Effect of Dimethylformamide on Post-Thaw Motility, Acrosome Integrity, and DNA Structure of Frozen Boar Sperm

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ABSTRACT

The beneficial effect of glycerol as a cryoprotectant, especially for sperm cryopreservation, has been shown in many studies. However, glycerol is toxic to living cells, and boar sperm in particular show greater sensitivity to glycerol than sperm from other domestic animals. Amides have been studied as alternative cryoprotectants for freezing stallion sperm. Sperm frozen in methylformamide or dimethylformamide as cryoprotectants show similar motility when thawed compared with sperm frozen in glycerol. We evaluated the cryoprotective effects of dimethylformamide on boar sperm freezing. To test the effect of amides, the concentration of boar semen was adjusted to 10^9sperm/mL, and seminal plasma was removed using Hulsen solution. After centrifugation, the pellet was diluted in modified-Modena B extender. Lactose-egg yolk (LEY) extender was used as the cooling extender. The freezing extender was madeed aaddition of the optimal amount of glycerol and amides to LEY-Glycerol-Orvus ES Paste extender, and this extender was used for the second dilution. Diluted sperm were frozen in liquid nitrogen using the 0.5 mL straw method. Sperm frozen in extender with glycerol as a cder ol were compared with those frozen in extender including the different amides. Sperm were tested for motility, viability, the sperm chromatin structure assay, and normal apical ridge after thawing. The percent of motile sperm diluted in glycerol was as high as that in the stallion study (61%). Dimethylformamide showed positive effects on sperm quality and was better than glycerol. Methylformamide provided similar sperm quality as glycerol. Therefore, dimethylformamide is useful for reducing cryoinjury in boar sperm and is expected to be useful as an alternative cryoprotectant.

(Key words : cryoprotectant, boar sperm, dimethylformamide, computer-assisted sperm analysis)

INTRODUCTION

Freezing and cold-storage of boar sperm are useful for preservation and reproduction of breeds and constitute an active area of research in the field of biotechnology (Bailey *et al.*, 2008; Grossfeld *et al.*, 2008; Medrano *et al.*, 2009; Rath *et al.*, 2009). However, there are several problems with this technology. During the freezing process, sperm get damaged by ice crystallization (Check *et al.*, 1994; Koshimoto and Mazur, 2002), osmotic disruption (Pukazhenthi *et al.*, 2000; Cotton *et al.*, 2010), and cytoplasmic disruption (Holt, 2000). Cryoprotectants are materials that decrease cryoinjuries. Glycerol is a cryoprotectant that has been shown to have beneficial cryoprotective effects and has been used in many studies examining sperm freezing (Kumar *et al.*, 2003). If the concentration of the original semen is too high, the semen is diluted in a specific extender, and the cryoprotectant is applied with the extender during the dilution process. However, glycerol has a harmful osmotic property to living cells (Hammerstedt and Graham, 1992; Squires *et al.*, 2004), and boar sperm in particular shows greater sensitivity to glycerol than sperm from other domestic animals (Fiser and Fairfull, 1990; Johnson *et al.*, 2000). In addition, use of high concentrations of glycerol has a negative impact on sperm quality (Buhr *et al.*, 2001; Grossfeld *et al.*, 2008; Purdy *et al.*, 2009).

The effect of amides on stallion sperm has been examined in another study (Squires, 2004). This study used skim milk-egg yolk (SMEY) extender, which is different than our extender, and tested six materials (methylformamide, dimethylformamide, formamide, acetamide, methylacetamide, glycerol) in-

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cluding five amides in the extender as cryoprotectants. This group evaluated the motility of thawed sperm after freezing them in one of the six cryoprotectants, which were each used at the same concentration. The motility of sperm frozen in methylformamide and dimethylformamide was similar to that of sperm frozen in glycerol. Thus, these two amides effectively reduce cryoinjury of stallion sperm. Our current study was done to compare sperm quality when boar sperm were frozen in these amides or in glycerol after thawing.

MATERIALS AND METHODS

1. Semen Collection

Semen was collected from mature Duroc boars (aged 12 to 36 months) using the gloved-hand method following the standard operating procedures at Kang-won University, Korea. The fertility of the boars and their semen quality has been previously demonstrated. Until the boars were used for daily sperm output, they were given days of sexual rest. Before the freezing process, the motility was examined, and the concentration for effective cryopreservation was determined. Only sperm with a motility greater than 80% were used. The concentration of the sperm was evaluated as follows: One milliliter of semen was diluted (1:400) in purified water, and the cells were counted three times using a hemocytometer.

The three counts were averaged as the final concentration of the semen. The optimal concentration of the semen was 10^9 sperm/mL, and was adjusted during the first dilution.

2. Cryopreservation and Thawing

The freezing method is different according to which extender is used. The extender we used was LEY-Glycerol-Orvus ES Paste (LEYGO) extender (89.5% LEY, 9% glycerol, 1.5% Equex-STM®, and 100 mM trehalose) and 0.55 M cryoprotectants (glycerol and amides). The collected semen was divided into four groups to be frozen in the four different cryoprotectants during the second dilution, and each group was classified precisely. After cooling to 15℃ over 1 hr, each of the four groups was diluted (1:3) with modified-Modena B extender (Lee *et al*., 2005), which is needed to remove the seminal fluid that is damaging during freezing. Diluted semen was centrifuged for 3 min at 2400 × g at 15℃ (Carvajal *et al*., 2004). The supernatant was discarded, and the remaining pellet was diluted in LEY extender to a concentration of 10^9/mL. The four groups were then cooled to 5℃ over 1 hr and diluted in the second extender, which included LEYGO extender and one of the four cryoprotectants (dimethylformamide, methylformamide, urea, or glycerol, each 0.55 M). The four tubes containing the resuspended sperm were loaded into 0.5-mL straws and placed 4 cm above LN_2vapor for 15 min before being plunged into liquid LN_2. Thawing of cells was done after storage in liquid LN_2. Cells in straws were thawed at 50℃ for exactly 15 s (Cordova-Izquierdo *et al*., 2006). Thawed samples were placed on slides to assess motility, viability, sperm chromatin structure using the sperm chromatin structure assay, and normal apical ridge.

3. Evaluation of Sperm Quality

1) Assessment of Sperm Motility

Motility and viability were assessed by computer-assisted sperm analysis (CASA, SAIS Automated Semen Analysis). The average pathway velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) were assessed in pre-warmed (37℃) condition as the sperm motility parameter.

2) Assessment of Normal Apical Ridge (NAR)

NAR reveals the rate of acrosomes that were undamaged after thawing of the sperm, and the degree of injury can be determined with this measurement. We used the Spermac stain for NAR, using the Spermac kit manufacturer's guidelines (Stain Enterprises, Onderstepoort, South Africa).

After smearing and drying thawed sperm, they were fixed in Fixative I containing formalin and then were stained with three steps: stain solution A for 5 min, stain solution B for 1 min, and stain solution C for 1 min. A total of 100 stained sperm cells were observed under a phase contrast microscope. Sperm with normal acrosomes are stained green and show a thin belt of dark-green color at the sperm head edge. On the other hand, damaged sperm are stained red, and the green belt on the head edge is cut. Using this procedure, we classified normal and damaged sperm. NAR was evaluated as the percentage of normal sperm with the above characteristics from the 100 sperm cells examined.

3) Assessment of Sperm Chromatin Structure (SCSA)

Samples of thawed boar sperm were placed on a glass slide and air dried. The slides were fixed with fixing fluid (Carnoy's fixative, 1:3 glacial acetic acid and absolute methanol) for 2 hr, and then stained with staining solution (10.0 mL 0.1%

(w/v) acridine orange (AO), 40 mL 0.1 M citric acid, and 2.5 mL 0.3 M sodium phosphate monobasic). The stained slides were incubated in a dark room for 5 minutes. AO-stained sperm were evaluated using a fluorescence microscope (Zeiss). Cells with intact DNA exhibit green staining over the head region. In contrast, abnormal sperm DNA emit yellow or red light. We evaluated 300 sperm/smear.

4. Statistical analysis

Statistical analysis for comparison of the differences were performed using Student *t*-test to test the significance at a level of $p<0.05$. Comparisons between each goup were performed with ANOVA (analysis of variance) test using the GraphPad Prism (for Windows, Release Prism 5, GraphPad Software Inc., USA). Data are reported as mean ± SD.

RESULT

1. Sperm Motility

The percent of motile sperm that had been frozen in dimethylformamide was higher than the percent frozen in other cryoprotectants ($p>0.05$). The use of urea resulted in a higher motility than glycerol. The percent of motile sperm that had been frozen in methylformamide was not significantly different than the percent frozen in glycerol, although it was slightly lower than glycerol (Table 1).

2. Normal Apical Ridge (NAR)

The NAR value of sperm frozen in all three amides was higher than the value of sperm frozen in glycerol (Table 2). As expected, NAR values of sperm frozen in dimethylformamide showed the highest value of the four groups. However, no significant difference ($p>0.05$) was observed between sperm frozen in dimethylformamide and those frozen in methylformamide. The NAR value of sperm frozen in urea was lower than the other two amides.

3. Sperm Chromatin Structure Assay (SCSA)

DNA damage was frequently observed in frozen and then thawed boar sperm. DNA fragmentation in sperm frozen in dimethylformamide indicated that this cryoprotectant provided the most positive effect among the four cryoprotectants, whereas there were no remarkable effects with methylformamide, glycerol, or urea (Fig. 1).

DISCUSSION

Glycerol has been used mainly for cell cryopreservation. Multiple studies have revealed its beneficial effect on sperm freezing by measuring after-thawing sperm qualities such as motility and viability (Bailey *et al*., 2008; Grossfeld *et al*., 2008; Rath *et al*., 2009). The efficacy of glycerol was better than other alternative cryoprotectants investigated. However, glycerol is harmful when used to freeze boar sperm, as glycerol is cytotoxic to living cells (Fahy, 1986; Grossfeld *et al*., 2008; Purdy *et al*., 2009).

In addition, boar sperm have distinct properties from the

Table 2. Effect of 4 different cryoprotectants on acrosome morphology

Cryoprotectant	Normal apical ridge (%)
	Total
Glycerol	62.90
Methylformamide	68.08
Dimethylformamide	70.31
Urea	59.59

Table 1. Effect of 4 cryoprotectants supplementation on sperm motility and on velocity parameters (means ± SD)

	Cryoprotectant			
	Glycerol	Dimetylformamide	Methylformamide	Urea
Sperm motility	97.68 ± 0.4	98.33 ± 0.14	97.96 ± 0.01	98.62 ± 0.02
VCL	412.37 ± 3.59	422.97 ± 12.18	403.04 ± 6.25	404.19 ± 8.07
VSL	152.22 ± 0.62	159.25 ± 6.01	146.38 ± 0.41	146.04 ± 5.09
VAP	176.06 ± 1.31	182.98 ± 6.97	169.41 ± 1.79	171.99 ± 6.69

*VCL (Curvilinear velocity), VSL (Straight-line velocity) and VAP (Average-path velocity).

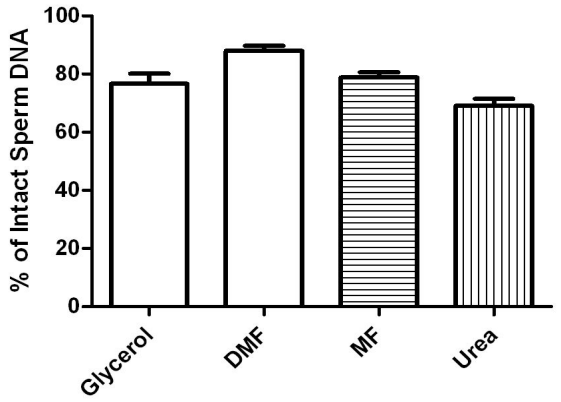

Fig. 1. The percentage of intact sperm DNA. Evaluation of sperm chromatin structure assay was conducted by acridine orange staining and observed by fluorescence microscope. (*DMF: dimethylformamide, MF: methylformamide)

sperm of other domestic animals. Sperm are produced in large volumes and are prone to damage from cold shock, sudden cooling stress, and cytotoxic materials. Because boar sperm have a greater sensitivity to the process of cryopreservation than the semen of other animals, specific complementary protocols for boar sperm are needed (Johnson *et al*., 2000; Grossfeld *et al*., 2008). In particular, boar sperm are highly sensitive to glycerol levels (Almlid and Johnson, 1988; Fiser *et al*., 1993).

Amides have long pairs of electrons, making amides effective cryoprotectants. In addition, because of the low molecular weight of amides compared to glycerol, amides can penetrate into cells easier than glycerol (Squires *et al*., 2004). The effect of amides was studied by Squires, 2004, who used stallion sperm. This group measured the quality of cryopreserved sperm, and showed that dimethylformamide and methylformamide were beneficial compared to glycerol. Then, the optimal concentration of urea for effective cryopreservation was examined. Similar to the Squires study, our results showed that dimethylformamide provided higher motility than any other cryoprotectant tested including glycerol. Thus, dimethylformamide has a positive effect on maintaining sperm Quality. Methylformamide provided a slightly lower motility value than glycerol, but this result was not significantly different among the different cryoprotectants. The high motility after thawing demonstrates the efficacy of the cryoprotectants and the freezing method because it indicates that sperm were not damaged by the freezing and thawing process. We used NAR to evaluate the damage level because it represents the rate of normal acrosomes after thawing. Damage to acrosomes leads to impaired fertility of sperm and is a negative measure of sperm quality (Graham *et al*., 1990; Gamer *et al*., 1994). The group with highest NAR value was the dimethylformamide group. The NAR value of sperm frozen in methylformamide was similar to that of sperm frozen in dimethylformamide. The NAR values of the urea group were similar to those from sperm frozen in glycerol, and no significant differences were found. We focused on the effect of dimethylformamide as a cryoprotectant and obtained promising results. The three amides (dimethylformamide, methylformamide, and urea) positively affected sperm quality, and in particular, dimethylformamide reduced damage from the freezing process. Therefore, we suggest the use of dimethylformamide for cryopreservation to minimize freezing damage to boar sperm and to obtain better results than glycerol.

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