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Research Article

Effects of Extender Osmolarity, Cooling Rate, Dilution Rate and Glycerol Addition Time on Post-thaw Ram Semen Characteristics and Fertilization

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ABSTRACT

Tree experiments were designed to optimize ram semen cryopreservation and to examine the effect of the extender B osmolarity, extension time with extender A and B, presence of glycerol in freezing media, cooling rate from 30˚C to 5°C, and dilution rate on post-thaw ram semen characteristics and fertilization. Ram ejaculates of thick consistency with rapid wave motion (>+++) and >70% initial motility were pooled. Pooled semen was diluted with Tris based extender using a two-step dilution method. Semen motility and morphology related parameters were assessed at the following five steps : (1) Fresh, (2) after dilution with extender A, (3) at 5°C, (4) after equilibration, and (5) after thawing at 37°C for 30 min. The extender osmolarity affected significantly post-thaw semen motility, defected acrosomes (DA) and total morphological defect (TMD), generally.

There was a significant interaction between the osmolarity, cooling rates, glycerol addition time and first dilution time, on post-thaw motility, DA and TMD. Higher fertilization rates were obtained in fresh semen and Group 15 compared to Group 21 ($P<0.05$). In conclusion, cooling rate, extender osmolarity, presence of glycerol, dilution time with extender A and B, and dilution rates interact on post-thaw semen parameters. Glycerol included freezing media improves post-thaw semen recovery. Also, increasing the extender osmolarity resulted better post-thaw semen motility especially in the group frozen without glycerol. Post-thaw semen parameters not affected by cooling and glycerol addition time regimes, generally. Obtained data showed that presence of glycerol in freezing media have a negative effect on embryonic development and it was observed that frozen ram semen at high osmolarity without glycerol could be used for *in vitro* fertilization.

Key Words: Ram semen, osmolarity, cooling rate, extender

ÖZET

KOÇLARDA SULANDIRICI OZMOLARITESI, SOĞUTMA ORANI, SULANDIRMA ORANI VE GLISEROL EKLEME ZAMANININ ERITME SONRASI SPERMATOLOJIK ÖZELLiKLER VE FERTiLiZASYON ÜZERiNE ETKiLERi

Koç spermasının dondurulmasını optimize etmek ve sulandırıcı B ozmolaritesi, sulandırıcı A ve B ile sulandırma zamanı, dondurma medyumunda gliserolün varlığı veya yokluğu, +30°C'den +5°C'ye soğutma oranı ve sulandırma oranının eritme sonrası spermatolojik özellikler ve fertilite üzerine olan etkilerini belirlemek amacıyla üç deney planlandı. İyi bir kitle hareketine(>+++) ve >%70 başlangıç motilitesine sahip olan koç ejekülatları çalışmaya dahil edilerek pooling yapıldı. Sperma motilitesi ve morfolojisi ile ilgili parametreler (1) taze, (2) sulandırıcı A ile sulandırdıktan sonra, (3) 5°C'ye soğutulduktan sonra, (4) ekilibrasyon sonrası ve (5) eritme sonrasında incelendi. Sulandırıcı ozmolaritesi eritme sonrası motiliteyi, kusurlu akrozom oranını ve toplam morfolojik bozukluk oranını önemli derecede etkiledi.

Ozmolarite,soğutma oranı, gliserol ekleme zamanı ve ilk sulandırma zamanı ile eritme sonrası motilite, kusurlu akrozom oranı ve toplam morfolojik bozukluk oranları arasında önemli bir ilişki bulundu. Grup 21 ile karşılaştırıldığında taze sperma ve grup 15'te önemli derecede daha yüksek fertilizasyon oranı elde edildi.

Sonuç olarak, soğutma oranı, sulandırıcı ozmolaritesi, gliserol varlığı, sulandırıcı A ve B ile sulandırma zamanı ve sulandırma oranları ile eritme sonrası spermatolojik özellikler arasında önemli bir etkileşim bulundu. Bunun yanında sulandırıcı ozmolaritesinin artırılması, özellikle gliserol katılmayan gruplarda daha iyi eritme sonrası motilite eldesi ile sonuçlandı. Eritme sonrası spermatolojik özellikler soğutma zamanı ve gliserol ilave zamanından etkilenmedi. Elde edilen sonuçlar, dondurma medyumunda gliserol varlığının embriyonik gelişim üzerinde olumsuzbir etkisinin olduğunu ve gliserol ilave edilmeksizin yüksek ozmolariteli sulandırıcılar ile dondurulmuş koç spermasının *in vitro* fertilizasyonda kullanılabileceğini gösterdi.

Anahtar Kelimeler: Koç sperması, ozmolarite, soğutma oranı, sulandırıcı

Introduction

Fresh ram semen has a short fertile lifespan, whereas acceptable fertility with cryopreserved semen is achieved only by laparoscopy, which limits the widespread of artificial insemination (AI) in sheep husbandry **(Coyan et al., 1992; Salamon and Maxwell, 1993)**. Although several researchers have developed different extenders and protocols for freezing ram semen, in general, the fertility results are not comparable to those obtained with fresh semen and natural mating **(Coyan et al., 1992; Fiser and Fairfull, 1984)**. The success of AI with frozen ram semen depends on the physiology of sperm transport and on the survival of spermatozoa in the female reproductive tract **(Coyan et al., 1992; Gunay et al., 2006; Sanches-Partida et al., 1992)**. Also, cooling and freezing rates **(Fiser and Failfull, 1984; Gunay et al., 2006; Maxwell et al., 1995; Woelders et al., 1997)**, extender osmolarity **(Soylu et al., 2007)**, type of cryoprotective agents **(Nur et al., 2010; Soylu et al., 2007; Yildiz et al., 2000)**, cryoprotective agent concentration **(Fiser and Fairfull, 1984; Woelders et al., 1997; Yildiz et al., 2000)**, dilution rates **(Maxwell et al., 1995; Sanches-Partida et al., 1992)**, temperature at which glycerol is added to the semen, equilibration time with cryoprotectant, and thawing rate interact with the success of AI with frozen semen **(Abdelhakeam et al., 1991; Ali et al., 1992; Fiser and Fairfull, 1984; Salamon and Maxwell, 1993)**.

The extenders composition assists in stabilizing the cell during the freezing and thawing process **(Kakadiya and Kavani, 1995; Liu et al., 1998)**. Placing mammalian semen in a high-osmolarity environment makes the spermatozoa lose water and shrink **(Liu et al., 1998)**. Also semen extender with high osmotic pressure provided better protection for post-thaw ram sperm against cooling damage on acrosome and morphological integrity than low osmotic media **(Soylu et al., 2007)**. Glycerol is commonly added to semen extenders for freezing mammalian semen, **(Abdelhakeam et al., 1991; Fiser and Fairfull, 1984)**. Although glycerol protects the spermatozoa from cryoinjury by removing the water found within the cell and by increasing extra cellular osmolarity **(Sayre and Lewis, 1997)**, the presence of glycerol in semen extender reduces motility (at 30°C and 5°C), DNA integrity **(Nur et al., 2010)** and fertilizing capability following intracervical insemination. Also presence of glycerol alternate acrosomal integrity and accelerates acrosome reaction **(Abdelhakeam et al., 1991)**. For these reasons, many studies were carried out on the amount of glycerol and the timing protocol for adding it to the extender and on exclusion of the glycerol from freezing media **(Abdelhakeam et al., 1991; Fiser et al., 1987; Nur et al., 2010; Soylu et al., 2007)**.

The aim of the present study was to monitor the effect of different extender osmolarity, dilution time, dilution rate, cooling rate and presence of glycerol on post-thaw ram semen characteristics and fertilization.

Materials and Method

Animals and semen collection

Semen was obtained from ten Kivircik rams at 3-5 years of age housed at the Laboratory of the Department of Reproduction and Artificial Insemination (Istanbul University, Faculty of Veterinary Medicine, Turkey). Semen was

collected by electrically-stimulated ejaculation for 5 times with a one day inter-semen collection interval (**Soylu et al., 2007; Varisli et al., 2008**). To collect semen, rams were restrained physically and lubricated probe was inserted into the rectum therefore, electrodes rest on the upper portion of ampullary region. An electrical stimulation was applied for 4 to 8 s. The electrostimulation was stopped briefly (3–4 s) while further massage was applied with the probe. This cycle was repeated until a 1 to 2 ml sample of semen was collected (usually three or four electrostimulations). Collected semen was placed in a warm water bath (30°C) and immediately evaluated for consistency, wave motion (0–5 scale) and percentage of motile spermatozoa (0–100%) (**Dhami et al., 1992**). Ejaculates having a thick consistency, rapid wave motion (3-5 in 0–5 scale) and >70% initial motility were pooled.

Semen dilution and processing

Semen extenders were prepared as described by **Fiser et al. (1987)** with some modifications. Extender A contained THAM (Tris (Hydroxymethyl) aminometane) 32.56 g, B-fructose 9.35 g, Citric acid 17.02 g, Penicillin G 500.000 IU and Dihydrostreptomycin 625 mg. Extender B contained Sodium Sitrate (dihydrate) 6.88 g, TES (N-Tris (hydroxymethyl-2-aminometane sulphonic acid) 58 g, Lactose 101.88 g, Fructose 5.06 g, Dihydro streptomycin 625 mg.

Extender A and B were prepared at the osmotic pressure of 350, 375, 400, 425 and 475 mOsm. The osmotic pressures of extenders were determined by freezing point depression osmometer (Advanced 3D3 Single Sample Osmometer, Advanced Instrument, INC Norwood MA USA), before adding cryoprotectants and egg yolk.

Tree experiments were designed to optimize ram semen cryopreservation and to examine the effect of the extender B osmolarity, extension time with extender A and B, presence of glycerol in freezing media, cooling rate from 30°C to 5°C, and dilution rate on post-thaw ram semen characteristics and fertilization.

Experiment 1: This experiment was designed to monitor effect of extender osmolarity, dilution time and cooling rates on post-thaw ram semen frozen without glycerol. Pooled semen was divided into six groups according to extender osmolarity, dilution time and cooling rates (Table 1). The final glycerol and dilution rate were, 0% and 1:2 (semen:extender) for all groups of experiment 1.

Experiment 2: This experiment was carried out to monitor effect of glycerol and time of addition, extender osmolarity, dilution time and cooling rates on post-thaw ram semen. For this pooled semen was divided into eight groups according to presence of glycerol and glycerol addition time, extender osmolarity, dilution time and cooling rates (Table 1). The final glycerol rates of the 7.-9 groups were 4% and the others were 0% and dilution rate was 1:2 (semen:extender).

Experiment 3: The effect of glycerol addition time, extender osmolarity, first dilution time with extender A and cooling rates on post-thaw ram semen diluted at 1:3 (semen:extender) was explored (Table 1). Pooled semen was divided to eight groups according to presence of glycerol, extender osmolarity, dilution time with extender A and cooling rates. All treatment groups in this experiment were diluted at 5°C with extender B. The final glycerol rates of 15 and 16. Groups were 4% and the others were 0% and dilution rate were 1:3 (semen:extender). For all experiment the final egg yolk was 10% and diluted semen were equilibrated at 5°C for 1 h.

Semen evaluation

All semen parameters were assessed at the following five steps: (1) Fresh, (2) after dilution with extender A, (3) to 5°C, (4) after equilibration, and (5) after thawing. Semen was frozen by the same person and each semen parameter was evaluated by the same person on each occasion throughout the entire study. Sperm motility was assessed subjectively using a phase-contrast microscope (x400) with a warm slide (38°C). Acrosomal structures and total morphological defect rate were evaluated using Hancock's formaline solution (**Hancock,**

1952). One drop of thawed semen was diluted with 1 ml of 37°C Hancock's formaline solution and examined by phase-contrast microscope at x1000 magnification under oil immersion (**İleri et al., 2000**) and 200 spermatozoa were counted per slide to determine the percentages of damaged acrosomes and total morphological defect.

At least three straws from each group were thawed at 37°C for 20 s in a water bath to evaluate post-thaw semen motility and morphology (5X3=15 straws). All experiments of the present study were repeated 5 times.

Fertility evaluation

For semen fertility evaluation, the best groups frozen with glycerol (Group 15) and that frozen without glycerol (Group 21), and fresh ram semen were used as a control group for *in vitro* fertilization.

Primary oocytes were harvested from ovaries of slaughtered ewes and were incubated *in vitro* for 26 hours at 39°C in a humidified atmosphere of 5% CO_2 in air. TCM 199 medium supplemented with Na pyruvate, FCS, FSH and LH was used as maturation medium (**Birler et al., 2002**). After *in vitro* maturation, oocytes (n=253) were divided into 3 groups randomly and fertilized with Group 15, Group 21 or fresh ram semen ($0.8x10^6$ spermatozoa/ml).

After 24 h of sperm oocyte incubation, the half of fertilized oocytes were fixed and stained for evaluation of fertilization and the remaining were SOF cultured for 8 days in anaerobic jar (5% CO_2+5% O_2+90% N_2) at 39°C and evaluated for embryonic development (**Birler et al., 2002**).

Table 1. Experimental design for semen processing in relation to extender B osmolarity, glycerol and dilution rates, cooling rate to 5°C and extension temperature.

Tablo 1. Sulandırıcı B ozmolaritesi, gliserol ve sulandırma oranları, 5°C'ye soğutma hızı ve sulandırma ısısı açısından spermanın işlenmesi ile ilgili çalışma dizaynı.

Experiment	Group	Extender B osmolarity (mOsm)	Final glycerol rate (%)	Final dilution rates (semen: extender)	Dilution at 30°C		Cooling to 5°C	Dilution at 5°C	
					Extender A	Extender B		Extender A	Extender B
Experiment 1	1	350	0	1/2	Yes	Yes	Slow	No	No
	2	350	0	1/2	Yes	Yes	Two steps	No	No
	3	350	0	1/2	No	No	Slow	Yes	Yes
	4	375	0	1/2	No	No	Slow	Yes	Yes
	5	400	0	1/2	No	No	Slow	Yes	Yes
	6	425	0	1/2	No	No	Slow	Yes	Yes
Experiment 2	7	425	6	1/2	Yes	Yes	Two steps	No	No
	8	425	6	1/2	Yes	No	Two steps	No	Yes
	9	425	6	1/2	Yes	No	Slow	No	Yes
	10	425	0	1/2	Yes	Yes	Slow	No	No
	11	425	0	1/2	Yes	Yes	Two steps	No	No
	12	425	0	1/2	No	No	Slow	Yes	Yes
	13	450	0	1/2	No	No	Slow	Yes	Yes
	14	475	0	1/2	No	No	Slow	Yes	Yes
Experiment 3	15	350	4	1/3	Yes	No	Slow	No	Yes
	16	350	4	1/3	Yes	No	Two steps	No	Yes
	17	425	0	1/3	Yes	No	Slow	No	Yes
	18	425	0	1/3	Yes	No	Two steps	No	Yes
	19	475	0	1/3	Yes	No	Slow	No	Yes
	20	475	0	1/3	Yes	No	Two steps	No	Yes
	21	425	0	1/3	Yes	No	Slow	Yes	Yes
	22	475	0	1/3	Yes	No	Slow	Yes	Yes

Slow cooling: Pooled semen were cooled from 30°C at <0.2°C/min to 5°C.
Two step cooling: Pooled semen were cooled from 30°C at <2°C/min to 16°C and from 16°C at <0.2°C/min to 5°C.

Statistical analyses

Data were subjected to analysis of variance (ANOVA, SPSS 10.0) by General Linear Model procedure using the extender osmolarity, cooling and glycerol rate, and dilution time as factors. Means of the obtained semen parameters were analyzed using Tukey Test with $P<0.05$ considered as significant. Fertility related results were analyzed with Fisher's Exact Test and embryonic development related results were analyzed with Chi square test.

Results

Experiment 1:

The percentage of motility, DA, and TMD for the pooled semen were 86.0%, 5.8%, 26.0%, respectively. Table 2 shows the effect of extender osmolarity, cooling rate and dilution time and their interactions on sperm parameters (ANOVA). As seen from the Table 2, there was no significant effect of the extender osmolarity, cooling rate and dilution time on equilibrated semen motility, DA and TMD ($P>0.05$).

Table 2. Effect of extender osmolarity, cooling rate, dilution time and their interactions on studied sperm parameters (Experiment 1).

Tablo 2. Sulandırıcı ozmolaritesi, soğutma oranı, sulandırma zamanı ve bunların etkileşimlerinin incelenen sperm parametreleri üzerindeki etkileri (Deney 1).

Factor	Equilibrated semen			Post-thaw semen		
	Motility (%)	DA (%)	TMD (%)	Motility (%)	DA (%)	TMD (%)
Osmolarity	NS	NS	NS	***	***	***
Cooling Rate	NS	NS	NS	**	NS	NS
Dilution time	NS	NS	NS	***	***	NS
Osmolarity x Cooling Rate x Dilution time	NS	*	NS	***	***	***

Table 3. The comparison of mean motility, DA and TMD (Mean±Standard Eror) after equilibration and thawing among groups (Experiment 1).

Tablo 3. Ekilibrasyon ve eritme sonrası motilite, DA ve TMD ortalamalarının (Ortalama ±Standart Hata) gruplar arasında karşılaştırılması (Deney 1).

Group	Extender Osmolarity (mOsm)	Cooling rate	Final Glycerol Rate (%)	Dilution temperature (ºC)	Equilibrated semen				Post-thaw			
					n	Motility (%)	DA (%)	TMD (%)	n	Motility (%)	DA (%)	TMD (%)
1	350	Slow	0	30	5	73.0±7.6^{a}	12.2±5.1^{a}	40.4±5.5^{a}	15	1.0±2.1^{d}	55.0±6.1^{ab}	75.0±4.9^{abc}
2	350	Two step	0	30	5	75.0±6.1^{a}	11.2±3.3^{a}	43.4±3.8^{a}	15	3.0±3.2^{d}	53.8±8.5^{ab}	74.4±6.2^{abc}
3	350	Slow	0	5	5	74.0±9.6^{a}	11.0±2.5^{a}	41.2±5.3^{a}	15	8.7±4.0^{c}	55.2±6.5^{ab}	77.4±6.9^{ab}
4	375	Slow	0	5	5	77.0±5.7^{a}	11.8±2.7^{a}	42.4±3.9^{a}	15	16.7±6.7^{b}	46.0±7.1^{bc}	71.0±5.2^{bc}
5	400	slow	0	5	5	76.0±8.9^{a}	14.0±3.4^{a}	46.4±8.3^{a}	15	14.7±7.7^{b}	56.8±8.1^{a}	81.6±8.4^{a}
6	425	slow	0	5	5	77.0±7.6^{a}	11.2±2.8^{a}	40.4±4.8^{a}	15	22.3±9.6^{a}	41.2±6.7^{c}	67.0±8.1^{c}

^{a,b,c,d} Values with different superscripts in the same column for different groups are significantly different ($P<0.05$).

The extender osmolarity, cooling rate and dilution time affected post-thaw semen motility (P<0.01). Osmolarity x cooling rate x dilution time interaction affected the motility, DA and TMD (P<0.001).

Table 3 shows the values of motility, mean percentage of acrosomal and total morphological defects after equilibration and thawing for each group in function of extender osmolarity, cooling rate and dilution time separately. For all groups the equilibrated semen motility, DA and TMD did not differ significantly.

In general, post-thaw motility, acrosomal and morphological integrity of the semen frozen with semen extender at 425 mOsm was better than those of 350, 375 and 400 mOsm, regardless of the cooling rates and dilution time. The post-thaw of slow cooled semen motility of the 425 mOsm (22%) were better than 350, 375 and 400 mOSm groups.

Table 4. Effect of extender osmalarity, cooling and glycerol rate, dilution time with extender A and B and their interactions on studied semen parameters (Experiment 2).

Tablo 4. İncelenen sperm parametreleri üzerine sulandırıcı ozmolaritesi, soğutma ve gliserol oranı, A ve B sulandırıcıları ile sulandırma zamanı ve bunlar arasındaki etkileşimin etkileri (Deney 2).

Factor	Equilibrated semen			Post-thaw semen		
	Motility (%)	DA (%)	TMD (%)	Motility (%)	DA (%)	TMD (%)
Osmolarity	**	NS	NS	NS	***	***
Cooling Rate	**	NS	***	NS	NS	NS
Glycerol	**	**	***	***	**	**
Dilution time with extender A	**	NS	**	NS	NS	NS
Dilution Time With extender B	*	NS	**	NS	NS	NS
Osmolarity x Cooling Rate x Glycerol rate x Dilution time with extender A and B	*	NS	***	***	***	***

*P<0.05, ,***P<0.01, ***P<0.001, NS: Not Significant, DA: Percentage of Defected Acrosome; TMD: Total Morphological Defect

Experiment 2

The percentage of motility, DA, and TMD for the pooled semen was 84.0%, 6.2% and 24.6%. Table 4 shows the effect of extender osmolarity, cooling rate, presence of glycerol and dilution time and their interactions on sperm parameters (ANOVA).

The equilibrated sperm motility was affected by extender osmolarity (P<0.01), cooling rates (P<0.01), glycerol rates (P<0.01), dilution time with extender A (P<0.01), and extender B (P<0.05). The percentage of defected acrosome of equilibrated semen was not affected by the all analyzed factors except glycerol rates (P<0.01). The equilibrated sperm TMD was affected by factor extender osmolarity (P<0.001), cooling rates (P<0.001), glycerol rates (P<0.01), dilution time with extender A (P<0.01), and extender B (P<0.01). Osmolarity x cooling rate x glycerol rate x dilution time with extender A and B interaction affected the motility (P>0.05) and TMD (P<0.001) of equilibrated semen and motility, DA and TMD of post-thaw semen (P<0.001).

Presence of glycerol in freezing media have an effect on post-thaw sperm motility (P<0.001), DA (P<0.01) and TMD (P<0.01). Post-thaw DA and TMD were affected by extender osmolarity (P<0.001).

Table 5. The comparison of mean motility, DA and TMD (Mean±Standard Eror) after equilibration and thawing among groups (Experiment 2).

Tablo 5. Ekilibrasyon ve eritme sonrası motilite, DA ve TMD ortalamalarının (Ortalama ±Standart Hata) gruplar arasında karşılaştırılması (Deney 2).

Group	Osmolarity (mOsm)	Cooling rate	Final Glycerol Rate (%)	Dilution time with A	Dilution Time With B	Equilibrated semen				Post-thaw			
						n	Motility (%)	DA (%)	TMD (%)	n	Motility (%)	DA (%)	TMD (%)
7	425	Two step	6	At 30°C	At 30°C	5	78.6±7.9^{a}	11.6±5.0^{a}	41.8±6.7^{bc}	15	16.7±7.5^{bc}	47.0±6.9^{ab}	70.4±5.0^{ab}
8	425	Two step	6	At 30°C	At 5°C	5	78.6±7.9^{a}	11.6±3.3^{a}	40.6±3.4^{c}	15	29.7±9.9^{a}	47.4±6.8^{ab}	69.6±10.7^{ab}
9	425	Slow	6	At 30°C	At 5°C	5	78.6±8.7^{a}	12.0±4.7^{a}	42.2±3.4^{bc}	15	22.0±9.6^{b}	40.4±2.9^{a}	64.6±3.3^{b}
10	425	Slow	0	At 30°C	At 30°C	5	74.2±9.7^{a}	8.8±1.8^{a}	51.2±5.3^{a}	15	1.3±2.3^{d}	48.2±7.6^{ab}	72.8±7.9^{ab}
11	425	Two step	0	At 30°C	At 5°C	5	76.6±4.2^{a}	9.6±2.6^{a}	48.0±3.7^{ab}	15	2.3±2.6^{d}	52.6±8.0^{a}	74.8±11.7^{ab}
12	425	Slow	0	At 5°C	At 5°C	5	77.2±4.7^{a}	11.6±4.6^{a}	43.4±4.8^{c}	15	22.9±9.8^{b}	42.4±4.3^{b}	66.8±2.6^{b}
13	450	Slow	0	At 5°C	At 5°C	5	73.0±10.4^{a}	10.8±2.6^{a}	51.6±3.4^{a}	15	14.3±8.0^{c}	55.6±8.1^{a}	78.0±8.0^{a}
14	475	Slow	0	At 5°C	At 5°C	5	69.0±7.4^{a}	10.2±3.8^{a}	44.0±7.1^{bc}	15	21.0±10.9^{b}	41.8±5.2^{b}	65.6±7.1^{b}

^{a,b,c,d} Vaiues with different superscripts in the same column for different groups are significantly different ($P<0.05$).

Table 5 shows the values of motility, mean percentage of acrosomal and total morphological defects after equilibration and thawing for each group in function of extender osmolarity, cooling rate, glycerol rate, and dilution time with extender A and B separately. The mean equilibrated semen motility and DA were not affected by semen processing (P>0.05). Better post-thaw motility were obtained with 6% glycerol supplemented Group 8 glycerolized at 5°C (P<0.05).

Experiment 3

The percentage of motility, DA, and TMD for the pooled semen was 81.0%, 2.6% and 21.6%. Table 6 shows the effect of extender osmolarity, cooling rate, presence of glycerol and dilution time and their interactions on sperm parameters (ANOVA).

The equilibrated sperm motility, DA and TMD was affected by extender osmolarity, glycerol rate and osmolarity x cooling rate x glycerol rate x dilution time with extender A interaction, significantly. Cooling rates and dilution time with extender A only affected the TMD of equilibrated semen.

Table 6. Effect of extender osmalarity, cooling and glycerol rate, dilution time with extender A and B and their interactions on studied sperm parameters (Experiment 3).

Tablo 6. İncelenen sperm parametreleri üzerine sulandırıcı ozmolaritesi, soğutma ve gliserol oranı, A ve B sulandırıcıları ile sulandırma zamanı ve bunlar arasındaki etkileşimin etkileri (Deney 3).

Factor	Equilibrated semen			Post-thaw semen		
	Motility (%)	DA (%)	TMD (%)	Motility (%)	DA (%)	TMD (%)
Osmolarity	***	***	***	***	**	***
Cooling Rate	NS	NS	**	NS	NS	NS
Glycerol	***	*	***	***	**	***
Dilution time with extender A	NS	NS	***	NS	NS	*
Osmolarity x Cooling Rate x Glycerol rate x Dilution time with extender A	***	**	***	***	**	***

*P<0.05, ,***P<0.01, ***P<0.001, NS: Not Significant, DA: Percentage of Defected Acrosome; TMD: Total Morphological Defect

Post-thaw semen motility, DA and TMD were affected by extender osmolarity, glycerol rate and osmolarity x cooling rate x glycerol rate x dilution time with extender A interaction, significantly. Cooling rate and dilution time have no effect on post thaw motility and DA (P>0.05).

Table 7 shows the values of motility, mean percentage of acrosomal and total morphological defects after equilibration and thawing for each group in function of extender osmolarity, cooling rate, glycerol and dilution time with extender A separately. The best results were obtained in Group 15 that 4% glycerol supplemented media. Presence of glycerol in freezing media improves post-thaw semen recovery.

Fertility evaluation

Fertility results and embryonic development of fresh semen and Group 15 and 21 were presented in Table 8 and 9, respectively.

Higher fertilization rates were obtained in fresh semen and Group 15 compared to Group 21 (P<0.05). There were no significant differences between groups for other fertility related parameters (P>0.05).

Table 7. The comparison of mean motility, DA and TMD (Mean±Standard Eror) after equilibration and thawing among groups (Experiment 3).

Tablo 7. Ekilibrasyon ve eritme sonrası motilite, DA ve TMD ortalamalarının (Ortalama ±Standart Hata) gruplar arasında karşılaştırılması (Deney 3).

Group	Osmolarity (mOsm)	Cooling rate	Final Glycerol Rate (%)	Dilution time with A	Equilibrated semen	Post-thaw						
					n	Motility (%)	DA (%)	TMD (%)	n	Motility (%)	DA (%)	TMD (%)
15	350	Slow	4	At 30°C	5	71.0±9.6^{a}	10.0±3.7^{ab}	30.2±5.7^{b}	15	53.3±6.5^{a}	25.6±5.9^{b}	45.8±6.0^{b}
16	350	Two step	4	At 30°C	5	69.6±9.4^{a}	11.4±3.4^{ab}	36.2±7.6^{ab}	15	50.3±13.2^{a}	36.8±9.5^{ab}	57.8±10.2^{a}
17	425	Slow	0	At 30°C	5	66.0±12.9^{a}	12.2±3.6^{ab}	38.0±5.5^{ab}	15	25.0±10.2^{cd}	34.2±5.5^{ab}	55.0±8.2^{ab}
18	425	Two step	0	At 30°C	5	62.0±10.4^{a}	12.4±5.4^{ab}	41.0±3.3^{a}	15	24.3±12.4^{cd}	37.6±6.5^{ab}	63.0±7.9^{a}
19	475	Slow	0	At 30°C	5	54.0±12.5^{ab}	14.6±6.8^{ab}	43.0±5.4^{a}	15	14.3±11.5^{e}	39.8±9.2^{a}	59.2±9.4^{a}
20	475	Two step	0	At 30°C	5	55.0±11.7^{ab}	16.4±5.3^{a}	43.2±8.1^{a}	15	22.0±8.0^{de}	36.4±8.1^{ab}	57.4±3.7^{a}
21	425	Slow	0	At 5°C	5	69.0±12.5^{a}	9.0±4.0^{b}	30.6±7.3^{b}	15	35.7±17.0^{ab}	34.0±8.9^{ab}	56.2±7.1^{ab}
22	475	Slow	0	At 5°C	5	45.0±13.7^{b}	14.2±5.7^{ab}	37.0±6.5^{ab}	15	33.0±12.5^{bc}	42.2±12.7^{a}	65.2±10.9^{a}

^{a,b,c,d} Values with different superscripts in the same column for different groups are significantly different ($P<0.05$).

Table 8. Comparison of *in vitro* fertilization results between groups.

Tablo 8. *İn vitro* fertilizasyon sonuçlarının gruplar arasında karşılaştırılması.

Group	Oocytes (n)	Fertilized (%)	Fertilized	
			Normal	Polyspermy
Fresh semen	28	26 (92.9)[a]	21 (80.8)	5 (19.2)
Group 15	22	21 (95.5)[a]	20 (95.2)	1 (4.8)
Group 21	29	14 (48.3)[b]	14 (100.0)	-

Table 9. Comparison of embryonic development results between groups.

Tablo 9. Embriyonik gelişim sonuçlarının gruplar arasında karşılaştırılması.

Group	Oocytes (n)	Cleaved rate (%)	Embryo				
			Degenerated Embryos	<8 Cell	8-16 Cell	Morula (%)	Blastocyst (%)
Fresh	72	68 (94.4)	2	10	19	29 (42.6)	8 (11.8)
Group 15	66	45 (68.2)	2	7	9	23 (51.1)	4 (8.9)
Group 21	36	28 (77.8)	-	7	3	15 (53.6)	3 (10.7)

Discussion

Ram semen has proven to be more difficult to cryopreserve than that of other farm animals **(Abdelhakeam et al., 1991; Liu et al., 1998).** Various extenders composition, cryopretective agents and cooling and freeze-thaw procedures have been described for cryopreservation of ram semen **(Abdelhakeam et al., 1991; Fiser et al., 1987; Gunay et al., 2006; Nur et al., 2010; Soylu et al., 2007)**. In the present study a series of experiments was design to optimize the ram semen cryopreservation with the objectives of different extender osmolarity, dilution time, dilution rate, cooling rate and presence of glycerol by monitoring post-thaw ram semen characteristics and fertilization. Fresh pooled semen motility, DA, and TMD obtained from the three experiments of the present study were in accordance with the results of some previous studies **(Gunay et al., 2006; Nur et al., 2010; Soylu et al., 2007).** The freeze-thaw process is detrimental to mammalian sperm viability, genome and functional integrity **(Hammadeh et al., 1999; Nur et al., 2010)**. The extenders composition assists in stabilizing the cell during the freezing and thawing process **(Kakadiya and Kavani, 1995; Liu et al., 1998)**. Placing mammalian semen in a high-osmolarity environment makes the spermatozoa lose water and shrink **(Liu et al., 1998)**. **Woelders et al. (1997)** had reported that media with high osmotic pressure provided better protection to sperm against cooling damage than low osmotic media. In the all group of the tree experiments it was observed that extender osmolarity have significant effect on post-thaw semen motility, DA and TMD generally and it could be concluded that extenders with high osmotic pressure (425 mOsm) is a better choice for ram semen freezing compared to extenders with low osmolarity.

For the groups of the experiment 1, pooled semen was diluted at 1:2 (semen:extender) rate and frozen without glycerol. Obtained semen parameters up to equilibration stage were similar ($P>0.05$). For the group 1 and 2 (350 mOsm), that were diluted at 30°C results in poor post-thaw semen characteristics compared to group 3-6 that diluted at 5°C firstly. For the extender osmolarity, the best post-thaw semen recovery were obtained in group 6 that diluted

with extender at 425 mOsm compared to 350, 375 and 400 mOsm. The first dilution time temperature had a significant influence on post-thaw semen motility and DA (P<0.001). Post-thaw semen recovery of the group 6 were better than finding of those **Nur et al. (2010)** and **Soylu et al. (2007)** frozen ram semen without glycerol.

The osmolarity of the first experiment were 350-425 mOsm and the glycerol rate were 0%. The osmolarity of the second experiment were increased (425-475 mOsm) due to failure of post-thaw semen recovery in experiment 1 and the freezing media were included 6% final glycerol rate in group 7-9. The osmolarity of the groups 7-12 was 425 mOsm, the group 13 was 450 mOsm and group 14 was 475 mOsm. The group 12 was processed as group 6; the best experiment 1, with extender B at 425 mOsm for comparison with the results of experiment 2. Similar results were obtained in both groups (Group 6 and 12). Extender osmolarity and presence of glycerol effected post-thaw semen recovery more than dilution time with extender A. Also second experiment indicated that glycerolization at 30°C did not alter post-thaw semen characteristics. These findings agree with **Salamon and Maxwel (1993)** and **Ali et al. (1992)**. Presence of glycerol in freezing media has an effect on post-thaw sperm motility (P<0.001), DA (P<0.01) and TMD (P<0.01). The beneficial effect of cryoprotectant-supplemented media on post-thaw mammalian semen has been reported in many studies (**De Leeuw et al., 1993; Nur et al., 2010; Salamon and Maxwell, 1993; Soylu et al., 2007**). **Nur et al. (2010)** reported that, presence of cryprotective agent in freezing media is obligatory for maintaining post-thaw cryosurvival of ram semen. In general, it was observed that exclusion of glycerol from freezing media results in reducing post-thaw motility and morphological integrity compared to semen frozen with glycerol in experiment group 2. Also the result of the experiment 2 showed that post-thaw DA and TMD were affected by extender osmolarity (P<0.001). Extender composition assists in stabilizing cells during the freezing and thawing process (**De Leeuw et al., 1993; Fiser et al., 1987**). Soylu et al. (**2007**) have reported that post-thaw sperm recovery was significantly better when sperm was frozen in hypertonic extender (400 mOsm) compared to low osmolarity (350 and 375 mOsm). In addition post-thaw semen motility (P<0.0001), DA (P<0.0001) and TMD (P<0.0001) of experiment 2 interact with extender osmolarity, cooling rate, glycerol rate, and dilution time with extender A and B.

For the groups of experiment 3, pooled semen were diluted at 1:3 (semen:extender). Post-thaw semen motility (P<0.001), DA (P<0.01) and TMD (P<0.001) were affected by extender osmolarity and glycerol, respectively. However, post-thaw semen characteristic were not affected by glycerolization time. **Fabbrocini et al. (2000)** have reported that post-thaw motility was better after late addition at 5°C of glycerol compared to at 30°C. **Ali et al. (1992)** have reported that glycerolization of ram spermatozoa could be done on any temperature with equally good results. Our results showed that glycerolisation at 30°C did not alternate post-thaw semen characteristics. These findings agree with **Salamon and Maxwell (1993)**.

Various extenders and cryopretective agents have been developed for the cryopreservation of mammalian semen (**Henry et al., 1993; Pérez-Pé et al., 2002; Schiller et al., 2000**). In the present study, we evaluated the effects of different extender osmolarity, dilution time and cooling rate on post-thaw ram semen characteristics frozen in a TRIS-based extender in combination with glycerol. **Nur et al. (2010)** have reported that freeze-thaw process is detrimental to post-thaw ram semen viability, as well as DNA and functional integrity. Although the glycerol was successful than the control group (glycerol omitted group) in maintaining post-thaw motility, presence of glycerol in freezing media negatively affects sperm morphology and DNA integrity and presence of cryprotective agent in freezing media is obligatory for maintaining post-thaw cryosurvival of ram semen. It was shown that media with high osmotic pressure provided better protection to sperm against cooling

damage than low osmotic media. In general, it was observed that exclusion of glycerol from freezing media result in reducing post-thaw motility and morphological integrity compared to semen frozen with glycerol in all experiment. **Soylu et** al. **(2007)** have reported that detrimental effect of glycerol on post-thaw semen carecteristics could be overcome by combining glycerol with sugars and by increasing the osmotic pressure of the extender used for semen cryoproreservation. We can conclude that an increased osmolarity of the freezing both and presence of cryoprotectants contribute to the protection of ram spermatozoa. These findings were in agreement with the results of **Woelders et** al. **(1997)** on bull semen and **Soylu et** al. **(2007)** on ram semen.

The cooling rates from + 30°C to + 5°C had a significant influence on post-thaw semen motility of the experiment 1 only. **Dhami et** al. **(1992)** have reported post-thaw motility of bull spermatozoa were influenced by cooling rate from +30°C to +5°C. **Bacinoglu et** al. **(2007)** have used the same cooling procedures and reported that the detrimental effect of glycerol on post-thaw semen motility was compensated by two step cooling rate regimes. In the present study, cooling rate from +30°C to +5°C has controversy effect on semen parameter up to equilibration time but not on post-thaw semen parameters. The best survival of human spermatozoa is obtained when the cooling velocity of room temperature up to 4/5°C is of 0.5 to 1.0°C/minute **(Henry et** al., **1993)**. The present study indicated that cooling rate from +30°C to +5°C was interacting with other studied factors (cooling rate, extender osmolarity, presence of glycerol, dilution time and dilution rates) on post-thaw semen parameters of all experiments.

The inability of differential staining to yield useful information on the fertilizing capacity of frozen-thawed semen conduct to the investigation of *in vitro* fertilization of oocytes as a method for quality assessment. In the present study frozen ram semen were evaluated by *in vitro* fertilization also. The best groups that frozen with media contained glycerol (Group 15) and that frozen without glycerol (Group 21), and fresh ram semen were used as a control group for *in vitro* fertilization. **Birler et** al. **(2002)** have reported that 88,3% percent cleavages were obtained from fresh ram semen fertilized sheep oocytes. The fertilization rate of fresh semen, Group 15 and group 21 were 92,9%, 95,5% and 48,3% and cleavage rates were 94,4%, 68,2% and 77,8%, respectively. Higher fertilization rates were obtained in fresh semen and Group 15 compared to Group 21 ($P<0.05$). Obtained data showed that frozen ram semen at high osmolarity without glycerol could be used for *in vitro* fertilization. There were no significant differences between groups for other observed fertility related parameters ($P>0.05$).

In conclusion, same factors as cooling rate, extender osmolarity, presence of glycerol, dilution time with extender A and **B**, and dilution rates interact on post-thaw semen parameters. Glycerol included freezing media improves post-thaw semen recovery. Also, increasing the extender osmolarity result in better post-thaw semen motility especially in the group frozen without glycerol. Post-thaw semen parameters were not affected by cooling and glycerol addition time regimes, generally.

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