® 6.4% glycerol or milk-5% egg yolk extender did not differ significantly for NRR-21 (36% vs. 33%), NRR-36 (35% vs. 33%) or PR (28% vs. 27%).

It was concluded that a simple two-step protocol using adjusted extension to achieve the final sperm concentration (200 x 10^6 total spermatozoa/straw) and avoiding centrifugation, could be used to freeze ram semen, also when freezing in static LN_2 vapors. This freezing method appeared as suitable for AI of large flocks in developing countries since it did not require any expensive equipment, yielded a good post-thaw sperm survival, and resulted in fairly acceptable fertility after a single cervical AI with FT-semen of ewes in spontaneous estrus under conditions of extensive animal husbandry. Similar fertility results could be reached with ram semen frozen either in milk-based extenders or in Bioexcell® (6.4% glycerol), the latter being a safer alternative compared to current extenders when considering introduction of genetic material.

Key words: sheep AI, cervical AI, post-thaw, semen evaluation, viability, capacitation status, semen-extender, milk-based extender, soy-bean lecithin extender.

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In memory of Francisco Haedo,
Gonzalo Córdoba, and
Horacio Lamarca

To the source of my strength and
love: Sandra, Lucía and Elisa ...

Abstract

Gil Laureiro, J. 2001. *Fertility of frozen ram semen under field conditions. With special reference to influence of extenders and freezing procedures.* Doctoral Thesis. ISSN 1401-6257, ISBN 91-576-5941-9.

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Key words: sheep AI, cervical AI, post-thaw, semen evaluation, viability, capacitation status, semen-extender, milk-based extender, soy-bean lecithin extender.

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Contents

Appendix

List of original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals **I-IV**:

I. Gil J, Söderquist L, Rodriguez-Martinez H. Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. Theriogenology 2000, 54:93-108.

II. Gil J, Rodriguez-Irazoqui M, Söderquist L, and Rodriguez-Martinez H. Influence of centrifugation or low prefreezing extension rates on the fertility of ram semen after cervical insemination. Theriogenology 2001, (in press).

III. Gil J, Lundeheim N, Söderquist L, and Rodriguez-Martinez H. Influence of extender, and temperature addition of glycerol on post-thaw sperm parameters in ram semen. 2001 (Submitted for publication).

IV. Gil J, Rodriguez-Irazoqui M, Lundeheim N, Söderquist L, and Rodriguez-Martinez H. Fertility of ram semen frozen in Bioexcell® after cervical artificial insemination. 2001 (Submitted for publication).

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Abbreviations

AI	artificial insemination
B/3.2	Bioexcell-3.2% glycerol
B/6.4	Bioexcell-6.4% glycerol
CASA	computer-assisted sperm analysis
CTC	chlortetracycline
CTC-AR	acrosome-reacted pattern, assessed by CTC
CTC-B	capacitated pattern, assessed by CTC
CTC-F	uncapacitated pattern, asssessed by CTC
FT	frozen-thawed
LM	linear motile spermatozoa
LN_2	liquid nitrogen
LR	lambing rate
M	milk-extender
MI	membrane integrity
M10	clarified milk-10% egg yolk extender
M15	clarified milk-15% egg yolk extender
M20	clarified milk-20% egg yolk extender
M5	clarified milk-5% egg yolk extender
NRR-21	non-return-rate after 21 days of AI
NRR-36	non-return-rate after 36 days of AI
P1	Protocol 1 (with centrifugation)
P2	Protocol 2 (adjusted semen extension)
P5°C	Protocol with the second-step extension at 5°C
P15°C	Protocol with the second-step extension at 15°C
PR-US	pregnancy rate by ultrasound
SM	subjective motility
TM	total motile spermatozoa
TRIS	tris-hydroximethyl amino methane
VAP	velocity average path
VCL	velocity circular line
VSL	velocity straight line

Introduction

Artificial insemination (AI) has been the biotechnology that has made the development of breeding programs in dairy cattle possible worldwide. Genetic progress in sheep may be increased by approximately 50% by its use (Clarke, 1986). However, AI in sheep has not been widely applied on a large scale, probably due to the often extensive management involved in sheep farming, and the difficulties that are involved when attempting accurate estrous detection during AI in large flocks. During the past years, different systems for using commercial AI in sheep have been developed, using either neat unextended semen, or extended semen that has been preserved in liquid form (fresh, or cooled to 5°C), or deep-frozen. Independent of the type of semen used, different locations (vaginal, cervical or intrauterine) have been used for semen deposition in the female genital tract (Evans and Maxwell, 1987; Halbert et al., 1990; see Salamon and Maxwell, 1995b, 2000).

Cryopreservation of semen is considered to be an optimal handling procedure for efficient and safe breeding programs as well as the introduction of new genetic material, which means that distances between the male and the females, including overseas transit, can be overcome. It also facilitates sanitary quarantine, sire insurance, genetic banking, etc. The use of frozen-thawed (FT) semen for AI in sheep has achieved consistent and acceptable fertility results after intrauterine semen deposition. However, fertility results usually are unacceptable when the FT-semen is deposited into the cervix (Salamon and Maxwell, 1995a, b). On the other hand, compared to the simple cervical insemination, intrauterine AI is both more time consuming (performed laparoscopically, or transcervically, as in the Guelph system; Halbert et al., 1990) and more costly, besides being a matter of concern for the welfare of the ewes (Campbell et al., 1996). The relatively low fertility after cervical AI with frozen semen calls for an improvement of the insemination technique itself.

Several approaches have been tested to circumvent the low fertility of FT-semen after cervical insemination. Hormonal treatments have been studied, either systemically applied to the females or added to the semen extender, attempting to improve sperm transport and/or the permeability of the cervix (Edqvist et al., 1975; Gustafsson et al., 1975; Memon et al., 1984; Buckrell et al., 1994). Another practical approach has been the deposition of highly concentrated sperm doses into the cervix (see Salamon and Maxwell, 1995b; Guillan and Maxwell, 1999a). However, an improvement in fertility rates requires insemination close to ovulation, particularly when FT-semen is going to be used (Holt, 2000). This implies the use of more than one estrous detection/day, which, under extensive rearing conditions, often represents both impractical and excessive handling of the ewes. The results reported so far vary widely, depending on, among other factors, flock management, insemination skills and, ultimately, the quality of the FT-spermatozoa in the AI dose. About half of the spermatozoa subjected to extension, cooling, freezing and thawing do not survive the procedures. Among

those spermatozoa surviving, a certain number presents a shorter life span and a lower fertilizing potential compared with the initial cells (Hammerstedt et al., 1990; Guillan et al., 1999; Guillan and Maxwell, 1999; Watson, 2000). Furthermore, FT-semen is lost from the reproductive tract at a faster rate than fresh semen (Guillan et al., 1999). This calls for the application of better procedures to freeze ram semen, including the use of more suitable extenders (Molinia et al., 1994, 1996; Salamon and Maxwell, 1995a; Sanchez-Partida et al., 1999; D'Alessandro et al., 2001), cooling rates (Byrne et al., 2000), thawing rates (Söderquist et al., 1997), etc. that would prevent sperm damage, and thus improve its potential fertility. Addition of seminal plasma during sperm handling has been shown to improve the quality of sperm parameters to the extent of restoring the changes in membrane status produced by the cold shock and the freezing-thawing procedures (Ollero et al., 1998; Barrios et al., 2000; Pérez-Pé et al., 2001). Furthermore, "normal" fertility results after cervical inseminations were obtained by restitution of seminal plasma to semen post-thaw (Maxwell et al., 1999). However, the steps forward must be of a practical nature, so that semen processing can be applied to extensive management procedures in countries where the infrastructure is not so well developed.

In Norway and Sweden, fertility rates of approximately 50% and higher have been reported after cervical insemination with FT-semen that was processed by a routine freezing protocol based on a two-step extension in a milk-based extender (Olafsson, 1980; Lillo, 1984; Grøtte, 1992; Olesen, 1993; Andersen Berg, 1999; Söderquist et al., 1997; 1999a, b). This routine protocol includes a first-step 5-6 fold extension with a fraction of the extender without glycerol, and a second-step 10-12 fold extension with a fraction of the extender with 14% glycerol to yield a final 7% glycerol concentration. The extended semen is then centrifuged to reconcentrate it so that approx. 200 x 10^6 spermatozoa are packed into each 0.25 mL plastic-straw. This centrifugation, however, not only complicates the processing of semen but may also impose further stress on sperm function and viability. Furthermore, it is likely that important sperm losses occur when the supernatant is removed after centrifugation (Söderquist et al., 1999a). Replacing the centrifugation by a simple semen extension to reach the aimed sperm concentrations appears to be an interesting alternative treatment.

However, the adoption of a "two step extension" methodology without centrifugation, would imply addition of the cryoprotectant glycerol with the second fraction after cooling to 5°C, why the cooling to 5°C is done with only half the final extension rate. Such a low proportion of the extender, when added to the semen during cooling can result in fewer amounts of protective compounds for the spermatozoa. Alternatives to solve this problem would be a "single step extension" procedure, or the addition of the second fraction before the temperature reaches 5°C. Semen extension in a "single step" has been routine when using other extenders, such as the Salamon's TRIS-base for pellets, whereby the cooling and equilibration are started at the same time (Evans and Maxwell, 1987). It has been shown that glycerol, despite its value as cryoprotectant, is metabolically toxic to spermatozoa, and noxious to membrane

integrity (MI), depending on the concentration and the temperature at which it is added, thus calling for a compromise if optimal results are to be achieved (Colas, 1975; Fiser and Fairfull, 1986b; Fahy, 1986; Fiser and Fairfull, 1989).

An early report found no differences in the revival of ram spermatozoa when the glycerol was mixed with the semen between 5°C and 15°C (Blackshaw, 1955). Adding the glycerolized fraction during the initial cooling, before the temperature reaches 5°C, would provide fresh media to spermatozoa, increasing the semen:extender ratio and diminishing the toxic effect of glycerol when sperm metabolism is somehow depressed. Thus, to reach these two goals, the temperature for addition of the glycerolized fraction must be approximately 15°C, to circumvent cold shock (Fiser and Fairfull, 1986a). Such a modified "two step extension" method with an earlier "second" step has not been tested for freezing ram semen in milk-based extenders with low pre-freezing semen:extender rates.

Semen extenders have been prepared, often empirically, to protect spermatozoa during semen processing and preservation. When used for preservation at low temperatures, they contain basic ingredients and additives to provide a suitable environment for spermatozoa, including energy substrates, buffers, antibiotics and cryoprotectants (reviewed by Salamon and Maxwell, 1995a; 2000; El-Alamy and Foote, 2001). The ingredients in an extender vary from pure chemical compounds to undefined products of animal or vegetal origin. Particularly, egg yolk and skim milk are two common components of animal origin that have been used for years to freeze ram semen (Olafsson, 1980; Lillo, 1984; Grøtte et al., 1992; Andersen Berg, 1999; Söderquist et al., 1997, 1999a, b). As well, *Aloe vera* gel (Rodriguez et al., 1988) or fruit resins (see Salamon and Maxwell, 1995a) are among those vegetal additives successfully used for freezing ram semen.

Extenders based on TRIS-citrate-fructose plus egg yolk have been widely used to freeze spermatozoa from several farm animal species, including the sheep (Andersen et al., 1973; see Salamon and Maxwell, 1995a), despite the species-to-species differences that exist (Holt, 2000). The protective component in the egg yolk has been demonstrated to remain in the supernatant after ultracentrifugation indicating that low-density lipoproteins are responsible for these protective effects (Watson and Martin, 1973; 1976). Milk has been used in extenders for bull and ram semen after the sperm-toxic components have been inactivated (by heat, dialysis, or cysteine-HCl treatmentM; Jones and Foote, 1972). Reconstituted skim milk powder is the base for the extender used in Sweden and Norway, to which egg yolk is added. Particles often remain un-dissolved or precipitate after heat-inactivation in such extenders and impair post-thaw semen evaluation, which is a prerequisite to studying the influence of different procedures for freezing semen. Thus, if clarified by centrifugation at 5°C from the big particles and overlaying lipids this extender might be an alternative to the standard milk extender by providing a clear media for the coming evaluation but also by having some beneficial effects on spermatozoa. To my knowledge, no reports are available concerning clarification of heavy particles and overlaying lipids in milk-based extenders for freezing ram semen.

Different concentrations of the skim milk powder for extenders have been reported (Fiser and Fairfull, 1986a). Since extender components usually interact with each other, an increase of egg yolk allows a lower glycerol concentration, thus diminishing the risk for toxic effects while maintaining cryoprotection. Higher egg yolk concentrations in a milk-based extender (11% w/v) to increase the protective components for low pre-freezing semen:extender ratios, like that used in the simplified protocol described above, have not yet been tested.

The addition of components of animal origin (e.g., milk or egg yolk) in a semen extender implies sanitary risks, not only through the inclusion of specific microbiological agents, but also by contaminants that may compromise the quality of the product (Bousseau et al., 1998; Thibier and Guerin, 2000). Furthermore, these additives are so diverse and often variable in their composition that quality certification is very difficult or even impossible to perform. Commercial extenders with an egg yolk substitute have recently become available for freezing bull semen (Biociphos® and Bioexcell®, IMV, L'Aigle, France), and some fertility reports for bull semen frozen in such extenders have been published (Hinsch et al., 1997; Gil et al., 2000a; Van Wagtendonk-de Leew et al., 2000). So far no reports are available concerning the value of this extender for freezing ram semen.

Any new method for processing ram semen needs to be evaluated before applying it practically under extensive field conditions. Therefore, testing, using suitable *in vitro* assays to evaluate the efficiency of cryopreservation in a certain extender is needed. These assays must focus on the assessment of sperm viability, including the determination of motility parameters, MI and acrosomal status (Saacke and White, 1972; Amann, 1989; Cross and Meitzel, 1989; Amann and Hammerstedt, 1993; Johnson et al., 1996; Rodriguez-Martinez et al., 1996). Furthermore, since sperm handling during cryopreservation has been shown to promote the destabilization of the sperm membrane, consequently mimicking capacitation or even eliciting the acrosome-reaction of the processed spermatozoa (Garde et al., 1993; Watson, 1995-2000; Perez et al., 1996; Guillan et al., 1997), such events must be evaluated after freezing-thawing.

The most widely used test for semen evaluation has been the subjective assessment of sperm motility, but results often show a rather high variability among operators. It is therefore wise to apply a more objective evaluation method as the one provided by computer-assisted sperm analysis instruments (CASA), which yields information of a wide range of motility traits, such as displacement velocities and patterns. Sperm MI has been evaluated by different methods, and those using combinations of fluorescent dyes are now well standardized and simple to perform (Garner et al., 1986; Garner and Johnson, 1995; Thomas et al., 1998). Sperm membrane stability that is assessed with the fluorescent antibiotic chlortetracycline provides information of the arrangement of calcium ions within the lipophilic membrane layer, in relation to the process of sperm capacitation (Tsien, 1989; Cross and Meitzel, 1989; Thundathil et al., 1999). Although a thorough *in vitro* evaluation provides valuable basic information about the status

of the cryopreserved spermatozoa, an assessment of the *in vivo* fertility is the ultimate proof for the fertilizing ability of the processed semen.

If the aim is to improve AI procedures for an extensive application of this biotechnology, primarily attempting the introduction of genes via frozen ram semen, a consideration of the diverse farming conditions present worldwide is imperative. Although sheep farming is done either in small or large flocks, the latter is the rule in many countries such as Uruguay, which holds over 13 million heads (SUL, 2000) raised under extensive conditions of management. Considering the application of AI technology with FT-semen on a large scale in Uruguay an adaptation of the freezing procedures and the AI management needs to be done. Use of AI with cervical deposition of FT-semen in spontaneous estrus is more feasible under extensive conditions, thus avoiding the use of hormones and providing the possibility of lowering the costs in terms of equipment and personnel compared to the laparoscopic insemination.

Aims of the study

The overall objective of this thesis was to simplify current procedures for freezing ram semen in mini-straws (0.25 ml). The work included the evaluation of several extenders and processing protocols, using a split-sample design, aiming at acceptable post-thaw sperm survival with potential fertilizing capacity *in vitro* as well as reasonable fertility after cervical AI of extensively managed Corriedale ewes in Uruguay.

Therefore, the sperm viability (motility, membrane integrity and capacitation status) of frozen-thawed ram spermatozoa was tested *in vitro*, to evaluate the effect of:

- Careful semen extension (without centrifugation) compared to semen extension and re-concentration by centrifugation to a final sperm concentration of 200 x 10^6 in the AI-dose, when to a
- Further extension and glycerolization at 15°C, instead of the routine procedure at 5°C.
- A TRIS-citrate-based extender compared to a standard milk-based extender (5% egg yolk) - clarified or non-clarified by centrifugation.
- Milk-based extenders with various concentrations of egg yolk (5, 10, 15, 20%).
- Extension with Bioexcell® with two different final glycerol concentrations (3.2 and 6.4%) compared to a clarified milk-5% egg yolk extender.

Furthermore, the fertility of Corriedale ewes was evaluated under extensive management conditions in Uruguay after a single cervical AI during spontaneous estrus with FT-ram semen, processed:

- in a clarified milk-egg yolk (5%) extender according to the protocol with centrifugation (control) compared to the adjusted semen extension (without centrifugation), and
- in Bioexcell® (6.4% final glycerol concentration) compared to the clarified milk-egg yolk (5%) extender (control).

Materials and methods

Animals and Semen Collection

Altogether, semen from 21 rams of two breeds (Gotlandic, Corriedale), and crossbred (Gotlandic x Finewool) aged between 10 mo to 4 yrs were used. The Gotlandic and crossbred rams were used in the Northern Hemisphere (**Papers I** and **III**) while housed at the Department of Obstetrics and Gynecology (Swedish University of Agricultural Sciences, Uppsala; 61°N/15°E), or at a neighboring ram AI-station of the Swedish Sheep Farmers Associations (Kungsängen, Uppsala: 61°N/16°E). The Corriedale rams used in the Southern Hemisphere (**Papers II** and **IV**) were located at the Experimental Station EEMAC–Paysandú (University of Uruguay; 32°S/58°W). Semen was daily collected using artificial vaginas, during the breeding season (Northern Hemisphere: November-December [1998 and 2000]; Southern Hemisphere: March April [2000 and 2001]). One to two consecutive ejaculates were collected per ram and later pooled and treated as a single ejaculate. The ejaculates were within normal ranges with regard to volume (0.75-2 ml), sperm concentration ($\geq 2.5 \times 10^9$ spermatozoa/ml), sperm motility (≥70%) and sperm morphology (≤10% total sperm abnormalities).

Extenders

Because of the two-step extension methodology used, all extenders had two different fractions (1 and 2): the first without glycerol, and the second with a glycerol concentration twice the final glycerol concentration. All extenders were prepared a week before each experiment as described below, and stored at –18°C until thawed and used in the experiments:

Extender M: (control: **Paper I**) non-fatty milk powder (11% w/v) and distilled water heated to 95° C for 10 min and then cooled to room temperature for further use. Fraction 1 was prepared by adding enough egg yolk to the milk-base extender to reach a concentration of 5% (v/v) and supplemented with antibiotics (0.03 g penicillin and 0.04 g streptomycin/100 ml). Fraction 2 was prepared by adding egg yolk and glycerol to the milk base, in order to obtain concentrations of 5% and 14% (v/v), respectively, and further supplemented with 224 mM of fructose and antibiotics as described above.

Extender M5: (treatment: **Paper I**, control: **Papers II-III-IV**) fraction 1 was prepared in the same way as extender M, except that it was clarified from particles by centrifugation at 3,310 g at 5° C for 20 min, repeating the treatment with the supernatant. Fraction 2 was centrifuged before adding the same glycerol and fructose concentration as in fraction 2 for extender M. The supernatant between the overlying lipoid layer and the compact pellet at the bottom was used as an extender in this study.

Extender TRIS: (treatment: **Paper I**) consisted of 263 mM TRIS, 85 mM citric acid, 73 mM fructose, and 20% (v/v) egg yolk. Fraction 1 consisted of extender clarified by centrifugation (as described for extender M5). Fraction 2 was the same as 1, but with the addition of glycerol (14% v/v). The antibiotic mixture used in this extender was the same as described in M.

Extenders M10, M15, and M20: (treatment: **Paper III**) were prepared with reconstituted milk powder as described for extender M5. All fractions 1 were then prepared by adding enough egg yolk to the milk-based extender to reach concentrations of 10% (M10), 15% (M15) and 20% (M20) (v/v). After clarification by centrifugation, fraction 1 of each extender was supplemented with antibiotics as described above in M. All fractions 2 were prepared by adding 224 mM of fructose, egg yolk (10, 15, 20% v/v) and glycerol (14% v/v) to the prepared milk-base, and supplemented with antibiotics as previously described.

Extenders B/3.2 and B/6.4: (treatment: **Papers III** and **IV**) were especially manufactured preparations of the Bioexcell® extender (courtesy of IMV, L'Aigle, France). For both extenders, fraction 1 was without glycerol, but fraction 2 of B/3.2 contained 6.4% (v/v) glycerol and B/6.4 contained 12.8% (v/v) glycerol. Thus, the final glycerol concentrations of each extender yielded half the concentration of the second fraction, i.e., 3.2% and 6.4%, respectively.

Semen Handling

The semen extension methodology applied in this thesis was based on a two-step extension, with two different fractions for all extenders used with the glycerol included only in the second fraction. After collection, the sperm concentration was measured for each ejaculate either with a hemocytometer (Bürker chamber, **Paper I**) or with a photometer (**Papers II-IV**). Thereafter, the ejaculates were placed in a portable water bath at 33° C, and split into as many fractions as extenders studied. Then, the semen was extended with fraction 1 of the respective extender.

For the *in vitro* studies (**Papers I** and **III**), ejaculates from all rams involved in each trial were pooled within extenders, mixing volumes containing equal sperm numbers from each ejaculate, and then, the pools were split into as many protocols as tested for freezing the semen in each study. For the *in vivo* studies (**Papers II** and **IV**), semen from each ram was frozen separately, as with the different treatments (**Paper II**: 2 protocols, **Paper IV**: 2 extenders).

For practical reasons, in those studies where the collection site was distant from the freezing facilities, the cooling to 5°C was done in an isothermal box with wrapped freeze-packs to mimic the cooling rate obtained in the water bath. In the *in vivo* studies, the equilibration was done after the manual packaging in mini-straws, in a water bath at 5° C.

Processing Protocols

After a primary extension (1+1) with fraction 1 of the respective extender, cooling was initiated, and the semen aliquots were submitted to one of the following freezing protocols:

Protocol 1: (control: **Papers I** and **II**, [P1]) **a)** Further extension (at 25° C) to a final ratio of 1+4 semen + extender-fraction 1. **b)** Cooling to 5° C within 1 h. **c)** A second-step of extension at 5° C to double the volume with fraction 2 of the extender. **d)** Equilibration at 5° C for 2 h. **e)** Centrifugation at 5° C (700g/10 min), removing enough supernatant to yield a calculated final concentration of $800x10^6$ cells/ml. **f)** Packaging in 0.25 mL mini-straws at 5° C. **g)** Freezing.

Protocol 2: (treatment: **Papers I** and **II** [P2]; control: **Papers III** and **IV** [P5°C]) **a)** Further extension (at 25° C) to $1.600x10^6$ cells/mL with extender-fraction 1. **b)** Cooling to 5° C within 1 h. **c)** A second-step of extension at 5° C to $800x10^6$ cells/mL with extender-fraction 2. **d)** Equilibration at 5° C for 2 h. **e)** Packaging in 0.25-mL mini-straws at 5° C. **f)** Freezing.

Protocol 15° C: (treatment: **Paper III** [P15°C]) **a)** Further extension to $1.600x10^6$ cells/mL with extender-fraction 1. **b)** Cooling to 15° C within 30 min. **c)** A second-step of extension started at 15° C to $800x10^6$ cells/mL with extender-fraction 2. **d)** Further cooling to 5°C within 30 minutes in a water-bath. **e)** Equilibration at 5° C for 2 h. **f)** Packaging in 0.25-mL mini-straws at 5° C. **g)** Freezing.

Freezing Procedures

For the *in vitro* studies (**Papers I** and **III**), freezing was done with a programmable freezer (Digitcool 5300, IMV, L'Aigle, France) where the temperature decreased from 5° C to -8° C at a rate of 3° C/min and from -8° C to -130° C at 25° C/min. The straws were then transferred to LN_2 for storage until evaluated. In total, four freezing operations were used in this study.

For the *in vivo* studies (**Papers II** and **IV**), the straws from the different treatments were frozen in an isotherm box, 4 cm above the level of the LN_2 (Evans and Maxwell, 1987). After 10 min, the straws were plunged into the LN_2 and transferred to a LN_2 container for further storage.

Post-Thaw Semen Evaluation

The straws were thawed in a water bath at 50° C for 9 sec, for evaluation during both the *in vivo* and *in vitro* studies. Prior to the evaluation, the thawed straws (2 to 5) were pooled in a test tube.

Final sperm concentration. The sperm concentration in the frozen-thawed straws was measured using a hemocytometer (Bürker chamber).

Subjective Motility (SM) was assessed post-thaw in a phase contrast microscope equipped with a warm stage (38°C, Nikon, Tokyo, Japan) from 5 μL aliquots of the diluted pooled sample in a Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel). The frequency of sperm motility was assessed to the nearest 5%, and an average of judgements from four different microscopic fields was used.

Computer Assisted Sperm Analysis (CASA) was performed on the same preparation of diluted semen and using the same microscope as used for SM (see above), with a Strömberg-Mika Cell Motion Analyzer SM-CMA (MTM Medical Technologies, Montreux, Switzerland). For each evaluation (**Papers I, II, III**), eight microscopic fields (sequences) were analyzed to include at least 200 spermatozoa. The proportion of total motile (TM) and linearly motile (LM) spermatozoa, straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average-path velocity (VAP, μm/s), and the extent of lateral sperm head displacement (LHD, μm) were determined. The CASA analysis was not performed in samples from the second *in vivo* trial (**Paper IV**) because of shipment restrictions following the Foot and Mouth Disease outbreak in Uruguay (April 2001).

Sperm Membrane Integrity (MI) was assessed with SYBR-14 and propidium iodide (SYBR-14/PI; Molecular Probes Inc., Eugene, OR, USA), as described by Garner and Johnson (1995). Two hundred spermatozoa were counted in a Laborlux-11® microscope (Leitz, Germany) equipped with a Paralens® (Becton-Dickinson, The Netherlands) objective set (x 60) and classified as 'intact' when stained green or as 'membrane damaged' when stained green-red or just red.

Capacitation Status was assessed in viable spermatozoa with chlortetracycline (CTC), as described by Gil et al (2000b). To assess only the viable spermatozoa, the sample was pre-stained with ethidium homodimer (23.3 μM EthD-1; Molecular Probes Inc., Eugene, OR, USA). Then, spermatozoa were fixed with glutaraldehyde (3% in cacodylate buffer, pH 7.4) prior to staining with CTC working solution. The evaluations were done using a Diaplan® microscope (Leitz, Germany) with epifluorescent optics. Two hundred viable spermatozoa were classified into three categories (Guillan et al., 1997; Gil et al., 2000b): the uniform fluorescent head (uncapacitated: CTC-F), the fluorescence-free band in the post-acrosomal region (capacitated: CTC-B), and the non-fluorescent head or a thin fluorescent band in the equatorial segment (acrosome-reacted: CTC-AR).

Insemination Procedures

The fertility trials involved a total of 1,270 AI's (one AI per ewe) of multiparous Corriedale ewes (2-4 parities), from two different flocks, during the breeding season in Uruguay (Southern Hemisphere: April 2000-2001). Flock 1 (300 ewes, **Paper II**) was grazing natural pastures at the Experimental Station

EEMAC–Paysandú (University of Uruguay, 32°S/58°W). Flock 2 (970 ewes, **Paper IV**) was grazing natural pastures in a private farm (Estancia "La Palma", Cerro Largo, Uruguay; 33°S/55°W). To detect ewes in estrus, 12 vasectomized rams with marker paint joined Flock 1 from 5 p.m. to 6 a.m. the following morning, when the marked ewes were separated from the flock for AI on the same afternoon (from 3:30 to 4:30 p.m.). The estrous detection in Flock 2 was done using 120 androgenized wethers (Testosterona Ultra Lenta Fuerte®: testosterone cyclopropionate, 200 mg/week/wether, Laboratorios Dispert, Montevideo, Uruguay) with marker paint. The ewes marked were separated from flock 2 twice a day and inseminated "over-the-rail" following an a.m./p.m. – p.m./a.m. schedule (7:00 a.m. – 6:00 p.m.). To shorten the AI program in the larger flock (Flock 2), ewes were injected twice (11 days in between) with 100 μg of $PGF_{2\alpha}$ (Glandinex®: Universal-Lab, Montevideo, Uruguay). Detection of estrus began 10 days after the second injection in order to skip the estrus that eventually followed the pharmacologically induced luteolysis. For insemination, the straws were thawed in a water bath at 50°C for 9 sec, and loaded into an insemination gun covered with a plastic sheath (Minitüb, Landshut, Germany). The external opening of the cervix was visualized with the aid of a tubular speculum equipped with light (Walmur-Veterinary Instruments, Montevideo, Uruguay) and the tip of the AI-gun carefully inserted as deep as possible into the cervix. At each AI, recordings were made of the identification of the ewe, the color (transparent, white, or cheese-like) and the amount (scarce, moderate, or abundant) of cervical mucus present in the vagina, the depth of insemination in the cervical canal (herein classified as "shallow" = <5 mm; "mid" = 5 - 10 mm; or "deep" = >10 mm), the ram, and the freezing method used. Weather conditions during the insemination period were also recorded.

Fertility Measurements

To detect ewes returning to estrus (**Papers II** and **IV**), the color used by the male teasers was changed to leave another color mark on the ewes than during the previous estrus detection (AI). Ewes not returning to estrus were considered pregnant, and recorded as the percentage of ewes that did not return to estrus at 21 (NRR-21) and 36 (NRR-36) days after AI. In flock 1 (**Paper II**), lambing rate (LR) and number of lambs born/ewe were recorded, while in flock 2 (**Paper IV**), pregnancy rate was confirmed by trans-abdominal ultrasonography (US, 3.5MHz sector probe, Pie Medical 1000, Maastricht, The Netherlands), 50 days after AI.

Experimental Design

For all trials, the semen was processed using a split-sample design. For the *in vitro* studies (**Papers I** and **III**), the semen from different rams was primarily pooled and then split into as many treatments as evaluated in each experiment.

The semen treatments that gave the best sperm viability results *in vitro* were chosen for evaluation in the *in vivo* trials (**Papers II** and **IV**). Semen from different rams were frozen separately, and the ewes were inseminated at random, attempting the use of an equivalent number of AI's per ram and freezing treatment.

Statistical Analysis

Data were analyzed using the Statistical Analysis System package (SAS Data were analyzed using the Statistical Analysis System package (SAS Institute Inc., Cary, NC, USA, 1989-96). In the *in vitro* studies (**Papers I** and **III**) the GLM or MIXED procedures were applied. For the *in vivo* studies (**Papers II** and **IV**), the GLIMMIX macro and paired *t*-test were used to analyze differences in sperm parameters between treatments. The GENMOD macro (**Paper IV**) was used for a logistic regression to observe the effect of all relevant variables, and a chi square test was used to measure the differences between specific and relevant variables. Analysis using Pearson correlation was used to determine relationships between sperm parameters and the fertility results (**Paper IV**). The results are presented as means ± standard deviations, or as least-square means ± standard error of the means. The level for statistical significance was set at $p<0.05$.

Results

Post-thaw sperm quality

Effect of Freezing Protocol

Sperm concentration: The protocol involving centrifugation to re-concentrate the sperm dose (P1) yielded a significantly lower concentration than the simplified protocol involving an adjusted semen extension (P2), ranging from 94 to 136 vs. 204 to 220 x 10^6 cells, respectively (P<0.001, **Papers I and II**). Due to this difference in sperm concentration, the total number of spermatozoa showing the best traits (SM, CASA parameters, MI, and CTC parameters) was higher in P2 than in P1, even though the percentage was lower for P2 than for P1. The photometric assessment of the sperm concentration yielded similar results as hemocytometry (**Papers II-III-IV**).

Subjective sperm motility (SM): The SM was better in P1 than in P2 (from 64% to 68% vs. 33% to 57%, respectively, P<0.05, **Papers I and II**). Conversely, the total number (x 10^6) of subjective motile spermatozoa was higher in P2 than in P1 (125 vs. 87, P<0.001, **Paper I**). The addition of glycerol with fraction 2 of the milk-based extenders at 15°C compared to 5°C did not affect motility post-thaw (69% for both P15°C and P5°C, **Paper III**). But when comparing extender B/3.2, B/6.4, and M5, the P5°C yielded better motility than P15°C (48% vs. 42%, P<0.05, **Paper III**).

CASA-assessed sperm motility: The sperm parameters assessed by CASA were higher in P1 than in P2 (NS-P<0.001, **Papers I and II**). The CASA parameters were almost equal in P15°C and in P5°C (**Paper III**). The absolute number (x 10^6) of TM was higher in P2 than in P1 (155 vs. 105, P<0.001, **Paper I**).

Membrane integrity (MI): The MI was higher in P1 than in P2 (56% to 76% vs. 31% to 49%, NS-P<0.05, **Papers I and II**). MI was lower than when glycerolization was done at 15°C compared to at 5°C (68 vs. 63%, P<0.01, **Paper III**). The total number (x 10^6) of MI spermatozoa was higher in P2 than in P1 (109 vs. 77, P<0.001, **Paper I**).

Capacitation status: A higher frequency of CTC-F spermatozoa were present in samples processed according to P1 than to P2 (56% vs. 48%, P<0.01, **Paper I**). Despite the higher percentage in P1 in samples from the *in vivo* trial, this difference was not significant (18% vs. 16%, NS, **Paper II**). A slightly higher frequency of CTC-F spermatozoa were recorded in P5°C than in P15°C (68% vs. 63%, NS, **Paper III**). The total number (x 10^6) of CTC-F was higher in P2 than P1 (47 vs. 37, P<0.05, **Paper I**).

A higher frequency of CTC-B spermatozoa were seen in P2 than in P1 in one of the trials, both as percentages and as total numbers per AI dose (39% vs. 29%, P<0.01; 39 vs. 19 x 10^6/straw, P<0.001; **Paper I**). This was not the case in samples from the *in vivo* trial, where similar percentages of CTC-B classified cells were present in P1 and P2 (77% and 73%, respectively, NS, **Paper II**).

P15°C compared to P5°C resulted in similar sperm capacitation values (50%, NS, **Paper III**). The percentages of CTC-AR spermatozoa did not differ significantly between P1 and P2 ranging from 5% to 12% (**Papers I** and **II**). The addition of glycerol according to P5°C resulted in lower values of CTC-AR than in P15°C (6% vs., 8%, P<0.01, **Paper III**).

Effect of Extenders

In total, 8 extenders were studied. Five of them were milk-based extenders that differed in either being clarified or not by centrifugation or by their egg yolk concentration. The others were two variants of Bioexcell® differing in glycerol content, and one was a TRIS-based extender.

Subjective sperm motility: This parameter showed higher values for milk extenders and Bioexcell® with 6.4% glycerol (B/6.4), with somewhat lower values in the TRIS-based extender, and lowest in Bioexcell® with 3.2% glycerol (B/3.2). The milk-based extenders (M, M5, M10, M15, and M20) did not distinctly affect the SM within each trial (**Papers I** and **III**). The SM in milk extenders ranged between 47% and 70%. The TRIS based extender (**Paper I**) yielded lower SM than the M5 (44% vs. 58%, P<0.01). Spermatozoa frozen in B/6.4 had a SM that was similar to M5 (47%, **Paper IV**), while B/3.2 gave lower (P<0.001) SM than M5 and its homologous B/6.4 (26%, 53%, and 58%, respectively, **Paper III**).

CASA-assessed sperm motility: The patterns of sperm motility post-thaw were better when semen was frozen in the clarified milk-based extender containing 5% egg yolk, followed by B/6.4. The clarified milk extender (**Paper I**) was consistently better for all parameters studied (total motility [TM], linear [LM], straight velocity [VSL], average velocity [VAP], circular velocity [VCL], lateral head displacement [LHD]) compared to the non-clarified milk extender (NS) and to the TRIS-based extender (P<0.001). There were no significant differences among milk-based extenders with different egg yolk concentrations and B/6.4 (**Paper III**). The results of TM and LM were lowest (P<0.001) for B/3.2 compared with M5 and B/6.4, but the velocity patterns differ (P<0.01) between M compared to B/3.2 and to B/6.4 (**Paper III**).

Membrane integrity: Basically, all extenders maintained acceptable MI levels, except for B/3.2. MI remained constant (approx. 51%) for milk- or TRIS-based extenders (**Paper I**). The M10 extender yielded significantly higher percentages of MI than M5 or M20 (69%, 64%, and 63%, respectively, P<0.05), but the difference was not significant compared to M15 (66%, NS) (**Paper III**). The difference in MI between M5 and B/6.4 varied between different trials. In one of them, the percentage of MI was better in M5 than B/6.4 and B/3.2 (57%, 46%, and 36%, respectively, P<0.001, **Paper III**), and in the other MI in M5 was similar to B/6.4 (38%, **Paper IV**).

Capacitation status: Extenders affected the capacitation status of frozen-thawed ram spermatozoa. The capacitation rates were higher in Bioexcell® and TRIS-based extenders than in the other extenders tested. The frequency of CTC-F did not differ significantly between the clarified and non-clarified milk extenders

(54% and 49%, NS), but was significantly higher than in the TRIS extender when compared to the milk extender (49% vs. 34%, P<0.001, **Paper I**). Increasing egg yolk concentrations in the milk-based extenders, from 5% to 20%, yielded decreasing percentages of CTC-F, from 51% to 41%, (P<0.01, **Paper III**), thus indicating a destabilizing effect of egg yolk on the plasmalemma. The percentages of CTC-F in the Bioexcell® extenders were almost equal, but in one trial both were lower than in M5 (30% vs. 44%, P<0.001, **Paper III**). This difference between M5 and B/6.4 was not seen in **Paper IV**, where they resulted in similar percentages (26%-28%, NS).

The percentages of CTC-B were similar in clarified and non-clarified milk extenders, albeit lower than in the TRIS extender (40% vs. 54%, P<0.001, **Paper I**). The M5 showed the lowest frequency of CTC-B compared to M10, M15, and M20, ranking from 44% to 53% (P<0.01, **Paper III**). The frequency of CTC-B was almost equal for M5 and B/6.4, but lower (P<0.001, **Paper III**) in extender B/3.2, (48% vs. 58% in one trial, and vs. 61% in another trial (**Paper IV**).

The percentage of CTC-AR did not differ significantly between clarified and non-clarified milk extenders, as well as between milk extenders and the TRIS-based extender (from 8% to 11%, **Paper I**, NS), or between M5, M10, M15, and M20 (from 4% to 10%, NS). The percentages of CTC-AR were lower in M5 than in B/3.2 and B/6.4 (8%, 10%, and 19% respectively, P<0.05, **Paper III**). This parameter did not differ between M5 and B/6.4 in the *in vivo* trial (10% to 13%, **Paper IV**).

*Effect of Interaction Extender * Protocol (in vitro trials)*

Sperm concentration: There was no effect of this interaction when using milk-based extenders, but sperm concentration was higher in samples processed according to P1 (re-concentration by centrifugation) in TRIS-based extender when compared to the clarified milk-based extender (150 vs. 130 x 10^6/straw, P<0.01, **Paper I**). This affected the total numbers of spermatozoa per straw with desirable sperm parameters (SM, CASA, MI, and CTC).

Subjective sperm motility: The SM was not affected by the interaction when using M or M5 extenders (either processed by P1 or P2, **Paper I**). This was also the case for M5, M10, M15, and M20, when comparing the addition of glycerol at two different temperatures with fraction 2 (NS, **Paper III**). The interaction affected the SM when using B/3.2, with higher values in P5°C than in P15°C (31% vs. 20%, P<0.05, **Paper III**).

CASA-assessed sperm motility: No significant differences were seen between clarified and non-clarified milk extenders vs. TRIS extender (**Paper I**). This was also the case when comparing the different egg yolk concentrations and the addition of glycerol at 5°C or 15°C (P5°C and P15°C, **Paper III**), as well as when comparing B/3.2, B/6.4, and M5. The exception was B/6.4, which had higher percentages of LM spermatozoa when processed by P5°C than by P15°C (51% vs. 39%, P<0.05, **Paper III**).

Membrane integrity: The MI was not significantly affected by the interaction of extenders M, M5, or TRIS with P1 or P2 (**Paper I**). This variable was affected when testing different egg yolk concentrations, with higher values for P5°C than for P15°C for M15 (70 vs. 63, $P<0.05$, **Paper III**). Extender B/6.4 performed significantly better when processed by P5°C than by P15°C (50% vs. 42%, $P<0.05$, **Paper III**).

Capacitation status: The percentage of spermatozoa with CTC-F pattern was not affected by the interaction of any protocol (P1, P2, P5°C, and P15°C) regardless of the extender used (milk, TRIS-based, or Bioexcell®, **Papers I** and **III**). This was also the case for the percentage of spermatozoa depicting the CTC-B pattern. The frequency of CTC-AR spermatozoa was affected by extenders M10 and M15 when processed by P15°C compared to by P5°C ($P<0.01$, **Paper III**). The categories assessed in B/3.2, B/6.4, and M5 were not influenced by the interaction.

Fertility after cervical AI with frozen-thawed (FT) semen

Fertility using two semen extenders (Bioexcell® with 6.4% glycerol vs. milk with 5% egg yolk and 7% glycerol, **Paper IV**) and two freezing protocols (re-concentration by centrifugation vs. extension without centrifugation, **Paper II**) was evaluated after a single cervical AI of ewes in spontaneous estrus, under extensive conditions of management.

Effect of Extenders

There was no statistically significant effect by the extender used in the NRR-21, NRR-36, nor the PR-US. The NRR-21 was 36% vs. 33%, the NRR-36 was 35% vs. 33%, and the PR-US was 28% vs. 27% for B/6.4 and milk-based extender with 5% egg yolk, respectively (NS, **Paper IV**).

Effect of Freezing Protocols

Fertility, when using only semen extension to achieve the final sperm concentration in the AI-dose (P2) did not differ from that of the control (P1), using re-concentration by centrifugation. In this case, the NRR-21 was 31% vs. 30%, the NRR-36 was 29% vs. 28%, and the LR 22% vs. 21%, for P1 vs. P2, respectively (NS, **Paper II**).

Effect of the ram

A variation between rams was present in all trials, albeit becoming statistically significant only in the second fertility trial ($P<0.05$, **Papers II** and **IV**).

Effect of the depth of cervical penetration at AI

Cervical insemination at different depths in the cervix did not affect fertility significantly (**Papers II** and **IV**). Extender B/6.4 gave significantly better NRR-

21 and NRR-36 ($P<0.05$) results with deeper inseminations, but results were not significant for the PR-US.

Effect of the weather

There was an association between weather and LR only in the first field-trial (**Paper II**). The lowest LR was observed when AI were performed in the most rainy day (63.7 mm rainfall) and the day after (14% and 11%, respectively).

General discussion

Any modification in the methods for processing ram semen needs to be tested before applying them in the field. Thus, laboratory *in vitro* assays are essential to test their efficiency. Among these assays, those focusing on sperm viability, such as motility patterns, membrane integrity and acrosomal status are considered to be of great value (Saacke and White, 1972; Amann, 1989; Cross and Meitzel, 1989; Amann and Hammerstedt, 1993; Johnson et al., 1996; Mortimer and Maxwell, 1999). Indeed, semen handling and particularly cooling and re-warming during freezing and thawing procedures have been shown to promote destabilization of the sperm membranes resulting in capacitation or acrosome exocytosis (Watson, 1995; 2000; Perez et al., 1996; Guillan et al., 1997). When assessing capacitation patterns, it is critical to assess only the sperm population actually viable after thawing (Cross and Meitzel, 1989). In this viable sperm population, those that are already capacitated (readily to acrosome exocytosis) probably have a reduced life span or at least a time dependent decrease in fertilizing potential (Garde et al., 1993; Watson, 1995; 2000; Maxwell and Watson, 1996; Perez et al., 1996). Therefore, when evaluating methods for freezing semen, those that yield the largest populations of uncapacitated viable spermatozoa should be considered the most useful ones.

Nevertheless, the ultimate test for evaluating the fertilizing potential of FT-semen still remains to be the assessment of fertility after AI in the field. The success of AI with FT-semen largely depends, apart from the post-thaw quality, on the biological context where it has to be used, such as female anatomy, and the timing of ovulation (Holt, 2000). This thesis aimed to test both the sperm viability and membrane status under laboratory conditions, as well as the field fertility of ram semen processed according to protocols with a variety of modifications during extension and cooling, as well as in the extenders.

It is often concluded that improvements seen *in vitro* are not necessarily seen *in vivo* (Molinia et al., 1996; Sanchez-Partida et al., 1999). In addition to the genetic and physiological status of the female, fertility is a multi-factorial trait affected by the environment, flock management, the insemination technique itself and (of utmost importance) by the post-thaw semen quality and its inherent fertilizing potential. The conditions of sheep husbandry in Uruguay are extensive. This situation is common for many other countries in the southern hemisphere where sheep farming in a large scale is customary. Thus, most of the above factors would largely affect fertility after application of AI and might, therefore be particularly considered.

Protocols

The routine protocol for freezing ram semen in Norway and Sweden, used as control for the modified methods studied herein, includes a re- concentration of previously extended spermatozoa by centrifugation. In Sweden, the centrifugation

step has been a matter of concern for some time, not only due to the possible stress inflicted onto the spermatozoa, but also because of the wastage of spermatozoa seen when removing the supernatant after centrifugation. Furthermore, those removed spermatozoa present in the supernatant, could be suspected to hold desirable attributes because of their capability to escape the centrifugal force and remain in or return to the supernatant at the end of centrifugation. The magnitude of the sperm loss by the centrifugation has been quantified (Söderquist et al., 1999a). Furthermore, pilot trials have shown that the quality of spermatozoa kept in the supernatant did not differ significantly from those in the pellet, following conventional centrifugation (Gil, unpublished results). As a consequence of these findings, it is only the necessary volume of the supernatant that is removed in order to reach a desired final concentration. The procedure is used extensively nowadays in Sweden (Söderquist et al., 1999b), thus avoiding the use of a third extension, a procedure still used in Norway (Andersen Berg, 1999). This adaptation of the methodology was used as the initial control protocol for this thesis (**Paper I**).

This control protocol, including centrifugation, requires the obvious use of a centrifuge, thus constraining the adaptability of the method for freezing under less favorable working conditions in developing countries. A possible alternative to avoid the centrifugation, whereby the semen extension was done by the adjusted addition of the extender knowing the sperm concentration in the ejaculate to the aimed final sperm concentration (200 x 10^6/straw), was tested herein. The procedure skips centrifugation, thus simplifying the processing of the semen. Such a procedure could be useful for freezing ram semen under the prevailing conditions in Uruguay, where sheep farming is performed extensively and proper facilities, electricity and necessary equipment are not always available.

The evaluation of spermatozoa processed by this simplified protocol (**Paper I**) showed that the low pre-freezing semen:extender ratio was detrimental for all the sperm parameters assessed (motility, membrane integrity and membrane stability) and confirmed previous reports of the harmfulness of such low dilution rates (Colas, 1975; Ashworth et al., 1994; Molinia et al., 1994). As a result, the hypothesis for using a less harmful protocol like that without centrifugation was initially to be rejected. However, the aimed concentration of 200 x 10^6 spermatozoa/straw was efficiently achieved, and demonstrated that substantial sperm losses could be avoided. This called for a re-calculation of the actual numbers of spermatozoa with progressive motility, intact membrane, uncapacitated pattern. The modified protocol largely compensated for the lower sperm quality by a higher final number of spermatozoa in the dose when compared to that measured in the controls (procedure including centrifugation).

The suitability of a semen:extender ratio depends on the extender used (D'Alessandro et al., 2001). Maxwell et al (1995) claimed that the best results were obtained at semen:extender ratios (v/v) between 1:4 and 1:8 when using a TRIS-glucose-citrate extender with egg yolk and glycerol (12% and 4% v/v, respectively). The authors used a single step extension procedure at 30° C.

Molinia et al (1994) reported that post-thaw motility was better if the dilutions were between 3 and 6 times the volume of the ejaculate. Increasing survival results were reported (reviewed by Salamon and Maxwell, 1995a), when raising the semen:extender ratio from 2- to 6-fold, albeit lowering the lambing rate after 3- to 5-fold extension. Rather than the semen:extender ratio, D'Alessandro et al (2001) reported that a milk-lactose-yolk extender yielded the best results at sperm concentrations of 100 to 500 x 10^6 spermatozoa/mL, with a clear effect of the composition of the extender. The procedure for the semen extension (single or a two step procedure) in relation to the addition of the cryoprotectant e.g. glycerol, is also of importance. In this sense, it seems difficult to compare different reports, where most of the studies did not correct the osmolar strength to the different extension rates. Thus the effect of the extension rate might be masked by the variation of the composition in the final extended semen (see Salamon and Maxwell, 1995a). In the present study (**Paper I**), the dilution rate for the control protocol was 1+4 (semen + extender) at the first step (at 33° C, fraction 1 without glycerol), and 1+9 after the second step (at 5° C, fraction 2 with glycerol). The tested protocol involved dilution rates of about 1+1.2 in the first step and 1+3.4 in the second step. It is unclear what the optimal extension rate would be for the extenders used here, but a low dilution rate is a prerequisite to achieve a proper sperm concentration in the final AI-dose.

Further studies that attempted to deal with the low pre-freezing semen:extender ratios were conducted in this thesis (**Paper III**). Some possible alternatives were preliminarily tested but rejected in pilot trials, e.g., the addition of fraction 2 with a glycerol concentration higher than 14% (thus allowing higher volumes of fraction 1 in the first extension step); a complete extension up to the final sperm concentration without glycerol; and later at 5°C, just the addition of undiluted glycerol (to get 7%). The alternative of adding fraction 2 with 14% glycerol in an earlier step at 15°C, before the temperature reaches 5°C, was finally selected for further evaluation. The rationale behind this temperature level was to reduce the reported toxic effect of glycerol on sperm metabolism and membranes (Colas, 1975; Fiser and Fairfull, 1989) as well as to increase the extender rate before the spermatozoa might be subjected to a possible cold-shock. Major modifications in the membranes due to "cold-shock" occur at temperatures below 15°C, mainly due to alteration in the lipid phase transition at the membrane level (Watson, 1981; Hammerstedt et al., 1990; Drobnis et al., 1993; Holt, 2000), and to ultrastructural modification (Holt and North, 1991). Although ram spermatozoa have been shown to be relatively resistant to rapid cooling above 15°C without a protectant, as egg yolk, cooling to lower temperatures requires the addition of cooling protectants, which are usually provided by the extenders (Fiser and Fairfull, 1986a).

In this thesis (**Paper III**), although not always statistically significant, all the sperm parameters assessed were better when the second fraction was added at 5°C rather than at 15°C. This effect was more evident with regard to the intactness of the membrane and the proportion of acrosome-reacted spermatozoa. Such an effect could be explained if glycerol promoted capacitation-like changes,

as reported (Slavik, 1987; Garde et al., 1993), or by its toxic effect on sperm metabolism (Fahy, 1986). In any case, addition of fraction 2 at 15°C, tested *in vitro*, did not represent any improvement to the sperm quality post-thaw. Another speculation was that by adding the glycerol at 15°C, while cooling was still taking place, changes in cell volume would occur simultaneously with the changes in the membrane lipids from a fluid- to a gel-like phase, thus destabilizing the plasma membrane. This might imply that some specific steps are better endured if the cells are in previous equilibrium with the environment. Probably, the lack of any benefit from adding the glycerolized fraction at 15°C revealed that the capacity of ram spermatozoa to sustain cooling seemed much larger than previously thought, if it was previously extended, even at low ratios, in an extender containing egg yolk and skim milk. Therefore, the simplified protocol with a second extension step at 5°C, was demonstrated to be an acceptable option to freeze ram semen, encouraging further trials concerning the evaluation of fertility after cervical AI.

Extenders

Cryopreservation of ram semen has been made possible by the use of different extenders to which glycerol is added as a cryoprotectant. Attempts to improve the post-thaw results, usually done on an empirical basis, involve the cooling and freezing rates, the package and, perhaps the most studied approach, the extenders. The diversity of compounds available is probably infinite, but some of them are basic and present in most available recipes, like the egg yolk and the reconstituted skim milk. Both ingredients seem to prevent "cold-shock" during processing, allowing a reduction in the glycerol concentration used. Therefore, it appeared useful to study, which one of these components render better sperm survival when using a protocol that includes low extension ratios prior to cooling.

In this thesis, eight extenders were compared (**Papers I-IV**). Large particles were seen in the control extender, probably caused by un-dissolved milk powder, making the motility evaluations (subjective and by CASA) more difficult. Thus, one of the extenders evaluated was a clarified version of the control where un-dissolved large particles were removed by centrifugation (**Paper I**).

Another extender commonly used to freeze bull semen, the TRIS-citric acid-fructose extender supplemented with 20% egg yolk, was also evaluated because it is easier to prepare compared to the milk-egg yolk extender (**Paper I**). This extender was also clarified from large particles by centrifugation and prepared in two fractions in the same way as the milk extender to fit into the two-step extension method hereby used. The TRIS-based extender has mostly been used in a one-step extension procedure for freezing pellets (Andersen et al., 1973), but there were no reports available of the use of a two-step method for freezing in 0.25 mL plastic straws. The other extenders evaluated were variations of the milk-based extender, with an increase of the egg yolk concentration to 10, 15, and 20%, respectively (**Paper III**).

The commercial extender Bioexcell®, available for bull semen, was also studied (**Paper II**). This product was considered to accomplish the criteria of avoiding components of animal origin in the extender (Thibier and Guerin, 2000). Apart from sanitary concerns, there is a possibility that milk and egg yolk would alter the chromatin structure of the spermatozoa assessed as the susceptibility to *in situ* DNA denaturation, and thus reduce sperm quality post-thaw (Karabinus et al., 1991). Furthermore, this commercial extender might provide a more standardized freezing medium for ram semen to be used as a control for further studies. Bioexcell® was specially manufactured for this study to fit the "two step" extension method used, with the first fraction free of glycerol and the second with two different glycerol concentrations (final concentration: 3.2% and 6.4%). They were considered to be two different extenders.

The results showed that all parameters studied were apparently higher in the clarified milk-based extender, but the difference was not statistically significant. This means that the clarification did not have any apparent advantage other than providing a clearer medium, which is important at the time of evaluation, especially when assessing sperm motility patterns by CASA (Mortimer et al., 1995). It was also found that increasing the egg yolk concentration above 10% in the milk extender does not convey any significant improvement to the sperm characteristics evaluated post-thaw. Furthermore, the use of 10% egg yolk improved sperm membrane intactness, but caused a dose-related simultaneous increase in sperm membrane destabilization. This confirms previous results, of a higher frequency of damaged acrosomes when increasing the concentration of egg yolk (Watson and Martin, 1973, 1976; Smith et al., 1979). The capacitation process represents a destabilization of the sperm membrane, which makes the spermatozoa more readily to the spontaneous acrosome exocytosis, thereby lowering the half-life of the cell population (Watson, 2000). The TRIS-based extender did not improve the results obtained in the milk-based extender. Bioexcell® with 6.4% glycerol concentration was consistently better than with 3.2% glycerol, for all parameters studied. Furthermore, Bioexcell® with 6.4% glycerol resulted in similar values to those seen in the clarified milk extender (control). The results obtained by the *in vitro* testing of these two extenders encouraged further studies to evaluate their performance in the field (fertility tests).

Fertility

The fertility trials involved two flocks with similar conditions of management, nutrition levels and sanitary status as well as a similar genetic base (Corriedale breed). The smaller flock (300 ewes, **Paper II**) was used to test the "simplified protocol" (extension without centrifugation). The largest flock (970 ewes, **Paper IV**) served to compare the semen frozen in Bioexcell® with 6.4% glycerol, with the milk-based extender (control).

The results in the first flock showed no differences in fertility between the simplified protocol and the standard protocol with centrifugation, thereby confirming the results obtained *in vitro*. This indicates that the simplified protocol with adjusted extension of the semen (resulting in higher numbers of viable spermatozoa), might be considered the procedure of choice when freezing ram semen under these field conditions. Although lower than in most of the Scandinavian reports (Olafsson, 1980; Lillo, 1984; Grøtte et al., 1992; Andersen Berg, 1999; Söderquist et al., 1999a), the lambing rate achieved was similar to those reported by others using the same (Holm et al., 2000) or different (see Salamon and Maxwell, 1995a, b, 2000; Sanchez-Partida et al., 1999) semen processing methodologies. This different result could be due to the differences in breeds, management conditions (e.g. rearing system, flock size, economical and social factors) affecting mainly the insemination procedure itself. In this study (**Paper II**), the identification of sheep in standing estrus was done once a day, thus, implying time intervals between 9 and 33 h from estrus detection to AI. It is known that the timing of insemination has been shown to be critical when using frozen-thawed semen, in any domestic species considered so far. The best results for cervical insemination in sheep have been reported for AI performed later in estrus, and consequently closer to ovulation, somewhere around 15 to 20 h after onset of estrus (Olesen, 1993; Andersen Berg, 1999; Söderquist et al., 1999a). Another factor that might influenced the results were the weather conditions at the time of the inseminations. It has been shown previously, that rainfall during mating and in the early post-mating period affects both ovulation rate and NNR in sheep (Doney and Gunn, 1972; Davis, 1973). Although I did not aim to study this effect on fertility, it could not be neglected since in the first field study (**Paper II**), the lowest percentages of lambing were registered when AI was done under heavy rains (63.7 mm of rainfall).

The detection and separation of sheep in estrus for AI twice a day is not always possible in practice under extensive management in large flocks. Such a routine to separate ewes in estrous twice daily for achieving a more optimal insemination time, was attempted in the second field trial (**Paper IV**), where ram semen frozen in two selected extenders was tested (clarified milk with 5% egg yolk and 7% glycerol vs. Bioexcell® with 6.4% glycerol). From the results obtained, Bioexcell® with 6.4% glycerol appears to be an alternative to the conventional milk-egg yolk extender to process ram semen for cervical AI in extensively managed large flocks. The pregnancy rates (28%) achieved in this fertility trial, were slightly better than the results from the previous first trial. The differences between them were probably due to the size of the flock and the routine for separation of ewes in estrus for AI.

The major source of variation in the field fertility of frozen-thawed ram semen has been reported to be the numbers of spermatozoa used for AI. Under commercial conditions, this is solved by the use of an excess of spermatozoa in each AI-dose, thus driving to a lack of relationship between the *in vitro* sperm quality and fertility (Eppleston and Maxwell, 1995). The numbers of viable spermatozoa in the AI-doses used in the present *in vivo* studies (**Papers II** and

IV) might have been large enough to mask any effect of the procedures or any effect of the extenders; differences that were seen *in vitro* in all trials. To point out *in vivo* the differences seen *in vitro*, critical minimal numbers of spermatozoa per insemination dose should have been used during the AI-trials. This is difficult to set when using commercial flocks, where despite the interest in new achievements, there is a high dependence on acceptable results, in order to warrant interest in the application of AI as a breeding tool.

General conclusions

In conclusion, the present studies indicate that:

- A low pre-freezing semen:extender ratio during processing of ram semen for deep-freezing appeared more detrimental to sperm viability than centrifugation (at 700g/10 min).
- The adjusted semen extension (without centrifugation) yielded the aimed concentration in the AI dose, thus compensating the proportionally lower quality post-thaw compared to the routine protocol with centrifugation, by higher numbers of viable spermatozoa.
- Both the clarified and the non-clarified milk extenders yielded similar *in vitro* results post-thaw. However, the clarified version was more advantageous for the *in vitro* sperm examination post-thaw.
- Further extension and glycerolization at 15°C did not represent any improvement to the routine procedure (5°C).
- The TRIS-citrate-fructose extender with 20% egg yolk was not as good as the milk-based extender to preserve post-thaw sperm viability using a two-step methodology.
- A low egg yolk concentration (5-10%) in the milk-based extender yielded the best *in vitro* results. There was a dose-related increase of sperm membrane destabilization.
- Bioexcell® with a final glycerol concentration of 6.4% appeared to be a suitable alternative to the milk-based extender.
- No significant difference in fertility was found between the semen frozen according to the protocol with centrifugation (control) and that with adjusted semen extension (without centrifugation) after single cervical AI in extensively managed Corriedale ewes.
- Fertility results did not significantly differ between semen frozen in Bioexcell® 6.4% glycerol and a milk-based extender (control) under the conditions mentioned above. However, Bioexcell (free from additives of animal origin) might represent a safer alternative to current extenders.

It is finally concluded that this simplified methodology is of value when considering introduction of genetic material by AI of large flocks in developing countries.

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