Fertility of Spermatozoa Cryopreserved with 2% Acetamide or Glycerol through Artificial Insemination in the Japanese White Rabbit

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Abstract: The rabbit is considered to be a valuable laboratory animal. We compared 2% acetamide and glycerol as cryoprotectants in egg-yolk diluent for ejaculated Japanese white rabbit spermatozoa to improve sperm cryopreservation methods. Fertility through artificial insemination, forward progressive motility and plasma membrane integrity of the post-thaw spermatozoa were examined. The rates of forward progressive motility and plasma membrane integrity of the spermatozoa frozen with acetamide ($27.1 \pm 8.3\%$ and $24.5 \pm 6.5\%$) were significantly ($P < 0.05$) higher than those of the spermatozoa frozen with glycerol ($16.3 \pm 10.9\%$ and $14.3 \pm 7.6\%$). Though there was no significant difference in the kindling rates, the litter size of females inseminated with spermatozoa frozen with acetamide (6.0 ± 1.1) were significantly ($P < 0.05$) higher than those of spermatozoa frozen with glycerol (3.0 ± 0.4). The results indicate that 2% acetamide has a higher cryoprotective effect than 2% glycerol for sperm cryopreservation in the Japanese white rabbit.

Key words: acetamide, fertility, glycerol, Japanese white rabbits, sperm cryopreservation

Introduction

The Japanese white rabbit (JWR) established in Japan is considered to be a valuable laboratory animal for research in teratology, immunology, microbiology and life sciences. In addition, valuable mutants [13, 30] including transgenic [5, 12, 26, 31] have been estab-

lished in the JWR. The need for reliable methods to bank rabbit genetic resources efficiently in the form of cryopreserved spermatozoa is commonly recognized. To date, glycerol has been extensively used as a cryoprotectant for sperm cryopreservation, since Polge *et al.* [23] reported its cryoprotective properties. However, spermatozoa frozen with glycerol resulted in

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extremely low fertility in early studies on freezing of rabbit spermatozoa [6, 20, 25, 28]. Since 1980, research employing with acetamide [4, 8, 16, 21] as a cryoprotectant has been encouraging. Although the JWR is common as a laboratory rabbit breed in Japan, there is a little information on cryoprotectants for the freezing of JWR spermatozoa [16].

In the present study, we compared fertility through artificial insemination (AI) of JWR spermatozoa frozen with 2% (w/v) acetamide or 2% (v/v) glycerol to improve methods for sperm cryopreservation with the ultimate aim of banking genetic resources of the JWR.

Materials and Methods

Animals

Nine buck rabbits (Aizu Jikkendoubutsu, Fukushima, Japan) as semen donors and 16 females (Kitayama Labes; Kbl, Nagano, Japan) for AI were used in the present study. All the rabbits of the JWR breed were sexually mature. They were housed in individual cages, fed with a commercial diet and given water *ad libitum*. The Animal Care Committee of Azabu University approved protocols for the use of the animals.

Spermatozoa

Buck semen was collected with an artificial vagina two to three times per week from the nine males. Immediately after semen collection, any gel plug was removed using Pasteur pipettes and forceps. Ejaculated semen with more than 60% motile spermatozoa was used for sperm cryopreservation in the present study. Sperm motility was evaluated by placing a small portion of it into an examination chamber (FA255, Fujihira Inc., Tokyo, Japan) on a micro-warm plate (Kitazato, Shizuoka, Japan) at 37°C, and examined by light microscopy at 100×. Sperm motility was defined as the percentage of spermatozoa that showed any sperm head movement. Forward progressive motility was the percentage of spermatozoa that moved linearly. The number of spermatozoa in samples was determined in duplicate by direct microscopic examination with a hemocytometer (a Thoma chamber).

Sperm cryopreservation

The collected semen was put in a 10-ml centrifuge tube (Eikenkizai Inc., Tokyo, Japan), was diluted with

the same volume of Modena [33], and centrifuged at 180 g for 10 min at room temperature (22–24°C) to wash and remove seminal plasma. The sperm pellet was diluted to 2×10^8 sperm cells/ml with cryopreservation medium-I [18]. Cryopreservation medium-I contained 23% (v/v) egg-yolk, 8% (w/v) lactose monohydrate, and antibiotics (1,000 U/ml penicillin G potassium and 1 mg/ml streptomycin sulfate, Sigma, St. Louis, MO, USA) at pH 7.2 adjusted with 10% (w/v) Tris(hydroxymethyl)aminomethane (TRIS, Sigma) solution. The tube containing semen was placed into a 50-ml glass beaker containing 50 ml water at room temperature (22–24°C) and the glass beaker was incubated at 15°C for 1 h. Then the glass beaker was refrigerated at 5°C for 1 h. The sperm suspension in cryopreservation medium-I was cooled to 5°C, then added to the same volume of cryopreservation medium-II which had been cooled to 5°C. Cryopreservation medium-II consisted of cryopreservation medium-I with 1.4% (v/v) Equex Stm (Nova Chemical Sales, Inc., Scituate, MA, USA) and 4% cryoprotectant. Therefore, 2% acetamide (w/v) and glycerol (v/v) were compared for sperm cryopreserving. The spermatozoa in the mixed cryopreservation medium cooled to 5°C was loaded into 0.5-ml plastic straws (Fujihira Inc., Tokyo, Japan) and exposed to liquid nitrogen (LN) vapor at –160°C, 4 cm above the LN level for 15 min. The straws were kept on 0.2-mm wire mesh on a perforated Styrofoam float. The straws were then plunged into LN and stored for at least one week. Thereafter, straws were thawed in a 37°C water bath for 10 s. Thawed semen in the straw was transferred into a 1.0-ml centrifuge tube and then incubated at 37°C. The rate of forward progressive motile spermatozoa was then evaluated microscopically.

Plasma membrane integrity of spermatozoa

The plasma membrane integrity of frozen/thawed spermatozoa was determined using a commercially available test kit (Live/Dead Sperm Viability Kit, Molecular Probes, Inc., Eugene, OR, USA) which differentiates between sperm cells with intact plasma membranes and those that are damaged according to the fluorescent staining pattern observed with a fluorescence microscope (Olympus, BX-50, Tokyo, Japan) [10]. The nuclei of spermatozoa with intact plasma

membranes have green fluorescence (stained with SYBR-14), whereas damaged plasma membranes have orange-red fluorescence (stained with propidium iodide). At least 300 sperm cells were counted in duplicate for each sample under microscopy at 200 $\times$.

Artificial insemination (AI)

AI was carried out with a curved glass pipette whose caliber, outside diameter and length were 4.0 mm, 6.0 mm and 24.0 cm, respectively. The bend was produced by heating the pipette in a flame at 5.0 cm from one end. To induce ovulation, female rabbits received an intravenous injection with 100 IU of human chorionic gonadotropin (hCG; Puberogen, Sankyo Yell, Tokyo, Japan) at 7 h before AI was performed. All the females were inseminated with 50 millions of post-thaw motile spermatozoa cryopreserved as described above, with inseminated volumes with varying (1.5 to 2.5 ml of the semen per female) depending on the straws. Pregnant females were transferred to cages containing nest boxes. After delivery, the litter size and condition of the offspring were recorded.

Statistical analysis

Statistical analysis of the data was performed using Excel 2004 (Microsoft, Redmond, WA, USA) with the add-in software Statcel2 [34]. The data are presented as mean $\pm$ SEM. The post-thaw forward progressive motility and plasma membrane integrity were arcsine-transformed before analyses, and differences between mean values were analyzed by Student's *t*-test. The kindling rates were analyzed by Fisher's exact test.

Results

We compared fertility in AI, forward progressive motility, and plasma membrane integrity of the post-thaw JWR spermatozoa frozen in cryoprotectant containing 2% acetamide or glycerol.

As shown in Table 1, the rates of forward progressive motility and plasma membrane integrity of the spermatozoa frozen with acetamide ($27.1 \pm 8.3\%$ and $24.5 \pm 6.5\%$) were significantly ($P < 0.05$) higher than those of spermatozoa frozen with glycerol ($16.3 \pm 10.9\%$ and $14.3 \pm 7.6\%$). Before sperm cryopreservation, the average forward progressive motility and plasma membrane integrity were $68.6 \pm 3.0\%$ and $74.8 \pm 1.7\%$,

Table 1. The post-thaw forward progressive motility and plasma membrane integrity of spermatozoa frozen with 2% glycerol or acetamide in the Japanese white rabbit

Cryoprotective agent	Post-thaw forward progressive motility	Sperm plasma membrane integrity
2% acetamide	$27.1 \pm 8.3\%^a$	$24.5 \pm 6.5\%^a$
2% glycerol	$16.3 \pm 10.9\%^b$	$14.3 \pm 7.6\%^b$

a vs. b: Values with different superscripts in the same column are significantly ($P < 0.05$) different.

respectively.

Although there was no significant difference in the kindling rates, the litter size of females inseminated with spermatozoa frozen with acetamide (6.0 ± 1.1) were significantly ($P < 0.05$) higher than those of spermatozoa frozen with glycerol (3.0 ± 0.4) in Table 2.

Discussion

In the present study, fertility, forward progressive motility, and plasma membrane integrity of rabbit spermatozoa frozen with 2% acetamide were significantly higher than those of spermatozoa frozen with 2% glycerol. The results indicate that 2% acetamide has a higher cryoprotective effect than 2% glycerol in the cryopreservation of JWR spermatozoa. To date, the effects of some cryoprotectants on post-thaw sperm characteristics have been compared by Hanada and Nagase [8] and Dalimata and Graham [4]. In the former study, 1.0 M lactamide, acetamide and DMSO were highly cryoprotective, and 1.0 M glycerol resulted in extremely low motility after thawing in the New Zealand white rabbit. Smith and Polge [27] also reported that less than 2% of the oocytes recovered from females inseminated with rabbit semen frozen in the presence of glycerol were fertilized. Although glycerol has been used successfully to freeze spermatozoa from several species, it is not an effective cryoprotectant for sperm freezing in the laboratory rabbit. The discovery of the cryoprotective actions of glycerol has allowed cryopreservation of spermatozoa [24], and although hundreds of potential cryoprotectants have subsequently been tested, glycerol has remained, almost without exception, the cryoprotectant of choice for spermatozoa from almost all species, especially in domesticated animals [3].

Table 2. Artificial inseminations with sperm frozen with 2% acetamide or glycerol in the Japanese white rabbit

Cryoprotective agent	No. of inseminated	No. of Kindling (%)	Litter size (range)
2% acetamide	8	7 (87.5%)	6.0 $\pm$ 1.1 ^a (1–9)
2% glycerol	8	4 (50.0%)	3.0 $\pm$ 0.4 ^b (2–4)

There was no significant difference in the kindling rates between females inseminated with 2% acetamide and glycerol ($P>0.05$).

a vs. b: Values with different superscripts in the same column are significantly ($P<0.05$) different.

However, despite extensive experimentation on sperm cryopreservation to optimize its use, the basis of the cryoprotective properties of glycerol and precisely why it should be more effective than other cryoprotectants still remains unclear. Glycerol is a permeating cryoprotectant, and is able to pass the cell membrane in general. The recent identification of the water channel protein Aquaporin (AQP7), which also facilitates glycerol transport at the late spermatid stage in the rat testis [9], may provide a specific route for glycerol to permeate sperm cells. Curry [3] has shown that spermatozoa from many livestock species have high permeability to water with low activation energy and insensitivity to inhibition by mercuric chloride [1, 7, 32], membrane characteristics consistent with the presence of the AQP7 channel. It has been difficult to ascribe a physiological role to the high water permeability of the sperm membrane and if AQP7 is found to be widely expressed in livestock species it may be that the transport of glycerol and not of water is its primary function. However, rabbit spermatozoa have relatively lower water permeability with higher activation energy than other domesticated animals, parameters associated with the lipid phase rather than channel-mediated water transport [2]. The rabbit is also one of the few exceptions in which acetamide, DMSO, and lactamide, but not glycerol, are the preferred cryoprotectants for sperm cryopreservation. In the present study, acetamide was also more suitable for sperm cryopreservation than glycerol in the JWR. Taken together, it is thought that this might be due to a lack of AQP7 as glycerol transporter, although currently there is no direct evidence to support this.

The cryoprotective effects of glycerol to spermatozoa are most evident at higher concentrations in

livestock species [3]. On the other hand, glycerol is reportedly toxic to spermatozoa during freezing and thawing in the mouse and rat, which are species closely related to the rabbit (*lagomorph*). Mouse spermatozoa seem unable to withstand more than about 1.75% glycerol [22, 29]. Also, it has been reported that glycerol is toxic to spermatozoa of rats [18] and kangaroos [15] during sperm cryopreservation. Caput and cauda epididymidal kangaroo spermatozoa appeared to suffer from glycerol-induced cryotoxic damage [15]. It appears that even a very short exposure to glycerol at higher temperatures is enough to induce a glycerol-induced cryotoxic effect. In addition, glycerol has a marked anti-fertility effect on fowl spermatozoa [14] without causing obvious morphological damage or impairing motility. Therefore, an important step forward will be the identification of an alternative cryoprotectant that is less detrimental than glycerol. On the other hand, it is thought that specifically acetamide has high compatibility with water, and that acetamide has high penetration into spermatozoa in the rabbit.

It is well known that there are strain differences in the fertility of cryopreserved spermatozoa in the mouse [11, 19]. Although the C57BL/6 mouse strain is extensively used for generating transgenic mice, the fertility with the cryopreserved C57BL/6 spermatozoa is extremely low compared to other inbred mouse strains [17, 19]. To date, there has been no report of the breed/strain difference in the fertility of laboratory rabbits' post-thaw spermatozoa. Thus, we thought that the differences in fertility needed to be examined in the laboratory rabbit. Furthermore, researches in which concentrations and combinations of cryoprotectants for spermatozoa are examined, are needed to improve sperm cryopreservation in the laboratory rabbit.

In conclusion, 2% acetamide had higher cryoprotective effects than 2% glycerol in cryopreservation of JWR spermatozoa.

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