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EFFECT OF THE PRIMARY COOLING RATE ON THE MOTILITY AND FERTILITY OF FROZEN-THAWED RABBIT SPERMATOZOA

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Abstract: In the present study, we examined the effect of primary cooling rates on the motility and fertility of frozen-thawed rabbit spermatozoa. Rabbit semen diluted with an egg-yolk acetamide extender was cooled from room temperature to 5°C at 4 different rates (–0.1, –0.2, –0.4, –0.8°C/min) as a primary cooling step, then semen was frozen in liquid nitrogen vapour. After thawing, sperm cooled at –0.1°C/min showed the highest motility (40.7±7.3%); there were no significant differences between the motilities of the –0.1, –0.2, and –0.4°C/min groups. The motility of frozen-thawed sperm cooled at –0.8°C/min (29.2±6.8%) was significantly lower than that of sperm cooled at –0.1 and –0.2°C/min. The viability (–0.1°C/min, 38.1±4.0%; –0.8°C/min, 24.3±7.3%) of frozen-thawed sperm was closely related to its motility (–0.1°C/min, 36.7±7.2%; –0.8°C/min, 22.3±4.7%). Quality of post-thaw motile sperm cooled at different rates was estimated by comparing the fertilisation ability of the –0.1 and –0.8°C/min groups following artificial insemination. There were no significant differences in pregnancy rates and mean litter sizes. These data suggest that cooling rabbit semen at rates ranging from –0.1 to –0.8°C/min affects the viability but not the fertilisation capacity of motile spermatozoa after thawing.

Key Words: rabbit, cryopreservation, cooling rate, motility, spermatozoa.

INTRODUCTION

Cryopreservation of sperm and embryos is becoming an important technique in domestic and laboratory animals for maintaining and preserving breeds on the verge of extinction. In rabbits, semen cryopreservation is a simpler and more economical alternative to embryo freezing. Until now, many protocols and extenders have been developed for cryopreservation of rabbit spermatozoa. However, frozen-thawed sperm cannot be used at a commercial level due to its decreased fertility and prolificacy compared to fresh semen (Mocé and Vicente, 2009). Sperm cryopreservation is important not only to preserve genetic resources, but also for transporting species between remote destinations. Transporting frozen semen instead of live rabbits is economical and avoids accidents such as animal death and escape (Liu *et al*., 2007). In view of the many potential future uses for cryopreservation of rabbit spermatozoa, it is essential to establish species-specific standard protocols.

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In general, rapid cooling and freezing of semen (cold shock) influences acrosome morphology and the percentage of post-thaw live and motile sperm (Blackshow, 1954; Gilmore *et al*., 1998; Watson, 2000). To avoid this severe irreversible damage, freezing semen in domestic animals requires a 2-step protocol (primary cooling and freezing) in which samples are suspended in an appropriate extender and gradually cooled from room temperature to 5°C, followed by freezing (Mocé and Vicente, 2002; Barbas and Mascarenhas, 2009). The appropriate conditions for cooling and freezing must be investigated for each species. Rabbit spermatozoa are characterised by their resistance to cold shock, so the primary cooling step during cryopreservation is usually 90-120 min in duration. Longer and shorter cooling times have been described (Mocé and Vicente, 2009), but the effects of different cooling rates on rabbit sperm have not been reported.

In this study, we examined the effect of the primary cooling rate on the motility and fertility of frozen-thawed rabbit spermatozoa. Our results will help establish an efficient protocol for semen cryopreservation in rabbits.

MATERIAL AND METHODS

Animals

Sexually mature male and female Japanese white (JW) rabbits (Japan SLC, Shizuoka, Japan) were housed individually in metal cages in a room maintained at a constant temperature (24±2°C), humidity (55±15%) and on a 12-h light-dark cycle (light on 08:00 to 20:00). They were fed 120 g commercial pellets (CRB-1; CLEA Japan Inc., Tokyo, Japan) per day and given free access to water. The experimental protocols were approved by the Saga University Animal Care and Use Committee, and performed according to the Saga University Guidelines for Animal Experimentation.

Semen collection

Semen was collected using an artificial vagina twice a week from one male rabbit (age, 9-14 mo); semen volume, sperm motility and concentration were measured using a haemocytometer. Sperm motility was defined as the percentage of moving sperm (total sperm-stationary sperm) in the semen sample. Semen with sperm motility >80% was used for further experiments.

Cooling and freezing procedure

The semen was diluted to 600×10^6 spermatozoa/mL with Tris-citric-glucose (TCG) buffer (Roca *et al*., 2000). When the sperm concentrations were $<600\times10^6$ cells/mL, the sample was centrifuged at $200\times g$ for 15 min (25°C) and adjusted to 600×10^6 cells/mL by discarding the supernatant. Semen was diluted 6-fold with an egg yolk-acetamide (EYA) extender (125 mM glucose, 105 mM lactose, 91 mM raffinose, 10 mM HEPES, 6% acetamide, 20% egg-yolk, 1000 U/mL penicillin G, 1000 U/mL streptomycin; pH 7.2; Chen and Foote, 1994; Dalimata and Graham, 1997). For the primary cooling step, sperm were cooled from room temperature (25°C) to 5°C at 4 different rates (–0.1, –0.2, –0.4, –0.8°C/min) using a thermo unit (CTU-N; TAITEC, Saitama, Japan), loaded into 0.5 mL-plastic straws (Fujihira Inc., Tokyo, Japan), and kept in a refrigerator at 5°C for 30 min. For the freezing step, all straws (3 to 10 at each time) were exposed to liquid nitrogen (LN_2) vapour (4-5 cm above the surface of the LN_2 level) for 15 min and then plunged into LN_2. Straws were thawed in a water bath at 37°C for 30 s, diluted with warmed TCG buffer (37°C), and used to investigate post-thaw sperm motility and viability.

Viability of frozen-thawed spermatozoa

The viability of post-thaw spermatozoa cooled at –0.1 and –0.8°C/min was compared using the propidium iodide (PI) staining method (Graham *et al*., 1990). Frozen-thawed sperm were stained with PI (12 μM) at 37°C for 5 min and observed under a fluorescence microscope. In each case, at least 300 sperm cells were observed; dead sperm appeared red.

Fertilisation capacity of frozen-thawed spermatozoa

We compared the fertilisation capacities of post-thaw spermatozoa cooled at –0.1 and –0.8°C/min. The experiment was performed twice. Semen was collected from 5 or 7 male rabbits for one experiment, pooled, and diluted 6-fold with EYA extender. Diluted semen was divided into 2 groups, cooled at –0.1 or –0.8°C/min, frozen using the previously described protocol, and kept in LN_2 until use. Artificial insemination (AI) was carried out with a glass inseminating pipette as previously described (Kaneda *et al*., 1993). Female rabbits (nulliparous does, 5-8 mo of age) were randomly divided into 2 groups not following the sexual receptivity (not classified according to colour and turgidity of the vulva). They were inseminated with 20×10^6 post-thaw motile sperm and simultaneously given 50 IU human chorionic gonadotrophin (hCG; Teikoku Hormone sMFG Co., Ltd, Tokyo, Japan) by intravenous injection to stimulate ovulation. Pregnancy rates and litter sizes were recorded.

Statistical analysis

All data are expressed as mean±standard deviation. Analysis of variance (ANOVA) was performed for multiple comparisons of motility at different cooling rates. The Tukey HSD post hoc test was applied when significant differences between cooling rates were observed by ANOVA. The pregnancy rate was analysed by Fisher's exact probability test and mean litter sizes were compared by Student's *t*-test. Significance was assumed at $P<0.05$.

RESULTS AND DISCUSSION

Characteristics of the fresh semen

Semen was collected from 17 male rabbits. The mean sample volume was 0.56±0.26 mL, the mean number of spermatozoa was $645.3\pm302.1\times10^6$/mL, and mean sperm motility was 84.7±3.6%.

Effect of primary cooling rate on motility of post-thaw spermatozoa

The time courses of the temperature changes controlled by the thermo unit are shown in Figure 1. In each case, temperatures decreased linearly from 25 to 5°C. The times needed to cool from 25 to 5°C at –0.1, –0.2, –0.4, and –0.8°C/min were 200, 100, 50, and 30 min, respectively.

Table 1: Mean±standard deviation of motility of frozen-thawed sperm cooled at different rates.

Cooling rate (°C/min)	No.	Motility (%)
–0.1	8	40.7±7.3[a]
–0.2	16	39.6±9.8[a]
–0.4	17	36.2±5.2[ab]
–0.8	17	29.2±6.8[b]

[a,b] There was a significant difference between the values with different superscripts in the same column ($P<0.01$).

Effects of the different cooling rates on the motility of the thawed spermatozoa are shown in Table 1. Post-thaw sperm cooled at –0.1°C/min showed the highest motility (40.7±7.3%). There were no significant differences between the motilities of frozen-thawed sperm cooled at –0.1, –0.2 and –0.4°C/min. Motility of post-thaw sperm cooled at –0.8°C/min (29.2±6.8%) was significantly (P<0.01) lower than that of post-thaw sperm cooled at –0.1 and –0.2°C/min. The post-thaw motility of the control semen packed into straws and cooled directly in cold water at 5°C was 16.1±6.4%; this was significantly lower than the motilities of the frozen-thawed sperm in all the groups cooled by the thermo unit (P<0.01-0.05).

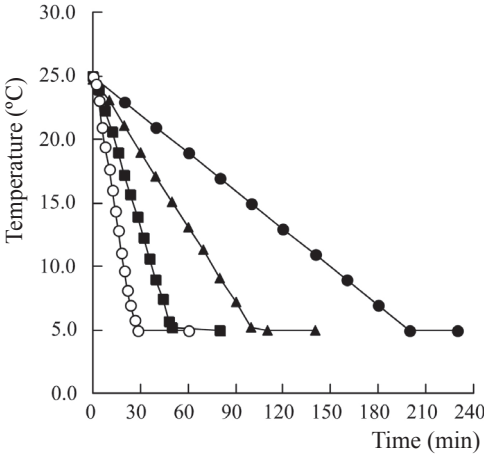

Figure 1: Time courses of temperature changes controlled by the cool thermo unit. As a primary cooling step, the tubes containing spermatozoa were cooled from room temperature (25°C) to 5°C at 4 different rates (–●–: –0.1, –▲–: –0.2, –■–: –0.4, –○–: –0.8°C/min).

Effect of primary cooling rate on viability of post-thaw spermatozoa

Post-thaw viability scores of spermatozoa in the –0.1°C/min group were significantly (P<0.01) higher than for sperm in the –0.8°C/min group (Table 2). The motility and viability of frozen-thawed sperm cooled at –0.8 and –0.1°C/min were closely related, indicating that the decreases in sperm motility associated with faster cooling rates were due to a decline in viability resulting from the freezing process.

Fertilisation capacity of post-thaw spermatozoa cooled at –0.1 and –0.8°C/min

To compare the quality of frozen-thawed motile sperm cooled at –0.1 and –0.8°C/min, we evaluated pregnancy rates and mean litter sizes after AI. There were no differences in pregnancy rates and mean litter sizes between the 2 groups. This experiment was done twice, and we found similar results with reproducibility. This suggests there was no difference in the fertilisation capacity of frozen-thawed motile sperm cooled at fast and slow rates, and that both had enough fertilisation ability for successful AI.

The motility of post-thaw spermatozoa can be influenced by several factors besides the cooling rate, kind of cryoprotectants, handling and breed of rabbit. Many researchers have studied the efficacy of different cryoprotectants for freezing rabbit sperm. We used an acetamide-based extender and a previously described method (Chen and Foote, 1994; Dalimata and Graham, 1997). Glycerol has been widely used as a cryoprotectant for several species, but is not effective

Table 2: Mean±standard deviation of motility and viability of post-thaw sperm cooled at –0.1 and –0.8°C/min.

Cooling rate (°C/min)	No.	Motility (%)	Viability (%)
–0.1	5	36.7±7.2[a]	38.1±4.0[a]
–0.8	5	22.3±4.7[b]	24.3±7.3[b]

[a, b] There was a significant difference between the values with different superscripts in the same column (P<0.01).

Table 3: Artificial insemination with post-thaw sperm cooled at –0.1 and –0.8°C/min.

Cooling rate (°C/min)	No. of inseminated	No. of sperm for AI (×10^6/doe)	No. of pregnant (%)	Mean litter size (Min-max)
–0.1	19	20	14 (73.7%)	4.5±3.1 (1-9)
–0.8	15	20	11 (73.3%)	3.9±2.8 (1-9)

AI, artificial insemination.

when freezing rabbit sperm; less than 2% of the oocytes recovered from does inseminated with rabbit semen frozen in the presence of glycerol were fertilised (Smith and Polge, 1950). Studies describing freezing techniques for JW rabbit sperm found that 1.0 M lactamide and acetamide were more suitable than 1.0 M glycerol and dimethyl sulphoxide (DMSO) as cryoprotectants (Kashiwazaki *et al*., 2006). DMSO, acetamide, and lactamide were found to be effective cryoprotectants for New Zealand white rabbit sperm (Hanada and Nagase, 1980); however, DMSO, ethylene glycol, and glycerol were not successful when freezing semen from Dutch Belted rabbits. The differing efficacies of the various cryoprotectants indicate that there are species-specific differences which must be accounted for when designing appropriate freezing protocols (Dalimata and Graham, 1997).

CONCLUSION

In this study, we examined the effect of 4 different cooling rates (from –0.1 to –0.8°C/min) on the motility and fertility of frozen-thawed rabbit spermatozoa and found that cooling rates affected the viability but not the fertilisation capacity of post-thawing motile spermatozoa. Therefore, a slow primary cooling step (slower than –0.4˚C/min is desirable) should be included in freezing protocols to maximise their efficiency.

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