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—Full Paper—

Comparison of Glycerol, Lactamide, Acetamide and Dimethylsulfoxide as Cryoprotectants of Japanese White Rabbit Spermatozoa

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Abstract. The rabbit is considered to be a valuable laboratory animal. We compared glycerol, lactamide, acetamide, and dimethylsulfoxide (DMSO) as cryoprotectants in egg-yolk diluent of ejaculated Japanese white rabbit spermatozoa for improvement of sperm cryopreservation methods. Rabbit semen was frozen with 1.0 M glycerol, lactamide, acetamide, or DMSO in plastic straws. Forward progressive motility and plasma membrane integrity of the post-thaw spermatozoa were examined. The rate of forward progressive motile spermatozoa in lactamide (37.8 ± 3.0%) was significantly ($P<0.05$) higher than in glycerol (17.0 ± 3.3%). In addition, the rates of sperm plasma membrane integrity in lactamide and acetamide (35.9 ± 3.3% and 30.2 ± 3.0%, respectively) were significantly ($P<0.05$) higher than in glycerol (17.0 ± 2.6%). The results indicate that 1.0 M lactamide and acetamide have higher cryoprotective effects than 1.0 M glycerol for cryopreservation of Japanese white rabbit spermatozoa.

Key words: Acetamide, Cryopreservation, Glycerol, Lactamide, Rabbit spermatozoa

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The rabbit is considered to be a valuable laboratory animal for research in teratology, immunology, microbiology, and medical and life sciences. In addition, many valuable mutants [1, 2] including transgenic [3–6], have been established in the rabbit. Therefore, a need has been recognized for reliable methods to bank rabbit genetic resources efficiently in the form of cryopreserved spermatozoa. Glycerol has been extensively used as a cryoprotectant for various cells, including mammalian spermatozoa and embryos, since Polge *et al*. [7] reported its cryoprotective properties. In early research on freezing of rabbit spermatozoa, spermatozoa frozen with glycerol resulted in extremely low fertility [8–11]. After 1980, studies employing acetamide [12–15] or DMSO [12, 16–22] as a cryoprotectant have been encouraging. However, there is a little information on cryoprotectants when freezing Japanese white rabbit spermatozoa.

In the present study, we compared 1.0 M glycerol, lactamide, acetamide, and DMSO as cryoprotectants in egg-yolk diluent for ejaculated spermatozoa of the Japanese white rabbit. Forward progressive motility and plasma membrane

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integrity of frozen/thawed spermatozoa were examined to improve methods of sperm cryopreservation for the Japanese white rabbit with the ultimate aim of banking genetic resources.

Materials and Methods

Animals

In the present study, 9 buck rabbits were used as semen donors, and 2 female rabbits were used as teaser females. All the rabbits were sexually mature, of the Japanese white breed (Aizu Jikkendoubutsu, Fukushima, Japan), housed in individual cages, fed a commercial diet, and watered *ad libitum*. The Animal Care Committee of Azabu University approved the protocols for use of the animals.

Spermatozoa

Semen was collected from the 9 males using an artificial vagina two to three times per week. Immediately after semen collection, gel plugs were removed using Pasteur pipettes and forceps. Only ejaculated semen with more than 60% motile spermatozoa was frozen. Sperm motility was evaluated by placing a small portion of the sperm into an examination chamber (FA255, Fujihira Industry Co., Ltd., Tokyo, Japan) on a micro-warmplate (Kitazato Supply Co., Ltd., Shizuoka, Japan) at 37 C, and then it was 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 the samples was determined in duplicate by direct microscopic examination using a hemocytometer (Thoma chamber).

Cryopreservation of spermatozoa

The collected semen was transferred to a 10-ml centrifuge tube (Eiken Kizai Co., Ltd., Tokyo, Japan) diluted with the same volume of Modena [23], and centrifuged at 180 *g* for 10 min at room temperature (22–24 C) to wash and remove the seminal plasma. The sperm pellet was diluted to 2 × 10^8 sperm cells/ml with Modena and then re-suspended. Freezing medium-I [24] 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 room temperature water (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. After the sperm suspension in freezing medium-I was cooled to 5 C, it was added to the same volume of freezing medium-II which had also been cooled to 5 C. Freezing medium-II consisted of freezing medium-I and 2.0 M cryoprotectant. Consequently, 1.0 M glycerol, lactamide (2-hydroxypropionamido), acetamide, and dimethylsulfoxide (DMSO) were compared as cryoprotectants for cryopreserving spermatozoa. The spermatozoa in the mixed freezing medium cooled to 5 C were loaded into 0.5 ml-plastic straws (Fujihira Industry) 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. The straws were thawed in a 37 C water bath for 10 sec. The thawed semen in the straws was transferred into 1.0-ml centrifuge tubes 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 the frozen/thawed spermatozoa was determined using a commercially available test kit (Live/Dead Sperm Viability Kit, Molecular Probes, Inc., Eugene, OR, USA) that differentiates between sperm cells with intact plasma membranes and those that are damaged according to the fluorescent staining pattern observed using a fluorescence microscope (BX-50, Olympus, Tokyo, Japan) [25]. The nuclei of spermatozoa with intact plasma membranes fluoresced green (stained with SYBR-14), whereas damaged plasma membranes fluoresced orange-red (stained with propidium iodide). At least 300 sperm cells were counted in duplicate for each sample under microscopy at 200×.

Statistical analysis

The data are presented as means ± standard error of the mean. Statistical analysis of the data was performed using Excel 2004 (Microsoft, Redmond,

Table 1. The post-thaw forward progressive motility and plasma membrane integrity of spermatozoa from the Japanese white rabbit frozen with 1.0 M glycerol, lactamide, acetamide, or dimethylsulfoxide (DMSO)

Cryoprotective agent	Forward progressive motility of post-thaw sperm	Sperm plasma membrane integrity (%)
1.0 M glycerols	17.0 ± 3.3%[a]	17.0 ± 2.6% [a]
1.0 M lactamide	37.8 ± 3.0%[b]	35.9 ± 3.3% [b]
1.0 M acetamide	28.3 ± 3.8%	30.2 ± 3.0% [b]
1.0 M DMSO	25.3 ± 3.5%	27.0 ± 2.4%

The data are presented as means ± standard error of the mean (n=9).

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

WA, USA) with the add-in software Statcel2 [26]. One-way analysis of variance (ANOVA) was used to compare means among treatments. Post hoc multiple comparisons were made using Tukey's test.

Results

We compared the effect of 1.0 M glycerol, lactamide, acetamide, and DMSO as cryoprotectants on post-thaw forward progressive motility and plasma membrane integrity of the frozen/thawed spermatozoa of the Japanese white rabbit. As shown in Table 1, the rate of forward progressive motile spermatozoa frozen with lactamide (37.8 ± 3.0%) was significantly (P<0.05) higher than that of spermatozoa frozen with glycerol (17.0 ± 3.3%). In addition, the rates of plasma membrane integrity spermatozoa frozen with lactamide and acetamide (35.9 ± 3.3% and 30.2 ± 3.0%, respectively) were significantly (P<0.05) higher than that of spermatozoa frozen with glycerol (17.0 ± 2.6%).

Discussion

In the present study, the progressive forward motility and plasma membrane integrity of rabbit spermatozoa frozen with 1.0 M lactamide were significantly higher compared to those of spermatozoa frozen with 1.0 M glycerol. The results indicate that 1.0 M lactamide had a higher cryoprotective effect than 1.0 M glycerol in the cryopreservation of Japanese white rabbit spermatozoa. To date, the effects of some cryoprotectants on post-thaw sperm characteristics have been compared by Hanada and Nagase [12] and Dalimata and Graham [15]. In the study by Hanada and Nagase, 1.0 M lactamide, acetamide, and DMSO were highly cryoprotective for the New Zealand white rabbit, and 1.0 M glycerol resulted in extremely low motility after thawing. 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 successfully used to cryopreserve spermatozoa from several species, it was not an effective cryoprotectant for rabbit sperm freezing.

Since the discovery of the cryoprotective actions of glycerol allowed freezing of spermatozoa [28] and although hundreds of potential cryoprotectants have been subsequently examined, glycerol has remained the cryoprotectant of choice for spermatozoa from almost all species, especially in domestic livestock [29]. However, despite extensive experimentation on freezing spermatozoa to optimize its use, the basis of the cryoprotective properties of glycerol and precisely why it would be more effective than other cryoprotectants remains unclear. Glycerol is a permeating cryoprotectant that is able to cross the cell membrane in general. Recent identification of the water channel protein Aquaporin (AQP7), which also facilitates glycerol transport at the late spermatid stage in the rat testis [30], may provide a specific route for glycerol to permeate sperm cells. Curry [29] has also shown that spermatozoa from many domestic species have high permeability to water with low activation energy and insensitivity to inhibition by mercuric chloride [31–33]. These are 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 amongst domestic species, it may be that the transport of glycerol, not water, is its primary function. However, rabbit spermatozoa have relatively lower water permeability and a higher activation energy than other domestic animals [34]. The rabbit is also one of the few exceptions in which DMSO, lactamide and acetamide, but not glycerol, are the preferred cryoprotectants for sperm freezing. In the present study, lactamide was also more suitable than glycerol for rabbit sperm freezing. Taken together, it is thought that this might be due to a lack of AQP7 as a glycerol transporter, although there is currently no direct evidence to support this.

The cryoprotective effects of glycerol are most evident at higher concentrations in domestic livestock species [29]. On the other hand, glycerol is reportedly toxic to spermatozoa during cryopreservation in mice and rats, which are closely related species to the rabbit (*lagomorph*). Mouse spermatozoa seem unable to withstand more than about 1.75% glycerol [35, 36]. Also, it has been reported that glycerol is toxic to spermatozoa in rats during sperm cryopreservation [24]. In addition, glycerol has a marked antifertility effect on fowl spermatozoa [37] without causing obvious morphological damage or impairing motility. The sensitivity of spermatozoa to these toxic effects varies with the species. For instance, the susceptibility of boar spermatozoa to acrosomal damage at relatively low concentrations of glycerol may explain the poor fertility of frozen/thawed boar spermatozoa compared with more resistant bull spermatozoa. There is at present no plausible explanation for these species differences in toxicity and the damage caused by glycerol to spermatozoa.

On the other hand, it is thought that lactamide and acetamide have specifically high compatibility with water, and lactamide, acetamide, and DMSO had high penetration into the rabbit spermatozoa. Therefore, it is thought that lactamide and acetamide have high cryoprotective effects, and the post-thaw forward progressive motile spermatozoa and/or plasma membrane integrity obtained using them were higher compared to glycerol in the present study. Although the cryoprotective effect of DMSO for freezing Japanese white rabbit spermatozoa was not confirmed in the present study, this may have been due to the small amount of data in the present study.

It is well known that there are considerable strain differences in the fertility of frozen/thawed spermatozoa in mice [38, 39]. The C57BL/6 mouse strain is extensively used for generating transgenic mice, but fertility with cryopreserved C57BL/6 spermatozoa is extremely low compared to other inbred mouse strains [40]. To date, there are no reports in the rabbit of breed/strain differences in the fertility of post-thaw spermatozoa. Thus, we believe that the differences in fertility need to be examined in the laboratory rabbit. Further studies are needed that examine the concentrations and combinations of cryoprotectants for spermatozoa and the fertility of post-thaw spermatozoa through artificial insemination to improve sperm cryopreservation for the laboratory rabbit.

In conclusion, 1.0 M lactamide and acetamide have higher cryoprotective effects than 1.0 M glycerol when used as cryoprotectants in cryopreservation of Japanese white rabbit spermatozoa.

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