Seminal Plasma Re-Addition to Ram Semen Ameliorates the Effect of Dilution Rate and Reduces Percentage of Acrosomal Damaged Sperm

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ABSTRACT

Ram spermatozoa are very sensitive to the effect of dilution rate during adjustment for artificial insemination dose for sheep. The objectives of this study was to determine the extent of protection that will be offered by the seminal plasma to ram sperm when added to the cryopreservation diluent, following low and high dilution rates before cooling semen to 5° C and freezing. In experiment 1, pre-cooling dilution with seminal plasma extender (group B) at low dilution rate was found to significantly ($p<0.05$) preserve the post-thaw percentage of acrosomal damaged sperm (19.6 ±0.52%) and maintained acceptable post-thaw sperm progressive motility (44.5 ± 1.57%) compared to milk base diluted semen in group A (53.0 ±1.11%) progressive motility and 41.0 ±1.90% acrosome damaged sperm). In experiment 2, following high dilution rate of semen, seminal plasma treated sub-groups C2 and D1, D2 were also found to significantly ($p<0.05$) reduce percentage of acrosomal damaged sperm (20.6± 0.99, 23.3 ±1.15 and 21.5 ±1.12% respectively) and maintained acceptable sperm progressive motility rates (47.5 ±0.01, 47.0 ±1.70 and 48.5± 1.67 % respectively). The study concluded that, addition of ram seminal plasma to ram semen freezing diluent or its pre-freezing supplementation, will preserve sperm acrosome membranes and maintains acceptable progressive motility rates both at high and low dilution rates.

Keywords: Ram Semen, Seminal Plasma, Dilution Rate, Acrosome Integrity

INTRODUCTION

Artificial insemination with frozen semen has not been widely adopted in sheep because of the extensive nature of sheep industry and the poor fertility obtained when cervical insemination technique is used with frozen semen. This is due to the fact that ram spermatozoa encounter problems during cryopreservation resulting in less viable spermatozoa post-thaw with less ability to traverse the cervical barrier (Hawk, 1983). However, some studies reported partial success in resolving these problems (Gil *et al.,* 2003, Morrier *et al.,* 2003). In sheep, the insemination dose necessary for cervical technique with frozen semen is ten times ($200x10^6$) more than that for cow (Salamon and Maxwell, 1995b, 2000).

In order to reach acceptable fertility with frozen-thawed semen after cervical insemination in sheep, adequate number of spermatozoa with attributes necessary to traverse the cervical barrier were needed (Salamon and Maxwell,2000), which in turn depended on the extender and method of dilution used (Ollero *et al.,* 1998).The dilution rates used in the afore mentioned studies were however high which would not meet the required number of spermatozoa in the insemination dose suitable for cervical insemination technique in sheep (D'Allessandro *et al.,* 2001, Gil *et al.,* 2002).

Ram spermatozoa are sensitive to the effects of low semen dilution rate. They became vulnerable to cold shock with negative effects on motility and plasma membrane integrity (Maxwell and Watson 1996, Watson 1995). The occurrence of cold shock as a consequence of the negative effects of semen low dilution rate, depends on type of cryoprotective agents, their level in the extender, dilution and cooling rates. Recently, inclusion of various levels of seminal plasma during cryopreservation of ram semen was reported to offer protection to ram sperm cells against the detrimental effects of cooling, freezing and thawing (Maxwell *et al* 1999; Perez-Pe *et al.,* 2002). It was also observed that, concentration of protective seminal plasma proteins were reduced to approximately 70%-80% during cryopreservation process. This reduction was followed by development of premature sperm membrane capacitation and capacitation-like events which may terminate with premature acrosomal reaction (Medeiros *et al.,* 2002).Therefore, protecting spermatozoa vital attributes while adjusting for cervical insemination dose during cryopreservation processes, may result to better post-thaw spermatological parameters and acceptable fertility following cervical insemination.

The aim of this study is therefore, to determine the effect of re-addition of seminal plasma at different stages of semen processing on sperm motility and acrosome membrane integrity following low and high semen dilution rates.

MATERIALS AND METHODS

Husbandry and procedure

The study was conducted at the Department of

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Reproduction and Artificial Insemination (AI), Faculty of Veterinary Medicine, Istanbul University between March and July in 2006. The experimental units involved six Kivircik rams, aged 4-6 years. The rams were kept under the same management, feeding and closed housing environment. They were provided with concentrate ration of 1kg/ram/day, offered good quality hay and clean drinking water made available for them throughout the period of study.

Semen collection, evaluation and processing

Semen was collected from the rams twice a week for a period of four months between March and July by electro-ejaculation method (Evans and Maxwell 1987). Sperm motility rate throughout the study was assessed subjectively using a phase-contrast microscope (x20). The acrosome integrity assessed as the absence or presence of a normal apical ridge was evaluated by mixing 5µml semen with 100µml formal saline fixative solution (Hancock, 1952) and examined using oil immersion lens (x100) under phase-contrast microscope. Haemo-cytometer method described by Evans and Maxwell (1987) was used for determination of sperm concentration.

Following assessment of spermatological characteristics of fresh semen immediately, semen were diluted at 26˚ C, then slowly cooled to 5˚ C at the rate of 0.3˚ C/min. Glycerolization of semen was done at 5˚ C within 50 minutes. Semen then equilibrated at 5˚ C for 2 hours before being loaded in to 0.25ml straw and frozen in liquid nitrogen vapor (-110° C) for 7 minutes and then stored in liquid nitrogen (-196° C). Frozen semen were thawed in water bath at 50° C for 10 sec and evaluated for post-thaw spermatological characteristics.

Seminal plasma harvesting and preparation
Preparation of semen extenders

Skim milk base extender: Skim milk base extender was prepared by dissolving 11g of 1% fat skim milk powder in 100ml distilled water to make 11% (w/v) solution. The solution was then heated to 95° C for 10 minutes (Evans and Maxwell 1987), cooled to room temperature and then 10% (v/v) of egg yolk was added to the solution. After thorough homogenization, the mixture was centrifuged in cold centrifuge at 3310 x g for 20 minutes according to the technique described by Gil *et al*., (2003). Following centrifugation, the middle clear solution was then separated from the upper and bottom parts by filtering through several layers of sterile gauze into a clean sterile glass beaker. 0.03 g/100ml penicillin, 0.04 g/100ml streptomycin and 0.024 g/100ml fructose were added to the filtrate which was then transferred into 14 ml plastic centrifuge tubes and stored under -20° C until used.

Preparation of glycerolized extender: glycerolized extenders (10%) glycerol, were prepared by addition of 10% glycerol (v/v) to the milk base extender and then stored under -20° C until used**.**

Preparation of seminal plasma extender: seminal plasma extender was prepared by addition of 20% seminal plasma to milk base extender (v/v).

Preparation of Hancock's formal saline solution: Hancock's formal saline solution was prepared according to Hancock (1952) and maintained under 5° C throughout the study period.

Experimental design

Experiment I

This experiment aimed to determine the effect of pre-cooling addition of seminal plasma following low semen dilution rate (1 + 1) and the extent of protection that will be offered by seminal plasma re-addition to ram sperm against the detrimental effects of cooling and freezing after final dilution rate (1 + 3).

Pooled semen was divided into two equal volumes. Each volume was then diluted at 26° C with skim milk base extender forming group A or diluted with skim milk base extender containing 20% seminal plasma forming group B.

Semen then cooled to 5° C at which temperature they were extended with skim milk diluent containing 10% glycerol to 1+3 dilution rate (Table 1). A period of two hours of equilibration was allowed before semen being packed into straws and frozen over liquid nitrogen vapour. Three straws from each group of 20 straws were thawed and evaluated for sperm progressive motility and acrosomes integrity.

Experiment 2

This experiment aimed at determining the extent of protection that will be offered by re-addition of seminal plasma before freezing at 5° C following high semen dilution rate, on sperm progressive motility and acrosome integrity of ram semen during cryopreservation.

Table 1: Formation of low dilution rate treatment groups

Processing Stage		Treatment Group	
Before Cooling		A	B
	Dilution Rate	1+1	1+1(semen+Diluent)
	Seminal plasma %	0	20
Before Freezing			
	Dilution Rate	1+3	1+3(semen+Diluent)
	Seminal plasma %	0	10
	Glycerol %	5	5

As in experiment 1, pooled semen was divided into two equal volumes, each of which was diluted to 1+1 (semen + extender) rate with skim milk base and 20% seminal plasma in skim milk base extenders forming main group C and D respectively (Table 2).

Semen was cooled to 5° C then immediately highly extended with 10% glycerol extender to 1 + 7 dilution rate. Each main group (C and D) again was divided into two equal volumes forming C1, C2 and D1, D2 groups. Groups C2 and D2 were again supplemented with 10% seminal plasma thus increased the pre-freezing level of seminal plasma in D2 to 15% and 10% in group C2 (Table 3).

Each experiment was replicated 10 times (n=10). Three straws / each group were examined for post-thaw sperm progressive motility rate and acrosomes integrity (n=40).

Table 2: Formation of high dilution rate treatment groups

%		Treatment group	
		C	D
Before cooling	Dilution rate	1+1	1+1 (semen+diluent)
	Seminal plasma level (%)	0	20
Before freezing	Dilution rate	1+7	1+7 (semen+diluent)
	Seminal plasma level (%)	0	5
	Glycerol (%)	5	5

Table 3: Pre-freezing formation of high dilution rate sub-groups

	Treatment Groups			
%	C1	C2	D1	D2
Seminal plasma	0	10	5	15
Glycerol	5	5	5	5

Statistical analysis

Data obtained in this study were presented as means ±SE. With the help of SPSS 10.0 statistical software package, one way analysis of variance (ANOVA) to evaluate the treatment effects was used and Duncan's Multiple Range test (Duncan,1955), LSD and t' test were used to determine significant differences between means at $p<0.05$ the level of significance.

RESULTS

Experiment 1

Following low semen dilution rate, the progressive motility of sperm following treatment showed no significant differences ($p>0.05$) between treatments before cooling and freezing (Table 4). However, statistically significant differences ($p<0.05$) between treatments were observed following freezing and thawing (53.0 ±1.11% in group A, and 44.5 ± 1.57% in group B). Addition of seminal plasma in group B appeared to reduce percentage of acrosomal damaged spermatozoa before cooling, freezing and thawing (Table 4).

Significant differences ($p<0.05$) were observed before freezing (8.0± 0.89% in group B and 17.9 ±1.10% in group A). After freezing and thawing, they were 19.6 ±0.52% in seminal plasma treated group B and 41.0 ±1.90% in milk base extender group A.

Experiment 2

At pre-freezing stage, the effect of high dilution rate on sperm progressive motility and acrosome integrity were not significant ($p>0.05$) among seminal plasma treated main group D, compared to untreated group C (Table 5).

Table 4: Effect of seminal plasma addition on sperm's progressive motility and acrosomes integrity

Parameters	Treatment groups	
	A	**B**
Progressive motility rate (%)		
Before cooling	86.0 ±1.23	87.0 ±1.53
After cooling	84.0± 1.45	86.0 ±1.80
Before freezing	82.0 ±1.53	85.0 ±1.83
Post-thawing	53.0± 1.11[a]	44.5 ±1.57[b]
Acrosomal Integrity (%)		
Before cooling	7.9± 0.85	4.1 ±0.48
After cooling	11.2± 1.07	6.3 ±0.56
Before freezing	17.9 ±1.10	8.0± 0.89
Post-thawing	41.0± 1.90[a]	19.6 ±0.52[b]

[a,b] values in the same raw with different superscripts are significantly different ($p<0.05$)

Table 5: Effect of seminal plasma addition on sperm's progressive motility and acrosomes integrity

Parameters	Treatment groups	
	C	**D**
Progressive motility rate (%)		
Before cooling	86.0±1.25	88.5±1.0 7
Before freezing	83.0±1.11	85.0 1.17
Acrosomes Integrity (%)		
Before cooling	8.2±0.53	5.1±0.43
Before freezing	13.0± 0.4[a]	8.5 ±o.6[b]

[a,b] values in the same raw with different superscripts are significantly different ($p<0.05$) according to Duncan's Multiple Range test.

Table 6: Effect of seminal plasma addition on sperm progressive motility and acrosome membrane integrity

Parameter	Sub-Groups			
	C1	C2	D1	D2
Progressive Motility Rate (%)				
Pre-Freezing	85± 0.8	87 ±1.1	87±1.1	88.5± 0.8
Post-Thawing	53±1.3[a]	48 ±0.01[b]	47±1.7[b]	48.5 ±1.7[b]
Acrosome Integrity (%)				
Pre-Freezing	17±1.2[a]	12 ±1.0[c]	11± 0.9[c]	13.2 ±1.4[b]
Post-Thawing	42±1.2[a]	21± 1.0[c]	23 ± 1.2[b]	21.5 ±1.1[c]

a,b,c, d values in the same raw with different superscripts are significantly different ($p<0.05$) according to Duncan's Multiple Range test.

However, after freezing and thawing, seminal plasma untreated group C1 showed significantly ($p<0.05$) higher progressive motility rate (53.0 ±1.33%), than groups containing various levels of seminal plasma (47.5 ±0.01% group C2, 47.0 ±1.70% group D1 and 48.5 ±1.67% group D2) as shown in Table (6).

Following high dilution rate, acrosomes damaged spermatozoa were significantly reduced in seminal plasma treated groups ($p<0.05$) C2, D1 and D2 than untreated group C1 (Table 6).

DISCUSSION

In sheep artificial insemination industry, interest for frozen ram semen was aroused with the adoption of intensive sheep breeding system. Cervical Artificial Insemination (AI) is the practical and cheap approach which is readily available for farmers. However, the relatively low fertility rate achieved with this method using frozen semen, limited its wider application.

Today, a trend for improvement of frozen ram semen for cervical insemination has been the goal in sheep industry and a research focus. Continuous search for a suitable extender that will preserve adequate number of spermatozoa with characteristics necessary to traverse the cervical barrier and results into acceptable fertility is needed (Holt, 2000).

The capability of sperm cell to resist cold shock generally known as freezability is an important attribute for ram spermatozoa to maintain the plasma and outer acrosome membranes intact throughout cryopreservation. This attributes varies among individual rams and between ejaculates of the same ram (Salamon and Maxwell, 1995a; Holt, 2000; Windsor 1997, Eppleston and Maxwell 1991).

In experiment 1, sperm progressive motility rates and acrosome integrity were preserved in seminal plasma supplemented group B, similar to the control group A. Spermatozoa motility and acrosome integrity were preserved during semen cooling to 5° C and throughout the equilibration period.

After freezing and thawing, when the level of seminal plasma was expected to be reduced by dilution to 10% (Table 1), significantly high post thaw motility rate and acrosome damaged spermatozoa was shown in group A compared to group B ($P<0.05$).

The role of milk protein lactalbumin in enhancing sperm motility and viability when added to semen extender in this result was supported by Ollero *et al.*, (1998),Gil *et al.*, (2000, 2003) and Watson, (1995). In addition, some sperm parameters were found to be better with clarified milk extender than none clarified in agreement with report of Gil *et al.*, (2000). However, D'Alessandro *et al.*, (2001) obtained lower proportion of motile spermatozoa post thawing when milk extender and low pre-freezing dilution rate were used. This indicates that milk extender does not support sperm motility frozen at low pre-freezing dilution rates.

In this study, despite the high proportion of motile sperm observed in group A after thawing, significant ($p<0.05$) number of spermatozoa with damaged acrosome membranes (41.0±1.9%) were observed compared to group B (Table4). Using the same extender, Gil *et al.*, (2003) reported less acrosomes damaged and high proportion of capacitated spermatozoa after thawing. Since capacitated spermatozoa submits readily to spontaneous acrosomal reaction, then it is logically to conclude that, the proportion of spermatozoa with damaged acrosome membranes is expected to increase after thawing which concurs with the findings of D'Alessandro and Martamucci (2005). However, this study did not consider the proportion of sperm which have undergone capacitation.

Addition of 20% seminal plasma to milk base extender in group B, maintains high sperm motility and acrosome membrane integrity after cooling to 5° C. However, following final dilution, seminal plasma level was reduced to 10% pre-freezing which was reflected in the slight reduction in spermatozoa motility rate but significant post-thaw reduction of acrosomal damaged sperm compared to group A ($P<0.05$). The obtained post-thaw motility rate in B was 44.5±1.57% and this fall within the range of 40%-60% considered to be high for ram spermatozoa motility rate after freezing and thawing (Salamon and Maxwell, 2000).

Although reduction of motility rate in seminal plasma groups was not that significant, yet it appeared that seminal plasma does slightly affect motility which contradicted the reports of Graham *et al.*, (1994), Bass, (1983), Moore *et al.*, (2005) and Bass, (1983) in their supporting seminal plasma maintenance of spermatozoa motility. They revealed that sperm motility promoting factor in seminal plasma may be rendered ineffective by prolonged exposure of spermatozoa to seminal plasma.

Sperm acrosome membrane integrity was significantly preserved in B ($P<0.05$) throughout cooling to 5° C, freezing and thawing (Table 4). This confirmed previous findings of Moore *et al.*, (2005) in which the occurrence of cooling induced capacitation and capacitation-like changes on sperm membranes were prevented in the presence of seminal plasma and that capacitated sperm membranes were again converted to normal state in presence of seminal plasma, termination of capacitation events by premature acrosome reaction is halted at early stages of cryopreservation.

In experiment 2, when semen was highly diluted (Table 2), significant post-thaw motility rate and acrosomes damaged spermatozoa was observed in sub-group C1 (Table 6). This agreed with the findings of Prathalingan *et al.*, (2006) in that high dilution rate did not have marked effects on spermatozoa motility following cryopreservation, but did reduce the proportion of spermatozoa with intact acrosome. At an intermediate pre-freezing semen dilution rate, milk base extender is shown

to display better protective effects on sperm motility (D'Alessandro *et al* 2001, D'Alessandro and Martemucci 2005).

Addition of 10% seminal plasma at 5° C in sub-groups C2 and D2, significantly improved the acrosomes integrity after freezing and thawing compared to sub-group C1 ($p<0.05$). This may indicate that, capacitation changes that occurred during cryopreservation process were prevented and reverted by the added seminal plasma, and accordingly, premature acrosomal reaction was prevented (Vadnais *et al.*, 2005) thus fertile life span of spermatozoa will be preserved for longer period. But sperm concentration desired for cervical insemination dose may need further re-adjustment with centrifuge.

Further increase in seminal plasma level by addition of 10% in group D2, significantly improved post-thaw sperm motility and acrosome integrity ($p<0.05$) which again indicates that, pre-freezing increase in level of seminal plasma, conferred better protection on ram spermatozoa during freezing and thawing.

CONCLUSION

This study concludes that during the process of stepwise adjustment of ram semen for cervical insemination dose (200 x 10^6 sperm/ml), inclusion of seminal plasma in the diluent for cryopreservation of ram semen or its re-addition pre-freezing, ameliorates the untoward effects of low and high extension rates through preservation of acrosome integrity and acceptable progressive motility rates.

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