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Effects of low molecular weight cryoprotectants on the post-thaw ram sperm quality and fertilizing ability

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ABSTRACT

The aim of the current study was to compare the cryoprotective effects of low molecular weight cryoprotectants 6% DMSO, 6% ethylene glycol, 6% 1,2 propanediol and 6% glycerol on the efficiency of ram semen cryopreservation and to test the *in vitro* fertilizing ability of frozen–thawed ram semen. Ejaculates with a thick consistency, rapid wave motion (3–5), and >75% initial motility were pooled. Sperm were diluted to a final concentration of 1/5 (semen/extender) in 6% glycerol, 6% DMSO, 6% ethylene glycol and 6% 1,2 propanediol using a two-step dilution method. Two hours equilibrated semen was frozen in 0.25-ml straws. As expected, the results of the current study showed that motility, HOST values and the rates of defective acrosomes in sperm were negatively affected by the cryopreservation procedure ($P < 0.01$). In conclusion, 6% DMSO had a deleterious effect on the post thaw ram sperm parameters and embryonic development. Ethylene glycol and 1,2 propanediol successfully protected acrosomal and DNA integrities. Glycerol provided a better cryoprotection to sperm motility and plasma membrane integrity. The results of *in vitro* fertilization, as assessed by the rate of blastocyst formation, in glycerol group were similar to 1,2 propanediol group.

1. Introduction

Cryopreservation is a destructive procedure for cell viability. During the freeze–thawing process, spermatozoa encounter a stressful status that includes osmotic stresses produced by adding and removing cryoprotectants, alterations in membrane lipids induced by shifting liquid crystalline to gel state, and intracellular and extracellular ice formation (Motamedi-Mojdahi et al., 2014). Intracellular ice crystallization during freezing process is the main cause of damage to the cells. In addition, sudden temperature changes such as cold shock, formation and dissolution of ice during the freezing thawing process affect the integrity and function of the acrosome, nucleus, mitochondria, axonema and plasma membrane (O'Connell et al., 2002; Nur et al., 2010). Therefore, the composition of extender, type of cryoprotective agents and optimum freezing and thawing rates are important factors for semen cryopreservation (Soylu et al., 2007; Rasul et al., 2007; Nur et al., 2010).

Glycerol (molecular weight: 92.10) is universally used for the cryopreservation of spermatozoa (Purdy, 2006; Rasul et al., 2007; Awad, 2011) and is commonly used for ram sperm (Silva et al., 2012). However, glycerol when used in high concentrations has some toxic effects and also may be the greatest osmotic damage on the plasma membrane because its permeability across the sperm plasma membrane is much lower than that of many other cryoprotectants (Guthrie et al., 2002; Awad, 2011). In addition, low-molecular-weight cryoprotectants such as dimethyl sulfoxide (DMSO), MW 78.13, ethylene glycol, MW 62.07, and 1,2 propanediol, MW 76.10, may prove less damage to spermatozoa as they equilibrate across the plasma membrane more readily than glycerol and will therefore induce much smaller and potentially less damaging cell excursions than glycerol (Moore et al., 2006; Awad, 2011; Silva et al., 2012).

The fertilization process involves complex biochemical and physiological events that cannot be measured solely by routine semen evaluation. The traditional evaluation of ejaculate quality has been based primarily on routine semen analyses (*i.e.*, motility, morphology, and acrosomal integrity), but these routine semen evaluations have a limited capacity for predicting the potential fertility of ejaculate (Kasimanickam et al., 2006). Therefore, advanced techniques for semen evaluation (*e.g.*, *in vitro* fertilization, cervical mucus penetration, DNA and plasma membrane integrity) should be implemented to increase the odds of accurate identification of high-quality sperm (Martí et al., 2008; Nur et al., 2010).

Considering the need to improve the quality of frozen ram sperm, the interest in identifying a more effective cryoprotectant

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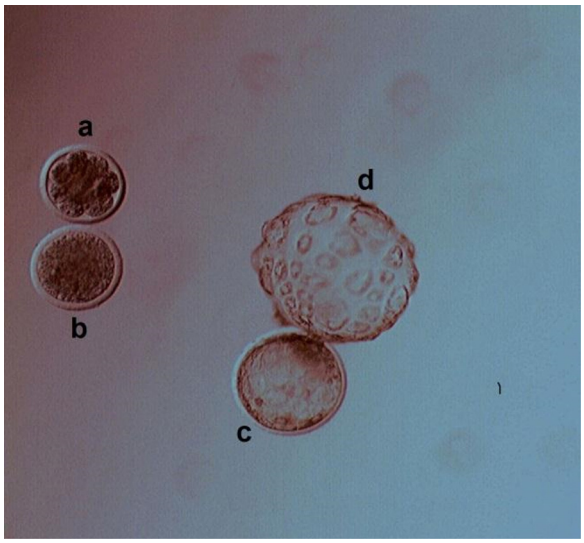

Fig. 1. Embryonic development on day 7.
a—8 cell stage, b—morula stage, c—blastosist stage (ekspanded), d—blastosist stage (hatched).

that can be used successfully during sperm cryopreservation arises. The aim of this study was to compare the cryopreservation effects of low molecular weight cryoprotectants DMSO, ethylene glycol and 1,2 propanediol glycerol, on post-thaw sperm parameters and embryo development after *in vitro* fertilization.

2. Material and methods

2.1. Animals and semen collection

Five rams (3–5 years old) maintained at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used as the sources of semen during the breeding season. Rams were maintained under uniform feeding and housing conditions also water was administered ad libitum. Semen was collected by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) (Nur et al., 2010). Ram semen was collected five times with a one-day interval between collections. Collected semen was placed in a warm water bath (30 °C) and immediately evaluated for consistency, wave motion (0–5 scale), and the percentage of motile spermatozoa (Nur et al., 2010). Ejaculates with a thick consistency, a rapid wave motion (3–5 on a 0–5 scale), and >75% initial motility were pooled (Fig. 1).

2.2. Semen dilution and freezing

Briefly, pooled ejaculates were diluted 1/2 (semen/extender) with extender A (27.1 g/l Tris (Sigma, USA), 10 g/l fructose (Sigma), 14 g/l citric acid (Merck, Darmstadt, Germany), 4 g/l penicillin G, 3 g/l dihydrostreptomycin and 20% egg yolk v/v) and than cooled to 5 °C within 60 min. The cooled semen was split into four equal aliquots. Each cooled sperm groups were then rediluted 1/1 (semen/extender) with cooled extenders B (extenders B, which were equal to extender A but supplemented with 38 g/l trehalose, 1.5 g/l EDTA (Aisen et al., 2005) and 6% (v/v) of one of the tested cryoprotectants: glycerol, DMSO, ethylene glycol or 1,2 propanediol in the final volume) (v/v). Extenders B were added in five steps at 10 min intervals in groups. The diluted samples were equilibrated at 5 °C for 120 min. Equilibrated semen was placed into 0.25 ml straws and frozen at 3 °C/min from +5 °C to −8 °C and at 15 °C/min from −8 °C to −120 °C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France).

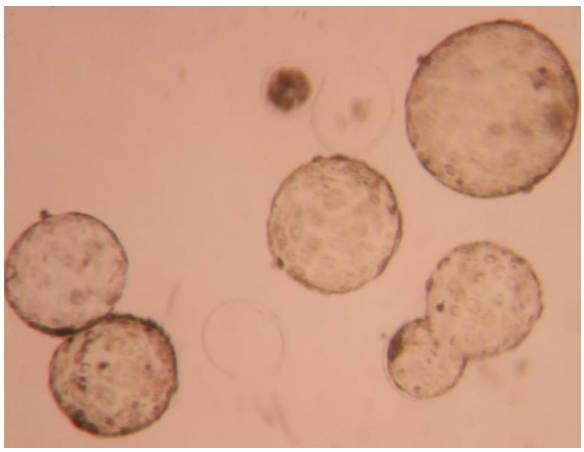

Fig. 2. Embryonic development on day 8.

The straws were then plunged into liquid nitrogen at −196 °C where they were stored for at least one month.

Three frozen semen straws from each group were thawed at 37 °C for 30 min a water bath to evaluate post-thaw semen motility, characteristics. All semen samples were frozen by the same person, and each of the studied semen parameters was evaluated by the same person on each occasion throughout the study (Fig. 2).

2.3. Semen evaluation

All semen parameters (motility, acrosome, morphology and plasma membrane integrity) were assessed at the following four time points: after dilution with extender A, at 5 °C, equilibration and post-thaw and also acrosome integrity (FITC PSA); and DNA fragmentation (TUNEL) were evaluated at post-thaw stage. Sperm motility was assessed subjectively using a phase-contrast microscope (Olympus BX51) (400X) with a warm slide (38 °C) (Soylu et al., 2007). Defected acrosome and other morphological defects "(OMD: head, midpiece and tail defects)" were assessed using the Giemsa staining method. At least 200 spermatozoa per smear were evaluated for morphological defects (Hafez, 1993).

2.4. FITC conjugated Pisum sativum agglutinin (FITC PSA)

Post-thaw acrosome integrity was assessed using by FITC PSA (Kawakami et al., 2002). Briefly, 20 μl of diluted semen was resuspended in 500 μl PBS and centrifuged at 800 × *g* for 10 min; the supernatant was then discarded. The spermatozoa pellet was resuspended in 250 μl PBS and one drop from resuspended spermatozoa was smeared on a glass microscope slide and air dried. Air-dried slides were fixed with acetone at 4 °C for 10 min, and the slides were covered with FITC PSA solution (50 μg/ml in PBS solution) in the dark room for 30 min. Stained slides were rinsed with PBS solution, covered with glycerol, and examined under a fluorescence microscope. At least 100 spermatozoa with a fluorescent acrosome were considered per smear and were evaluated for acrosome integrity.

2.5. The hypoosmotic swelling test (HOST)

Hypoosmotic swelling test was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 10 μl of semen with 100 μl of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) (Soylu et al., 2007) at 37 °C for 60 min. After incubation, 20 μl of the mixture was spread with a cover slip on a warm slide. 200 sperm cells were evaluated under magnification 1000× with phase-contrast microscopy. Sperm with swollen