

## Resuspending ram spermatozoa in seminal plasma after cryopreservation does not improve pregnancy rate in cervically inseminated ewes

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### Abstract

The role of seminal plasma (SP) components on the maintenance of motility, viability and fertilising ability of frozen–thawed spermatozoa is of considerable interest. However, differences observed in constituents of SP among males could explain differences in fertility obtained in vivo. Two experiments were designed to examine the effects of seminal plasma on fertility from cervically inseminated frozen–thawed semen. The objective of Experiment 1 was to investigate if source or type of SP influences pregnancy rate. Seminal plasma was collected from rams previously classified as having either High (HSP;  $n = 3$ ) or Low (LSP;  $n = 3$ ) fertility in vivo. Artificial SP (fructose/sodium solution with 10% BSA; ASP) was made. Frozen semen from the same 6 rams was thawed and inseminated (Control) or resuspended either in HSP, LSP or ASP (20% in semen) prior to insemination of ewes ( $n = 284$ , over 2 farms). The overall pregnancy rate was 28.1%. Treatments (Control, ASP, HSP and LSP) were not significantly different ( $P > 0.3$ ). There was no difference between HSP and LSP ( $P > 0.5$ ), and no effect of using ASP compared to ram SP ( $P > 0.7$ ), on pregnancy rate. As there was no effect of SP on pregnancy rate a repeat experiment (Experiment 2) was designed to test the effect of washing and selecting motile sperm prior to resuspending in phosphate-buffered saline (PBS) containing SP on pregnancy rate. Frozen–thawed semen from each of 2 rams was centrifuged through a density gradient, pellets were centrifuged through a wash medium and the sperm concentration/ram was counted. Sperm cells were resuspended in: (1) control PBS, (2) PBS containing 30% HSP or (3) PBS containing 30% LSP to give  $100 \times 10^6$  motile sperm in 0.25 mL. Control straws were thawed and inseminated directly. Ewes ( $n = 223$  over 2 farms) were inseminated 57 h post-sponge withdrawal and those not returning to oestrus were slaughtered 29–50 days post-insemination for pregnancy determination. In Experiment 2, the pregnancy rate for Control, PBS, HSP and LSP were 15.4%, 2.3%, 0% and 0%, respectively, for Farm 1 ( $P > 0.05$ ) and 17.8%, 11.0%, 3.9% and 12.4%, respectively, for Farm 2.

Under the conditions of the current study, addition of SP from different donors of either High or Low fertility status to frozen–thawed ram semen post-thawing did not improve pregnancy rate in ewes. ASP had no effect on pregnancy rate in ewes when added to frozen–thawed semen. Washing and selection of motile sperm prior to resuspension in PBS with or without SP (30%) before insemination had a negative effect on pregnancy rate in cervically inseminated ewes. Hence, the addition of seminal plasma or some of its constituents to semen does not appear to improve pregnancy rate in cervically inseminated ewes.

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## 1. Introduction

Cryopreservation changes the behavioural and functional capacity of spermatozoa, leading to a reduction in motility, reduced ability of sperm to traverse the cervix and decreased viability in the female reproductive tract. This usually results in unacceptably low conception rates in ewes inseminated into the cervix with frozen–thawed semen [1–3].

Seminal plasma has been shown to suppress capacitation [4,5] and to decapacitate previously capacitated spermatozoa resulting in a reduced ability to fertilise oocytes *in vivo* [6]. Across a variety of domestic species it has also been reported to maintain sperm motility and viability [7–9] and increase sperm resistance to cold shock damage [10–12] by providing specific components that stabilise the membrane of frozen–thawed spermatozoa [9,13]. However, detrimental effects of seminal plasma on sperm motility [14,15], viability [16–19] and survival after freeze–thawing [20,21] have also been reported.

Maxwell et al. [22] examined the effects of resuspending ram spermatozoa in 20% or 30% seminal plasma post-thawing and found that penetration of spermatozoa through cervical mucus was improved and fertility after cervical insemination of ewes was significantly increased. However, this technique, as described by Maxwell et al. [22], is not easily applicable under field conditions due to labour and time constraints and modifications to the technique would be beneficial. Mortimer and Maxwell [23] subsequently reported that frozen–thawed spermatozoa resuspended in artificial seminal plasma or ram seminal plasma had improved movement and increased plasma membrane stability compared to those resuspended in PBS and suggested that this was due to components of the medium. Furthermore, Catt et al. [24] reported metabolites or ionic components, other than proteins, as the most essential constituents of seminal plasma for maintenance of sperm cell viability. Artificial seminal plasma however, has not been tested previously as a resuspension medium in fertility trials.

Various authors have reported variability in the seminal plasma composition among species, among males within a species and even among ejaculates of the same male (bull: [8,25]; ram: [8]; boar: [26,27]; stallion: [28]). Several authors have also suggested that seminal plasma contains constituents that may also improve male fertility [22,25]. We have previously observed differences between rams in fertility (range 17.7–45.2%) following cervical insemination of ewes using frozen–thawed semen [29], which may be attributed to

differences in seminal plasma components among individual males.

With this background, the objective of the current study was to examine the effects of resuspending frozen–thawed ram spermatozoa in seminal plasma, from rams previously identified as being of High or Low fertility [29], or in artificial seminal plasma on pregnancy rate in cervically inseminated ewes.

## 2. Materials and methods

### 2.1. Semen collection and processing

Ejaculates were collected from individual rams using an artificial vagina (IMV, France) and motility was assessed by wave motion and graded [30]. Any sample with a wave motion below 3 was discarded. Sperm concentration was calculated using a photometer (Minitub, Germany) and ejaculates with a concentration below  $3 \times 10^9$  spermatozoa/mL were discarded. Suitable ejaculates were pooled on a per ram basis prior to processing.

Semen was processed and frozen as described by Papadopoulos et al. [30]. Briefly, semen was diluted with a skim milk/egg yolk based extender (Extender 1: 11% skim milk; 5%, v/v, egg yolk) at 32 °C. Samples were then cooled to 5 °C for 45 min. A second extender (Extender 2: 11% skim milk; 5%, v/v, egg yolk; 14%, v/v, glycerol; 4% fructose) was then added in two equal steps, with an equilibration time of 45 min per step, to a final concentration of 7% glycerol. The samples were centrifuged at 5 °C for 10 min at  $700 \times g$ . The volume of supernatant was reduced by the amount required to give a final concentration of  $800 \times 10^6$  spermatozoa/mL. The remaining mixture was loaded in 0.25-mL Minitub™ (Minitub, Tiefenbach, Germany) straws ( $200 \times 10^6$  spermatozoa/straw) and cooled in a liquid nitrogen programmable freezer (Planar Series II, Planar Products Ltd., Middlesex, UK). Straws were cooled from 5 to –10 °C at –5 °C/min and from –10 to –130 °C at –50 °C/min and were then plunged directly into liquid nitrogen at –196 °C for storage.

### 2.2. Preparation of seminal plasma

Rams with the most divergent fertility estimates (3 High and 3 Low), based on *in vivo* fertility data obtained over 3 years were selected from a pool of 18 rams [29,31] (Table 1). Ejaculates from these rams were collected, centrifuged ( $6500 \times g$  for 15 min at 4 °C) and the clear supernatant recovered. Ejaculates were pooled on a per ram per day basis over 3 days, filtered (0.22 µm

Table 1

Estimates of fertility in vivo (pregnancy rate) following cervical insemination using frozen–thawed semen ( $200 \times 10^6$  spermatozoa per AI dose) for 6 rams

Ram category <sup>a</sup>	Ram ID	No. of ewes inseminated	Estimated fertility <sup>b</sup> (%) (90% confidence interval)
High	14	69	45.2 (30.8–60.4)
High	18	69	38.9 (25.7–54.0)
High	12	72	35.3 (22.9–50.1)
Low	19	72	24.3 (14.7–37.5)
Low	11	70	23.3 (14.0–36.1)
Low	10	70	17.7 (10.1–29.0)

<sup>a</sup> Categorized on the basis of mean fertility.

<sup>b</sup> Fertility determined over 3 years and was based on pregnancy diagnosis by laparoscopy, rectal ultrasound scanning or slaughter 26–75 days after cervical AI (Donovan et al., unpublished data).

filter) and frozen in 2 mL aliquots. One aliquot per ram per day of collection was then thawed at room temperature and seminal plasma from the 3 High fertility rams (High seminal plasma: HSP) was pooled, vortexed and stored at  $-20^\circ\text{C}$  in 2 mL aliquots. This was repeated for the 3 Low fertility rams (Low seminal plasma: LSP). Samples were thawed at room temperature prior to use.

Artificial seminal plasma (ASP), containing 38 mM sodium citrate, 10 mM sodium chloride, 50 mM potassium chloride, 1 mM sodium dihydrogen phosphate, 6 mM calcium chloride, 5 mM magnesium chloride and 30 mM fructose supplemented with 10 mg/mL BSA [32], was prepared and frozen in 2 mL aliquots.

### 2.3. Synchronisation of ewes

All ewes were synchronised with intravaginal sponges (30 mg fluorogestone acetate, Chronogest) for 12 or 13 days and received 400 IU eCG at sponge withdrawal. Ewes were cervically inseminated at 57 h after sponge removal [30]. The same operator carried out all inseminations. Raddled rams were used to detect ewes returning to oestrus and all unmarked ewes plus any cases with doubtful raddle marks were slaughtered 29–50 days post-insemination (p.i.). Pregnancy was determined by dissection of reproductive tracts.

### 2.4. Experimental design

All experiments were carried out during the breeding season. Two experiments were designed to examine the effects of seminal plasma or its constituents in the sperm re-suspension medium on subsequent pregnancy rate. The objective of Experiment 1 was to evaluate the effects of resuspending frozen–thawed ram spermatozoa directly into SP from either High (HSP) or Low

(LSP) fertility rams or in artificial seminal plasma (ASP), on pregnancy rate of cervically inseminated ewes. A Control treatment, insemination with untreated frozen–thawed semen, was also included. The experiment was replicated at two locations. The results from Experiment 1 were not as expected and a further experiment was designed to explore whether washing sperm free of cryoprotectant was responsible for the improved pregnancy rate observed by others [22]. Experiment 2 was designed using  $100 \times 10^6$  washed motile sperm resuspended in either PBS containing seminal plasma (30%, v/v) from High (HSP) or Low (LSP) fertility rams or in PBS with no seminal plasma. The experiment was replicated at two locations.

#### 2.4.1. Experiment 1

A total of 125 multiparous ewes (comprising Cheviot, Suffolk, Suffolk-cross and Scottish Blackface-cross Border Leicester types) were used at Lyons Research Farm (Farm 1,  $53^\circ18'N$ ) and 160 multiparous ewes (comprising Scottish Blackface, Belclare  $\times$  Scottish Blackface, Belclare, Texel and Cambridge) were used at the Teagasc Research Centre (Farm 2,  $53^\circ17'N$ ). Frozen straws of semen from 3 High and 3 Low fertility rams (Table 1) were thawed in a water bath at  $70^\circ\text{C}$  for 8 s. Each of the frozen–thawed straws (250  $\mu\text{L}$ ) was emptied directly into a round-bottom bacteriological glass tube maintained in a water bath at  $33^\circ\text{C}$  containing 62.5  $\mu\text{L}$  of HSP, LSP or ASP at a final concentration of 20% (v/v) in semen. The sample was vortexed and maintained at  $33^\circ\text{C}$  for 5 min before being drawn into a 0.5 mL straw (IMV, France) and used to inseminate one ewe. A control sample (0.25 mL straw) containing no seminal plasma (Control) was inseminated directly after thawing. Frozen–thawed semen from each ram was used to inseminate a total of 22 ewes at Farm 1 (6 ewes for each of HSP, LSP and ASP treatments and 4 Control

ewes) while at Farm 2 a total of 26 ewes were inseminated per ram (6 ewes for each of HSP, LSP and ASP treatments and 8 Control ewes). Care was taken to avoid any confounding of ewe breed and treatment and all treatments were distributed evenly throughout each AI session.

#### 2.4.2. Experiment 2

A total of 115 multiparous ewes (comprising mainly of Cheviot, Suffolk, Texel and Scottish Blackface crosses) were used at Farm 1 and 118 multiparous ewes (comprising Scottish Blackface, Belclare × Scottish Blackface and Charollais × Scottish Blackface) were used at Farm 2. Seminal plasma was thawed from the same 3 High or 3 Low fertility rams as used previously (Experiment 1) and diluted with filtered (0.22 µm filter) PBS to a final concentration of 30% (v/v) SP supplemented with 3 mg/mL bovine serum albumin (BSA). The PBS control was supplemented with 10 mg/mL BSA.

Frozen straws of semen ( $n = 10$ ) from each of 1 High and 1 Low fertility ram (Table 1; ram 18 and ram 19, respectively) were thawed in a water bath at 70 °C for 8 s and pooled on a per ram basis and maintained at 37 °C in a water bath. Semen for each ram was layered evenly over a Percoll density gradient [3 mL 45%, v/v, over 3 mL 90%, v/v, Percoll (Pharmacia AB, Uppsala, Sweden)] and centrifuged at  $600 \times g$  at RT for 15 min. The supernatant was discarded, the pellet was resuspended in 4 mL of Tyrodes medium [33] and centrifuged for a further 6 min at  $250 \times g$  at RT. The recovered pellets were combined on a ram basis and the concentration determined. Cells were diluted with (1) control medium of PBS, (2) in PBS containing 30% HSP or (3) in PBS containing 30% LSP to yield an end concentration of  $100 \times 10^6$  motile sperm in an inseminate volume of 0.25 mL. Random samples were evaluated by phase contrast light microscopy (Nikon Diaphot, Japan) for progressive motility prior to insemination. Control straws were thawed and inseminated directly. The procedures for assigning ewes to treatment were as for Experiment 1.

#### 2.5. Statistical analysis

Data from Experiment 1 were analysed using the Genmod procedure of SAS (SAS Institute Inc. Cary, NC, USA). Mean values for pregnancy rate for each treatment with the upper and lower 95% bounds, were derived from estimates of effects on a logit scale. The initial model had effects for ewe breed, ram (High,

Low), treatment and Day of insemination (2 days). There was no effect of ram or ewe breed or interactions and these effects were eliminated from the final model and data were further analysed for Day and treatment effects only. Linear contrasts were evaluated to test the effect of processing, type of SP (HSP versus LSP) and difference between PBS and SP. In Experiment 2, because of the low pregnancy rate achieved on Farm 1, the data from the two farms were analysed separately. Data from Farm 1 were analysed using the frequency procedure of SAS (Fishers exact test) and the number of ewes pregnant for each treatment. Data from Farm 2 were analysed using the Genmod procedure of SAS.

### 3. Results

#### 3.1. Experiment 1

The differences among individual rams did not approach statistical significance ( $P > 0.5$ ), reflecting in part the small number of ewes per ram. Consequently ram type was used in all subsequent analyses. In these analyses there was no evidence for significant effects due to ewe breed within location ( $P = 0.49$ ) or for the interaction between ram type and treatment ( $P = 0.60$ ) and these terms were dropped from the final model which had terms for location, treatment, ram type and the interaction between location and treatment. The location differences (22.2% at Farm 1 versus 29.2% at Farm 2) were not significant ( $P = 0.21$ ) but the ram type effect approached significance (30.1% versus 21.5% for High and Low, respectively;  $P = 0.09$ ). The estimates of pregnancy rate for each treatment (with upper and lower confidence limits) are given in Table 2. The overall

Table 2

Estimate for the pregnancy rate of ewes cervically inseminated with frozen–thawed semen from either High or Low fertility rams (Table 1 resuspended in different seminal plasma treatments prior to artificial insemination)

Treatment	No. of ewes inseminated	Estimated fertility <sup>a</sup> (%) (90% confidence interval)
ASP	70	28.3 (20.24–37.92)
HSP	72	31.4 (22.99–41.13)
LSP	70	26.9 (19.10–36.52)
Control	73	17.4 (9.79–28.92)
Significance		$P = 0.37$

ASP: Artificial seminal plasma; HSP: High seminal plasma (from High fertility rams); LSP: Low seminal plasma (from Low fertility rams).

<sup>a</sup> Pregnancy of ewes that were unmarked by raddled rams was confirmed by slaughter 29–50 days post-AI.

Table 3

Pregnancy rate of ewes cervically inseminated with frozen–thawed semen resuspended in phosphate buffered saline (PBS) containing seminal plasma (SP) from either High (HSP) or Low (LSP) fertility rams or in PBS without SP compared with a Control treatment where semen was inseminated directly

Treatment	No. of ewes inseminated		Pregnancy rate <sup>a</sup> (%)	
	Farm 1 <sup>b</sup>	Farm 2 <sup>c</sup>	Farm 1 <sup>c</sup>	Farm 2 <sup>c,d</sup>
HSP	22	23	0.0	3.9 (0.5–24.9)
LSP	23	23	0.0	12.4 (3.7–34.4)
PBS	44	43	2.3	11.0 (4.3–25.3)
Control	26	29	15.4	17.8 (7.2–37.7)

<sup>a</sup> Pregnancy rate was based on pregnancy diagnosis at slaughter 29–50 days post-AI.

<sup>b</sup> Farm 1: Lyons Research Farm.

<sup>c</sup> Farm 2: Teagasc Research Centre, Athenry.

<sup>d</sup> Least squares means with 95% confidence interval in parentheses.

difference among treatments (ASP, HSP, LSP and Control) was not significant ( $P = 0.37$ ; Table 2). The contrast between the three treatments that involved resuspending spermatozoa in seminal plasma (HSP, LSP and ASP) and the Control treatment was not significant ( $P = 0.13$ ). The interaction between treatment and location was not significant ( $P = 0.15$ ) but when this interaction was partitioned into orthogonal components, using linear contrasts, the only element that approached significance ( $P = 0.06$ ) was the difference between locations for the contrast of ASP (31.4% at Farm 1 and 25.4% at Farm 2) and Control (8.9% at Farm 1 and 31.1% at Farm 2).

### 3.2. Experiment 2

The numbers of ewes per treatment and pregnancy rate are given in Table 3. On Farm 1, the pregnancy rate for Control semen (ewes inseminated directly with frozen–thawed semen) and PBS treated semen ( $100 \times 10^6$  motile sperm added to PBS prior to insemination) was 15.4% and 2.3%, respectively. No pregnancies were obtained from either High or Low seminal plasma treatments. On Farm 2, there was no effect of ram or ewe breed ( $P > 0.05$  in both cases). There was a significant effect of Day of insemination on pregnancy rate ( $P < 0.01$ ) and there was no interaction between day and treatment. The overall pregnancy rate for Control, PBS, HSP and LSP was 17.8%, 11.0%, 3.9% and 12.4%, respectively (Table 3). There was no difference between control and SP treatments ( $P = 0.31$ ). When results for Day 1 (of 2 days) were considered alone the pregnancy rate for Control, PBS, HSP and LSP were 30.4%, 23.9%, 11.1% and 22.2%, respectively, but these differences were not significant ( $P > 0.05$ ).

## 4. Discussion

The main finding from this study is that pregnancy rate was not increased when frozen–thawed ram spermatozoa were resuspended in ram seminal plasma collected from the rams used in this study or in artificial seminal plasma (ASP) prior to cervical artificial insemination. These results do not support previous findings by Maxwell et al. [22] who suggested that factors in seminal plasma were responsible for improved pregnancy rate following the resuspension of frozen–thawed spermatozoa in a medium containing seminal plasma. However, the rams used in the present study were not selected on the basis of the beneficial effects of their seminal plasma on in vitro sperm function, as in the study of Maxwell et al. [22]. Therefore, as the use of SP in Experiment 1 did not improve pregnancy rate, it was hypothesised that differences in fertility observed by Maxwell et al. [22] may be a consequence of different sperm preparation and selection procedures. Frozen-thawed spermatozoa were centrifuged through a discontinuous density gradient to remove the freezing diluent and select a highly motile sperm population for insemination. Other studies of the effects of seminal plasma on spermatozoa relative to fertility, involved centrifugation or Percoll density gradient separation procedures, prior to addition of seminal plasma (ram: [13,22,23]; bull: [25,34]). Such procedures could remove potential toxic effects of glycerol [35], which have been reported to induce sperm capacitation or other factors which would promote lipid peroxidation of sperm membranes. This may be a necessary step in making spermatozoa available to interact with components of seminal plasma before exposure to the female reproductive tract [13,22] and subsequent fertilisation events. We altered the

protocol in Experiment 1 to eliminate time-consuming steps such as centrifugation and counting of spermatozoa prior to resuspension in seminal plasma, as such procedures would not be practical at farm level. As these results were not consistent with those found previously [22] it was hypothesized that washing and selection of motile sperm was removing harmful effects of freezing diluent. The results from Experiment 2 suggest that washing and selection of spermatozoa by centrifugation through a density gradient and resuspension in PBS containing seminal plasma (from either High or Low fertility rams) at 30% (v/v) inseminated at a dose of  $100 \times 10^6$  sperm had a negative effect on fertility.

The entire procedure from thawing of semen through centrifugation, counting, resuspension in treatment to final insemination of ewes took on average 60 min, which meant that although the most motile cells were selected, spermatozoa may have been compromised by the length of time incubated. There is considerable evidence that dilution, incubation, cooling and freeze-thawing affect function of spermatozoa and these functional changes enable spermatozoa to effect immediate fertilisation in vitro, or in vivo if semen is deposited near the site of fertilisation [36]. However, it has been suggested that there is a decline in sperm motility, viability and longevity [3], possibly resulting in premature capacitation and death when spermatozoa are deposited cervically [36]. We suggest that the length of the procedure in Experiment 2 as well as dilution effects and a decreased inseminate dose compromised sperm cells and ultimately pregnancy rate.

Several authors have also reported differences among individuals in components of seminal plasma [25,27]. In an in vitro study, the penetration of zona-free oocytes by sperm from bulls of Low fertility was improved by seminal plasma from bulls of High fertility and penetration by High fertility spermatozoa was decreased by exposure to Low fertility seminal plasma [34]. However, in the current in vivo study, there was no evidence to suggest that seminal plasma from High fertility rams, selected on the basis of pregnancy rate, was any different to seminal plasma from rams of Low fertility.

A possible explanation for the apparent conflicting results with respect to the addition of seminal plasma is the relative ratio of capacitating and decapacitating, or beneficial and harmful, proteins in seminal plasma, and their variation between species or individuals and within individual males over time. Whole seminal plasma is a complex mixture of organic and inorganic components, as well as proteins with potential positive

and negative effects. The concentrations of these proteins vary from male to male and can appear and disappear depending on environmental factors such as season of collection, temperature, nutrition and stress [36].

In conclusion, under the conditions in these experiments, seminal plasma did not improve pregnancy rate in ewes when using frozen-thawed ram spermatozoa for cervical insemination. There were also no beneficial effects of centrifugation and removal of the freezing diluent after thawing.

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