RAMSES (RAM Semen Extender Study)

The development of modern long-life storage diluent for fresh ram spermatozoa

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1. Background and general project update

Sheep breeding in the UK remains largely an extensive natural mating system involving small flocks with little biosecurity and disease control. The limited artificial insemination (AI) that takes place is of little direct impact despite enabling farmers to benefit from genetic improvement programmes and to increase their biosecurity. This is due in part to the technical limitations of the semen preservation methods currently available. Semen freezing and laparoscopic AI procedures work well, but the level of technical expertise and attendant costs mitigate against its widespread adoption. Furthermore, laparoscopic AI is the only technique that can provide acceptable pregnancy rates with frozen-thawed semen. The development potential for the widespread use of AI across the industry therefore depends on fresh semen inseminated cervically. Cervical insemination using fresh semen generally yields around 50-60% conception rates, and the interest in AI using fresh semen has been growing in Wales following the awareness of the potential of AI for breed improvement during the WEGS AI Scheme. However, there has, to date, been no reliable extender for fresh ram semen that provides more than a few hours storage at ambient or chilling temperatures, and this has imposed a major limitation to the development of cervical AI services in the sheep industry.

This project was undertaken as part of a 3-year Defra LINK project which aimed to solve this problem by developing a suitable diluent for longer-term fresh semen storage and to validate its effectiveness by extensive field trials in Wales. The project has been evaluating the potential of a series of factors to enhance the survival of ram semen through *in vitro* laboratory studies. These studies, using commercial diluents, a protein (HSCP70) isolated from the oviduct of sheep and other factors (including antioxidants), were been conducted by The Royal Veterinary College, Institute of Zoology and Sheffield University through the LINK project. The most promising components were then selected for field trials to be conducted in Wales by Innovis and the Institute of Biological, Environmental and Rural Sciences (IBERS). This element of the project has been supported by Hybu Cig Cymru and is reported here.

The academic partners had previously demonstrated that two diluents, RSD-1 and INRA96 could support ram sperm motility *in vitro* for more than 5 days. The first field trial was conducted in 2006/7 and has been reported previously to HCC. 1000 ewes were synchronised and inseminated with $200x10^6$ spermatozoa held in RSD-1 or INRA96 at 5°C for either up to 6 h or up to 30 h (nominally Day 0 and Day1). The results of that first study were quite clear: storage of ram semen in these two diluents for 24hours at 5°C was not capable of sustaining fertility *in vivo* in spite of there being excellent motility of the sperm after storage and in spite of the excellent sperm functional attributes that had been observed in the laboratory. Diluent RSD-1 was poorer than INRA96 at sustaining fertility at 24 hours (11.1% *vs.* 14.9% respectively). It was clear that 24h storage of ram semen at 5°C in INRA96 or RSD-1 was very disadvantageous, and this comprised the original project plan which had intended to use INRA 96 at 5°C as the base to which the potential for HSC70 or other factors would be added in order to determine the improvement in fertility *in vivo*.

The second field trial planned for 2007/8 was postponed due the outbreak of Foot and Mouth disease, and this allowed time for a review of other commercial extenders to be undertaken and for the laboratory studies to be completed.

2. Field Trial 2008/9

During 2007/8, data provided by IMV Technologies in France indicated that INRA96 was best used following storage at 17℃ rather than at 5℃, and preliminary results from a study in Greece also indicated that a new commercial extender, Ovixcell, had achieved significant levels of fertility when used with fresh ram semen stored for 24 and 48 hours. The absence of data on these products in the UK meant that the subsequent protocol for the second Welsh trial, conducted during 2008/9, needed to take this into account.

Laboratory studies during this period had also shown that a cocktail of potential antioxidant additives were beneficial when added to INRA96 *in vitro* in controlling the production of hydrogen peroxide, a damaging metabolite of lipid oxidation and an indicator of the extent of peroxidation. They had also shown that the sheep oviduct is also capable of producing active proteins which can modulate sperm activity and viability *in vitro*. This work selected and focused on Heat Shock Proteins 70 and 90 (HSP70 and 90), Heat Shock Cognate Protein 70 (HSC70) and clusterin. HSC70 was found to sustain viability of ovine spermatozoa in vitro at 39℃ for 48h. Although it proved difficult to purify sufficient quantities of ovine HSC70 for use *in vivo*, recombinant cattle HSC70, which is structurally very similar to HSC70 from the sheep oviduct epithelial cells, was shown to enhance sperm viability when added to INRA96 *in vitro*. Sufficient quantities of this recombinant protein were subsequently obtained and provided for use in the *in vivo* trials.

The aim of the second field trial conducted between October 2008 and April 2009 was therefore to investigate the ability of commercial extenders INRA96 and Ovixcell to sustain ram sperm fertility for up to 24h and to determine the ability of heat shock cognate protein (HSC70) to enhance fertility when used in conjunction with each of these extenders.

2a Experimental design:
800 Welsh mule ewes were used in two batches of 450 which were prepared for insemination during two consecutive weeks. The ewes were allocated at random to make a balanced design throughout the insemination period and across all groups of ewes. Ewes were synchronised using progestogen sponges for 12 days and 400 IU eCG at sponge removal, with cervical inseminations were performed at ~54±2 h after sponge removal.

The ewes were inseminated with semen diluted in one of the diluents, either with (+) or without (-) HSC70: INRA 96 (- / +) and Ovixcell (- / +) 24 hours after collection.

Semen was collected from 12 Inverdale-texel rams, assessed by microscopic examination for adequate quality (80-90% motile, vigour score 4 on scale of 0-5). Semen from 6 of these rams was pooled and sperm concentration determined using an Accucel. The pooled semen split and diluted to give insemination doses of 200 X 10^6 sperm in 0.25 ml (*i.e.* 800 x 10^6/ml) for INRA diluent and 400 x 10^6 sperm in 0.25 ml (*i.e.* 1600 X 10^6/ml) for Ovixcell diluent.

Semen diluted in INRA96 (- / +) was cooled slowly and stored aerobically at 17ºC. Semen diluted in Ovixcell (- / +) was cooled slowly over 2.5hours and stored aerobically at 5ºC in tubes. Tubes were filled to minimise the amount of air above the semen. HSC70 was provided frozen by RVC in 50ug packages and was added to portions of diluents to give a final concentration of 8ug/ml.

Insemination was performed with ewes restrained within an AI crate using a speculum and standard plastic insemination equipment ~54 h after sponge removal. The external os of the cervix was visualised and the inseminate placed into the external os. Lambing results were subsequently recorded as ewe ID at lambing, treatment, single or twin, live birth or dead.

2b Results
The final results for the trial are presented in Table 1 below.

Treatment Group	Ewes Inseminated	Ewes Lambed	Mean litter size/ewe lambing	
			Total	Alive
INRA(-) at 24h	181	13 (7%)	1.38	1.31
INRA(+) at 24h	201	23 (11%)	1.52	1.43
OVI(-) at 24h	200	56 (28%)	1.70	1.68
OVI(+) at 24h	200	53 (27%)	1.55	1.49

Table 1. Overall pregnancy rates and litter sizes for ewes within the RAMSES trial. Pooled semen was stored in either the INRA or the OVI diluents, with (+) or without (-) addition of HSC70, for 24h prior to insemination.

Chi-squared analysis of the pregnancy rate data indicated that the overall pregnancy rates for ewes inseminated with semen preserved in the INRA diluent for 24h was significantly ($P<0.001$) lower than that ewes inseminated with fresh chilled semen preserved in Ovixcell. However, the addition of HSC70 to either diluent did not significantly improve pregnancy rates. While there appear to be some small differences in litter size between treatments, these differences were not statistically significant.

3. Conclusions
Given the statistical requirements for around 200 ewes per treatment group it was only possible to examine a limited number of variables with the second field trial. Given the poor performance of the extenders used in field trial one, and the absence of knowledge of the performance of INRA96 to support storage of diluted semen at 17ºC or of Ovixcell to support storage of diluted semen at 5°C, a detailed trial using just one of the extenders as the base was not considered to be sensible use of such a large-scale resource. The inclusion of each of these extenders was therefore considered essential. This did however mean that an investigation of different concentrations of HSC70 was not possible within the same trial.

Results from the second field trial conducted in 2008/9 indicated that neither Ovixcell nor INRA96 at 17^{o}C were capable of sustaining levels of fertility that would be considered acceptable for widespread use on farm. Ovixcell alone did achieve significantly ($P<0.001$) higher conception rates (28%) than that of ewes inseminated with semen preserved in INRA96 (7%). However, it should be noted that the semen concentration recommended for the commercial use of Ovixcell is twice that of INRA96, and this may account for some (if not all) of the difference in the conception rates between the two extenders. These results were consistent with the pattern obtained from field trial one in which INRA96 at 5^{o}C and RSD-1 did not sustain significant levels of fertility *in vitro*.

The addition of a single concentration of HSC70 to either diluent had no significant beneficial effects on pregnancy rates. Furthermore, when the data were pooled over diluent types and comparisons made with and without the addition of the HSC70, there was again no significant difference in pregnancy rates. The increased sperm concentration recommended for use with Ovixcell may have compounded the dose requirements for HSC70 compared to INRA96.

Although there appear to be some differences in mean litter size between the individual treatment groups, Chi-squared analysis indicated that there were no significant differences between treatments in the proportion of ewes producing single and multiple litters. Semen appeared to remain motile during the insemination timescale but it is possible that the requirements for pooling the very large volumes of semen required may have reduced the function of those sperm.

5. Overall conclusions

Despite laboratory evidence which indicates that HSC70 isolated from the sheep oviduct and various ram semen extenders are able to enhance sperm viability *in vitro*, the results from each of the field trials conducted in Wales during 2006/7 and 2008/9 have indicated that these *in vitro* findings do not appear to translate into enhanced functional capabilities *in vivo*. The levels of fertility achieved after 24 hours did not exceed 28% and the diluents used, with or without the HSC70, do not therefore lend themselves to more widespread commercial use at this time. It is possible that an *in vitro* assay is required which better reflects the sperm's capability for fertilisation *in vivo* than the mobility tests that are currently used. This would enable a wide range of concentrations of HSC70 and conditions to be screened effectively before use in field trials. The statistical and ethical limitations of the study meant that it simply wasn't possible to include the range of experimental variables that would show unequivocally that HSC70 would perform *in vivo* as well as it did *in vitro*, and that was the scientific breakthrough that this project sought to provide so that cervical insemination using fresh ram semen could be developed into a viable commercial option for sheep farmers in Wales.

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