J Anim Reprod Biotechnol 2023;38:38-46
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.38.1.38

JARB
Journal of Animal Reproduction and Biotechnology
Check for updates

Original Article

Sperm motility and viability of experimental animals using different cryopreservatives

Won Yong Park[1,2,§] and Byoung Boo Seo[1,*]

[1]Department of Animal Science, Daegu University, Gyeongsan 38453, Korea
[2]Preclinical research center Daegu-Gyeongbok Medical Innovation Foundation (DGMIF), Daegu 41061, Korea

Received February 22, 2023
Revised March 18, 2023
Accepted March 20, 2023

***Correspondence**
Byoung Boo Seo
E-mail: bbseosd@daegu.ac.kr

[§]Current affiliation: Liora Fertility Center, Busan 47245, Korea

Author's Position and Orcid no.
Park WY, MS,
https://orcid.org/0000-0001-6254-9224
Seo BB, Professor,
https://orcid.org/0000-0002-1344-2145

ABSTRACT In this study, an experiment was conducted in order to determine what cryopreservatives (CPVs) were more effective in supporting the motility and viability of sperm from experimental animals. The sperm of mice, rats, beagle dogs, and rabbits were frozen using different CPVs, including DMSO, TYB, and Sperm CryoProtec. The results from freezing the sperm of each laboratory animal in Sperm CryoProtec showed a high level of sperm motility and viability in sperm samples from mice, rats, and beagle dogs melted at the end of the first week. For rabbits, a high level of motility was observed in sperm thawed during the first week, whereas a high level of viability was observed in sperm thawed during the second week. The results of analysis of sperm motility and viability using different CPVs according to laboratory animals showed a significantly higher level of sperm motility (26.28%) and viability (36.20%) for mice in Sperm CryoProtec and the lowest levels of motility and viability were observed in DMSO ($p < 0.05$). Significantly higher levels of motility (27.94%) and viability (37.94%) were observed for rats in Sperm CryoProtec compared with TYB, which showed the lowest levels of motility and viability ($p < 0.05$). The study findings described above suggest that the selection of appropriate cryopreservatives is required for each experimental animal. This is because there are differences in the levels of sperm motility and viability of experimental animals depending on the CPVs that are typically used for freezing human sperm, including Sperm CryoProtec and TYB.

Keywords: cryopreservatives (CPVs), sperm cyroprotec, sperm motility, sperm viability, test yolk buffer (TYB)

INTRODUCTION

Fertility rates have continued to show a dramatic decline worldwide, including South Korea. According to data from Statistics Korea, the average fertility rate dropped to 1.1 births between 2015 and 2020, the lowest reported birth rate among OECD countries. Greece, Japan, and Canada have reported some of the lowest fertility rates; the increasing number of couples with infertility and subfertility problems is one of the reasons for low fertility. Masoumi et al. (2015) reported that the causes of infertility were female etiological factors in 30-55%, male etiological factors in 20-40%, and unknown etiology in 5-15%. The causes of male infertility were semen disorders in 44.6%, genetic factors in 29.8%, vascular disorders in 17.2%, and spermatogenesis agents in 11%. Consequently, cryopreservation of healthy sperm cells with suitable motility or embryos created by *in-vitro* fertilization has been

increasingly utilized for preservation of fertility (Vanden Meerschaut et al., 2014; Mocé et al., 2016; Bosch et al., 2020).

Different cryopreservatives (CPVs) are used for freezing sperm. Among them, dimethyl sulfoxide (DMSO) is the cryoprotective agent used most often in the cryopreservation of a variety of cell types including sperm. However, because DMSO is considered toxic to cells, cell damage might occur during the cryopreservation process (Kim et al., 2015).

Flow of intracellular water out of cells occurs by osmosis and formation of ice crystals during cooling may cause significant damage to sperm cells. Therefore, rather than using a single cryopreservative agent, CPVs are typically composed of more than one cryoprotectant including sucrose, ethylene glycol, glycerol, and ficoll (Mochida et al., 2013). Higher survival of sperm has been reported with use of Sperm CryoProtec, a glycerol and glycogen-based cryopreservative solution, compared with low temperature cryopreservation (McGonagle et al., 2002). Use of a combination of egg yolk resulted in increased viability of sperm compared with use of glycerol alone. Better motility of sperm was obtained after freezing and thawing with use of Test yolk buffer (TYB), a CPV composed of egg yolk and glycerol, compared with use of glycerol alone. According to the World Health Organization (WHO)'s list for use in evaluation of morphological abnormalities, a higher number of amorphous and round head defects were observed along with a lower number of abnormal tail defects with use of TYB than with glycerol (Hallak et al., 2000). A variety of CPVs have been developed and are being utilized in different types of subjects including humans, experimental animals, livestock, companion animals, and others. CPVs are most commonly applied to livestock such as cows (Guthrie et al., 2002; Magnusson et al., 2008; Blässe, et al. 2012), swine (Kwon et al., 2004; Choi et al., 2007; Kang et al., 2014), horses (Ball and Vo, 2001; Mantovani et al., 2002), chickens (Choi et al., 2012), and goats (Kim et al., 2006; Seifi-Jamadi et al., 2017).

Despite accumulation of a large amount of experimental data from studies on humans and livestock, only a small amount of data has been obtained from experiments using laboratory animals such as dogs and rabbits, excluding mice and rats. Conduct of studies on cryopreservation of dog sperm from popular and significant breeds is expected to show a gradual increase. However, data on dogs are rare because obtaining IACUC approval for use of dogs in experiments is difficult due to the Animal Protection Act and concern for animal welfare. Rabbits have been commonly used in development of defect models such as calvarian defect, articular defect, and anterior cruciate ligament (ACL) defect models, but are rarely used in experiments on cryopreservation of sperm.

The aim of this study was to examine sperm motility and viability by thawing frozen sperm at weekly intervals after cryoperservation of sperm from experimental animals using different CPVs including Sperm CryoProtec, TYB, and DMSO.

MATERIALS AND METHODS

Sentinel animals

Twelve-week-old male ICR mice (Hyochang Science, Korea) and Sprague-Dawley (SD) rats (Hyochang Science, Korea) were used in this study. All experiments using mice and rats were conducted in accordance with the regulations of the Daegu University Institutional Animal Care and Use Committee (DUIACC-2019-010-0221-009). All animals were allowed an adaptation period of one week from the day of their arrival in the laboratory. The animals were housed under a 12-hr light/12-hr dark lighting schedule (lights automatically turned on at 07:00) in a controlled environment at a temperature of 21-24℃ and humidity of 25-30%. Two-year-old male Dogs (beagle) and twelve-month-old male rabbits (NZW) left as surplus animals after conduct of experiments were used in the Laboratory Animal Center of Daegu Gyeongbuk Medical Innovation Foundation (DGMIF) Dog (Beagle) IACUC Approval No.: DGMIF-19121701-00, Rabbits (NZW) IACUC Approval No.: DGMIF-19111203-00). Each animal experiment was performed in 3 replicates.

Culture medium

The basic culture medium used in this study was Ham's F-10 (Sigma-Aldrich) mixed with 20% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 1.5 mL of 40 mg/mL gentamicin (Shin Poong Pharm. Co., Ltd, Korea). The CPVs used included Sperm CryoProtec (Nidacon Co. Ltd, Sweden), TYB (Fujifilm Irvine Scientific, Inc. USA), and DMSO (Sigma-Aldrich). PureSperm CryoProtec 90 and 40 (Nidacon Co. Ltd), Sperm Washing Medium, and Isolate (Fujifilm Irvine Scientific) media solutions were

used for washing the sperm pellet.

Pre-culture

The day before the experiment, the cells were added to a 2-well dish (Fujifilm Irvine Scientific) containing 3 mL of Ham's F-10 and coated with mineral oil (Sigma-Aldrich); the plate was incubated at 37℃ for 18 hours for activation in a 5% CO_2 incubator (Thermo Fisher Scientific, Inc. USA).

Sperm collection

Collection of semen samples from mice and rats was performed in the Cell Culture Room, College of Natural and Life Sciences of Daegu University, and from dogs and rabbits in the Preclinical Research Center, Laboratory Animal Center of DGMIF. Following anesthetization of mice and rats with 240 mg/kg of Avertin (Aldrich chemical Co. USA), the epididymis was removed and precultured in a 2-well dish using the method reported by Nakagata et al. (2020); the sperm pellet of the cauda epididymis was clipped using iris scissors (Nopa, Ac 450/10) to allow sperm to swim out for 15 min at 37℃ in a 5% CO_2 incubator. Then, 2 μL of the sperm sample was placed on a Makler counting chamber (Fujifilm Irvine Scientific) for determination of sperm motility and viability. Dogs and rabbits were anesthetized by intravenous (IV) administration of Zoletil (Virbac S.A. France) and Rompun (Bayer Healthcare, Germany). Dogs were euthanized by injection of 1 mL/kg of pentothal sodium (Choongwae Pharmaceutical, Korea) through an intravascular catheter (22G, Korea Vaccine, Korea). Rabbits were induced with anesthesia and euthanized by intracardiac injection of 3 mL/kg of KCl (Jeil Pharmaceutical, Korea). The cauda epididymis was isolated, and blood and fat were removed by washing with 37℃ saline solution (Dai Han Pharm Co. Ltd., Korea). The sperm sample was collected according to the same procedures used in mice. Sperm showing viability at 80% or above were used in this study.

Freezing

Sperm CryoProtec mixed in a 1:1 dilution with semen was added to cryovials, which were then sealed (Corning Co. Ltd., USA), and refrigerated at 4℃ for 20 min. The sample was then stored frozen in liquid nitrogen after pre-freezing at -4℃ under liquid nitrogen steam for 10 min. TYB mixed in a 1:1 dilution with semen was added to conical tubes (Corning Co. Ltd.) and mixed gently for 30 sec to avoid formation of bubbles. The mixture remained in the stationary phase for 10 min and was then stored frozen at -70℃. DMSO mixed in a 1:1 dilution with semen was loaded into cryovials, held stationary in the jar, and stored at -70℃.

Thawing

1) Sperm CryoProtec

Sperm CryoProtec remained in the stationary phase for 30 min by addition of 0.3 mL of PureSperm Wash 90 and 40 to conical tubes. Then, 6 mL of Ham's F-10 were added to a Petri dish (Doowon Meditech Co., Ltd., Korea), followed by activation for 30 min with the cover lid open. Frozen cryovials were thawed for 2 min in a 37℃ water bath for measurement of sperm viability. Semen adhering to the lid and the inside wall surface of cryovials was collected using 1 mL of Ham's F-10 and loaded into conical tubes containing PureSperm Wash. The conical tubes were centrifuged (Effendorf, Germany) at 1,300 rpm for 20 min. A 1 mL portion of the pellet was transferred to new conical tubes and mixed with 4 mL of Ham's F-10. These tubes were centrifuged at 1,300 rpm for 15 min, followed by addition of 0.2 mL of Ham's F-10 above the pellet so as not to disturb the culture media after isolating the supernatant. Swim-up was conducted for 30 min at 37℃ in a 5% CO_2 incubator by tapping the pellet at the bottom of the tube with the lid slightly open.

2) TYB

Cryovials of TYB freezing media were thawed at room temperature for 15 min, followed by placement of sperm in the isolated layer and loading with 2 mL of sperm washing medium or Multipurpose Handling Medium (Fujifilm Irvine Scientific). After centrifugation at 1,300 rpm for 15-20 min, the supernatant was carefully removed by leaving the pellet untouched and the pellet was pipetted and transferred to a new tube. Following addition of 2-3 mL of sperm washing medium, the tube was centrifuged at 1,300 rpm for 8-10 min to isolate the supernatant. Again, 2-3 mL of sperm washing medium was added to the tube and the supernatant was removed after centrifugation at 1,300 rpm for 8-10 min.

3) DMSO mixtures

Semen samples frozen in DMSO were thawed for 2 min

in a 37℃ water bath, and 1 mL of Dulbecco's Phosphate Buffered Saline (D-PBS, Sigma-Aldrich) was loaded into a 15 mL conical tube. After centrifugation at 1,500 rpm for 10 min, the supernatant was removed. The sample was suspended in 0.3 mL of Ham's F-10 and swim up was allowed for 30 min at 37℃ in a 5% CO_2 incubator.

Measurement of sperm motility and viability

Using the sperm pellet collected from the epididymis via swim-out, 2 μL of the sperm sample was placed in a Makler counting chamber. Measurement of motility was performed only in sperm located in the counting chambers. The equation for calculation of motility is as follows:

$$\text{Sperm motility (\%)} = \frac{\text{Total motile sperm count}}{\text{Total sperm count}} \times 100$$

For evaluation of sperm viability, 10 μL of semen was mixed with 10 μL of Eosin Y stain (Sigma-Aldrich) and dried on a slide glass. The specimen was observed under a microscope, and spermatozoa showing red staining in the head was considered dead and remained unstained while alive (Fig. 1). For determination of sperm viability, sperm counting was repeated three times in each vial by dividing semen obtained from the epididymis of a single animal into three vials, and the average number of sperm counted was used.

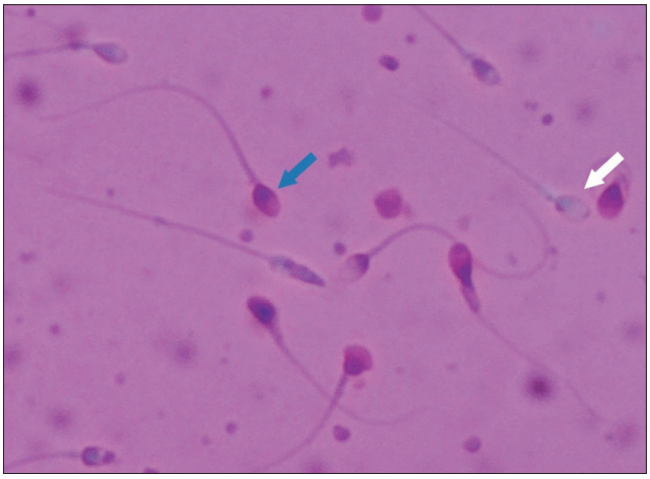

Fig. 1. Eosin Y Staining of beagle sperm (× 200). *white arrow: live sperm, blue arrow: death sperm.

Statistical analysis

Standard deviation (± SD) of discontinuous variables and significance were determined using SAS (statistics analytical system, version 9.4, USA). Testing of significant difference between the treatment groups was performed using Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Motility and viability of sperm from experimental animals thawed at weekly intervals after freezing in Sperm CryoProtec

Sperm samples from mice, rats, dogs, and rabbits were frozen in Sperm CryoProtec and thawed at weekly intervals over four weeks from the first day of the experiment. Sperm motility and viability are shown in Table 1.

The highest levels of sperm motility were detected during the first week of thawing at 31.58%, 37.50%, 23.53%, and 26.47% in mice, rats, dogs, and rabbits, respectively ($p < 0.05$), and showed a gradual decrease over time; the lowest level of motility was observed during the fourth week ($p < 0.05$).

Significantly higher levels of sperm viability were ob-

Table 1. Motility and viability of sperm after thawing of frozen experimental animals sperm using sperm CryoProtec

Sperm activation	Species	1 week	2 week	3 week	4 week
Motility	Mouse (n = 12)	31.58^{a}	27.94^{b}	26.67^{b}	18.94^{c}
	Rat (n = 16)	37.50^{a}	27.00^{b}	26.32^{b}	20.00^{c}
	Dog (n = 3)	23.53^{a}	20.02^{b}	19.61^{b}	15.22^{c}
	Rabbit (n = 6)	26.47^{a}	21.69^{b}	18.18^{c}	12.45^{d}
Viability	Mouse (n = 12)	37.94^{a}	36.58^{a}	36.67^{a}	33.94^{b}
	Rat (n = 16)	47.50^{a}	37.94^{b}	36.32^{b}	30.00^{c}
	Dog (n = 3)	39.61^{a}	28.53^{c}	35.02^{b}	20.22^{d}
	Rabbit (n = 6)	33.18^{b}	36.47^{a}	31.69^{c}	22.45^{d}

$^{a-d}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

served during the first to third weeks in mice and the first week in dogs but diminished progressively over time ($p < 0.05$). The highest level of viability was detected in rabbits at 36.47% during the second week but showed a significant decrease during the third and fourth weeks at 31.69% and 22.45%, respectively ($p < 0.05$).

Motility and viability of sperm from experimental animals thawed at weekly intervals after freezing in TYB

Sperm samples from mice, rats, dogs, and rabbits were frozen using TYB and thawed at weekly intervals for four weeks from the first day of the experiment. Sperm motility and viability are shown in Table 2.

Significantly high levels of sperm motility, ranging between 21.50–21.74% in mice and 24.00–25.50% in rats ($p < 0.05$), were detected during the first and second weeks, with the highest level at 35.29% in dogs and 40.91% in rabbits ($p < 0.05$). The highest level of sperm viability was observed during the first week in all livestock species ($p < 0.05$), which decreased to the lowest level during the third week (28.42%) in mice, the fourth week (17.50%) in rats, the second week (19.29%) in dogs, and the fourth week (27.55%) in rabbits ($p < 0.05$).

Motility and viability of sperm from experimental animals thawed at weekly intervals after freezing in DMSO

Sperm samples from mice, rats, dogs, and rabbits were frozen using DMSO and thawed at weekly intervals for four weeks from the first day of the experiment. Sperm motility and viability are shown in Table 3.

Significantly high levels of sperm motility were detected during the first week in rats (33.33%) and rabbits (31.74%) ($p < 0.05$), and between the third and fourth weeks in mice (18.79–20.00%) and during the third week in dogs (31.50%) ($p < 0.05$).

A significantly high level of sperm viability was detected between the second and third weeks in mice (28.79–30.00%), between the first and second weeks in rats (37.50–38.33%), during the first week in dogs (41.50%), and between the first and third weeks in rabbits (35.50–36.77%) ($p < 0.05$). The lowest level of viability was observed during the fourth week regardless of livestock spe-

Table 2. Motility and viability of sperm after thawing of frozen experimental animals sperm using TYB

Sperm activation	Species	1 week	2 week	3 week	4 week
Motility	Mouse (n = 12)	21.74^{a}	21.50^{a}	16.67^{b}	18.42^{b}
	Rat (n = 16)	24.00^{a}	25.50^{a}	12.50^{c}	21.05^{b}
	Dog (n = 5)	35.29^{a}	15.75^{bc}	16.63^{b}	14.29^{c}
	Rabbit (n = 7)	40.91^{a}	31.88^{b}	33.33^{b}	17.55^{c}
Viability	Mouse (n = 12)	41.74^{a}	31.67^{b}	28.42^{c}	31.50^{b}
	Rat (n = 16)	41.05^{a}	35.50^{b}	29.00^{c}	17.50^{d}
	Dog (n = 5)	50.29^{a}	19.29^{d}	21.63^{c}	25.75^{b}
	Rabbit (n = 7)	50.91^{a}	43.33^{b}	41.88^{b}	27.55^{c}

$^{a-d}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

Table 3. Motility and viability of sperm after thawing of frozen experimental animals sperm using DMSO

Sperm activation	Species	1 week	2 week	3 week	4 week
Motility	Mouse (n = 6)	15.41^{b}	11.59^{c}	20.00^{a}	18.79^{a}
	Rat (n = 4)	33.33^{a}	27.50^{b}	25.30^{c}	19.57^{d}
	Dog (n = 3)	21.00^{c}	28.61^{b}	31.50^{a}	17.54^{d}
	Rabbit (n = 3)	31.74^{a}	25.50^{b}	11.46^{c}	26.77^{b}
Viability	Mouse (n = 6)	21.41^{b}	30.00^{a}	28.79^{a}	21.59^{b}
	Rat (n = 4)	38.33^{a}	37.50^{a}	35.33^{b}	19.47^{c}
	Dog (n = 3)	41.50^{a}	33.61^{b}	31.00^{c}	31.54^{c}
	Rabbit (n = 3)	36.74^{a}	35.50^{a}	36.77^{a}	21.46^{b}

$^{a-d}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

Table 4. Motility and viability after thawing of frozen laboratory animal sperm using different cryopreservatives

Treatment	After thawing laboratory animal sperm activation (%)							
	Motility				Viability			
	Mouse	Rat	Dog	Rabbit	Mouse	Rat	Dog	Rabbit
Sperm[1)]	26.28a	27.94a	19.59b	19.69c	36.20a	37.94a	30.84b	30.94b
TYB	19.58b	20.76b	20.49b	30.91a	33.33b	30.76b	29.24b	40.91a
DMSO	16.44c	26.43a	24.66a	23.86b	25.44c	32.68b	34.41a	32.61b

[a-d]Means with the different superscripts in the same columns are significantly different at $p < 0.05$ by Duncan's multiple range test.
[1)]Sperm CryoProtec.

cies ($p < 0.05$).

Motility and viability of sperm from experimental animals thawed at weekly intervals after freezing in different CPVs

Sperm samples from laboratory animals were frozen using different CPVs including Sperm CryoProtec, TYB, and DMSO, and thawed at weekly intervals. The average post-thaw motility and viability are shown in Table 4.

Motility and viability of 26.28% and 36.20% were detected in mouse sperm frozen using Sperm CryoProtec, with a significantly higher level of viability compared with other CPVs ($p < 0.05$). A significantly higher level of motility of rat sperm was detected in Sperm CryoProtec with 27.94% and in DMSO with 26.43% ($p < 0.05$). The highest level of viability was detected in Sperm CryoProtec with 37.94% ($p < 0.05$). Motility and viability of 24.66% and 34.41% were detected in dog sperm, with the highest level detected in DMSO ($p < 0.05$). Rabbit sperm showed a high level of motility (30.91%) and viability (40.91%) in TYB ($p < 0.05$).

DISCUSSION

McGonagle et al. (2002) reported that a significant difference in sperm motility was observed between experimental groups after thawing, ranging from 9 to 44% ($p < 0.05$). Motility of 21% was detected in the group with glycerol alone and 35% and 37% in the groups with combined CVPs containing glycerol- egg yolk-citrate-glucose and egg yolk-glycerol-glucose-tris diluents, respectively, higher in the group with combined CPVs than in the group with glycerol alone ($p < 0.05$). After thawing, motility showed a range similar to that observed in the current study. Critser et al. (1988) examined motility using different human sperm preservation mediums (HSPM) with varying content of glycerol (0%, 2.5%, 5.0%, and 7.5%) and sucrose (0, 25, 50, and 100 mM). The results of the analysis showed that the highest level of motility was 41.50% in HSPM containing 5.0% glycerol and 25 mM sucrose, and the lowest level of motility was 12.40% in HSPM without glycerol and sucrose ($p < 0.05$), demonstrating a significant difference. These outcomes were comparable to the results of this study showing a similar range between 12.45% and 37.50%.

Hammadeh et al. (2001) suggested that morphologically better motility and activity of sperm could be obtained with use of TYB medium compared with HSPM ($p = 0.001$). The results of the hypoosmotic swelling (HOS) test also indicated that the level of motility was significantly higher in TYB (50.6 ± 28.4) than in HSPM (43.3 ± 21.3) ($p = 0.001$). In particular, lower values for condensed chromatin were observed when freezing with TYB compared with use of typical cryoperservatives. This finding was confirmed to be attributable to the fact that occurrence of DNA damage during mitosis is minimized when chromatin levels are high, enabling better control of gene expression and DNA replication. Bhattacharya et al. (2006) reported a higher level of sperm motility and higher active sperm counts after thawing semen samples frozen in TYB (45.7 ± 16.6) compared with HSPM (36.5 ± 29.7). These results were comparable to those of this study where sperm motility of 12.50%-40.91% was detected.

In this experiment, the increase in sperm motility and viability appeared to occur as a result of the removal of sludge around Ham's F-10 and sperm by washing sperm twice using Isolate, Multipurpose Handling Medium, or Sperm Washing Medium when thawing the samples in TYB.

As suggested in several previous studies, DMSO has strong toxicity. In their toxicity experiment with DMSO using mouse embryo, Kim et al. (2015) reported a significant difference in the rate of embryonic development with

a substantially lower rate in DMSO compared with glycerol and propanediol (PROH) ($p < 0.05$), and a higher level of toxicity was observed for DMSO compared to the other two CPVs. Galvao et al. (2013), who analyzed cell death using the retinal ganglion cell-5 (RGC-5) cell line of rats at different concentrations of DMSO, 0, 1, 2, 4, and 8%, reported a significantly lower difference in all experimental groups at a concentration of 8% ($p < 0.001$). The high toxicity of DMSO was the same as that observed in this study. In agreement with the studies reported by Kim et al. (2015), a relatively lower level of motility and viability appear to be attributable to the toxicity of DMSO, which causes damage to spermatozoa compared to those of other CPVs. In addition, as a result of a lower frequency and amount of sperm washing during freezing compared with the other two CPVs, Sperm CryoProtec and TYB are predicted to cause sperm damage due to contamination.

Based on the findings of this study, conduct of additional experiments using extender media in combinations of different media or substances for use in sperm-washing and to offset the toxicity of DMSO is anticipated.

Galvao et al. (2013) reported a significant difference in toxicity depending on the concentration of DMSO ($p < 0.0001$). DMSO was found to cause a decrease in sperm viability by reducing mitochondrial respiration and ATP concentration, leading to cell apoptosis. In addition, in a study on the toxic effects of DMSO and ethylene glycol, Kim et al. (2015) reported a significant decrease in the number of blastocysts (68.1 ± 24.1), apoptosis rates (15.4 ± 1.5), and the developmental rate of normal embryos (14.7%) due to toxicity compared to the control group ($p < 0.0001$). Yang et al. (2004), who evaluated the toxicity of DMSO and PROH using hybrid mouse embryos, reported that a significantly higher number of dead cells (14.2 ± 1.5) was observed with use of DMSO compared to PROH (11.2 ± 1.4) ($p < 0.001$). A lower developmental rate of embryos and number of blastocysts were observed with use of DMSO (53.9%, 67.4 ± 24.9) compared with PROH (63.1%, 75.9 ± 27.0), however, the difference was not significant.

Lee et al. (2003), who performed slow freezing of dog semen extended with Tris-egg yolk buffer, reported viability ranging between 54 and 78% in sperm samples thawed at 37℃ and 55℃, which concurs with the results of this study. Hammadeh et al. (2001) reported that human spermatozoa frozen in TYB showed sperm motility of 13.3 ± 9.9% and viability of 30.8 ± 21.2% after thawing, comparable to the results of this study.

CONCLUSION

In this experiment, we carried out an experiment to determine whether cryopreservatives (CPVs) were more effective in promoting the motility and viability of the sperm of experimental animals. Different CPVs, including DMSO, TYB, and Sperm CryoProtec, were used to freeze the sperm of mice, rats, beagle dogs, and rabbits and the sperm samples were thawed in one-week intervals. After freezing the sperm of each laboratory animal in Sperm CryoProtec, the results showed that the sperm motility and viability were high in mouse, rat, and beagle dog sperm samples thawed at the end of the first week. For rabbits, the motility was high in sperm thawed in the first week, whereas the viability was high in sperm thawed in the second week. The study findings presented above suggest that appropriate cryopreservatives need to be chosen for each experimental animal. This is because the sperm motility and viability of experimental animals differ depending on the CPVs that are typically used to freeze human sperm, including Sperm CryoProtec and TYB.

In this study, various factors were identified to affect sperm motility and viability such as age of animals, environmental factors, experience of technicians, duration of sperm freezing and others, and the number of animal samples reduced data bias and provided more accurate measurements. For these reasons, additional studies are warranted to further investigate effective culture media, CPVs, length of time, and techniques.

Author Contributions: Conceptualization, W.Y.P. and B.B.S.; methodology, W.Y.P. and B.B.S.; investigation, W.Y.P. and B.B.S.; data curation, W.Y.P. and B.B.S.; writing—original draft preparation, W.Y.P. and B.B.S.; writing—review and editing, B.B.S.; supervision, B.B.S.; project administration, B.B.S.; funding acquisition, B.B.S.

Funding: This research was supported by the Daegu University Research Grant, 2018.

Ethical Approval: All procedures were performed in accordance with the protocol approved by the Daegu University Animal Experimental Ethics Committee (approval

number: DUIACC-2019-010-0221-009) and DGMIF Animal Experimental Ethics Committee (approval number: DGMIF-19121701-00 for beagle and DGMIF-19111203-00 for rabbit).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Ball BA and Vo A. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22:1061-1069.

Bhattacharya J, Kundu S, Varghese AC, Bhattacharya SM, Bhattacharyya AK, Agarwal A. 2006. A comparative study on TEST-Yolk buffer and human sperm preservation medium on post thaw characteristics of human sperm from prefreeze specimens. Fertil. Steril. 86(3 Suppl):S200.

Blässe AK, Oldenhof H, Ekhlasi-Hundrieser M, Wolkers WF, Sieme H, Bollwein H. 2012. Osmotic tolerance and intracellular ion concentrations of bovine sperm are affected by cryopreservation. Theriogenology 78:1312-1320.

Bosch E, De Vos M, Humaidan P. 2020. The future of cryopreservation in assisted reproductive technologies. Front. Endocrinol. (Lausanne) 11:67.

Choi JS, Kim SW, Shin DB, Ko YG, Do YJ, Kim DH, Kong IK, Park SB. 2012. Effects of N-methylacetamide on the viability, fertility and hatchability of cryopreserved Ogye (Korean native black fowl) semen. Korean J. Poult. Sci. 39:291-295.

Choi WC, Yang MH, Lee YS, Cheong HT, Yang BK, Lee DS, Park CK. 2007. Effect of thawing temperature on sperm characteristics of frozen semen in miniature pig. Reprod. Dev. Biol. 31:175-179.

Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. 1988.Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil. Steril. 50:314-320.

Galvao J, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF. 2014. Unexpected low-dose toxicity of the universal solvent DMSO. FASEB J. 28:1317-1330.

Guthrie HD, Liu J, Critser JK. 2002. Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol. Reprod. 67:1811-1816.

Hallak J, Sharma RK, Wellstead C, Agarwal A. 2000. Cryopreservation of human spermatozoa: comparison of TEST-yolk buffer and glycerol. Int. J. Fertil. Womens Med. 45:38-42.

Hammadeh ME, Greiner S, Rosenbaum P, Schmidt W. 2001. Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. J. Androl. 22:1012-1018.

Kang HH, Lee JW, Kang MJ, Kim KH, Moon SJ. 2014. In vitro development of porcine oocytes following intracytoplasmic injection of freeze-dried spermatozoa with trehalose. J. Emb. Trans. 29:51-57.

Kim H, Yu DJ, Choe C, Seong HH. 2015. Toxic effects of ethylene glycol on mammalian embryo survivability. Reprod. Dev. Biol. 39:77-81.

Kim HJ, Choe JY, Choi SH, Son DS, Choi SH, Sang BD, Han MH, Ryu IS, Kim IC, Kim IH, Im KS, Kim SJ, Cho SR. 2006. Comparison of diluents on liquid storage of Korean native goat spermatozoa. Korean J. Emb. Trans. 21:339-344.

Kwon IK, Park KE, Niwa K. 2004. Activation, pronuclear formation, and development in vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa. Biol. Reprod. 71:1430-1436.

Lee YR, Lee SL, Kang TY, Choe SY. 2003. Viability and acrosomal status changes following post-thawing canine spermatozoa. Korean J. Emb. Trans. 18:51-59.

Magnusson V, Feitosa WB, Goissis MD, Yamada C, Tavares LM, D'Avila Assumpção ME, Visintin JA. 2008. Bovine oocyte vitrification: effect of ethylene glycol concentrations and meiotic stages. Anim. Reprod. Sci. 106:265-273.

Mantovani R, Rora A, Falomo ME, Bailoni L, Vincenti L. 2002. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. Reprod. Nutr. Dev. 42:217-226.

Masoumi SZ, Parsa P, Darvish N, Mokhtari S, Yavangi M, Roshanaei G. 2015. An epidemiologic survey on the causes of infertility in patients referred to infertility center in Fatemieh Hospital in Hamadan. Iran. J. Reprod. Med. 13:513-516.

McGonagle LS, Goldstein M, Feldschuh J, Foote RH. 2002. The influence of cryoprotective media and processing procedures on motility and migration of frozen-thawed human sperm. Asian J. Androl. 4:137-141.

Mochida K, Hasegawa A, Li MW, Fray MD, Kito S, Vallelunga JM, Lloyd KC, Yoshiki A, Obata Y, Ogura A. 2013. High osmolality vitrification: a new method for the simple and temperature-permissive cryopreservation of mouse embryos. PLoS One 8:e49316.

Mocé E, Fajardo AJ, Graham JK. 2016. Human sperm cryopreservation. Eur. Med. J. 1:86-91.

Nakagata N, Mikoda N, Nakao S, Nakatsukasa E, Takeo T. 2020. Establishment of sperm cryopreservation and in vitro fertilisation protocols for rats. Sci. Rep. 10:93.

Seifi-Jamadi A, Ahmad E, Ansari M, Kohram H. 2017. Antioxidant effect of quercetin in an extender containing DMA or

glycerol on freezing capacity of goat semen. Cryobiology 75: 15-20.

Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. 2014. Assisted oocyte activation following ICSI fertilization failure. Reprod. Biomed. Online 28:560-571.

Yang KC, Kang HG, Lee HC, Lee HH, Ko DS, Yang H, Park WI, Park EJ, Kim SS. 2004. Toxic effect of cryoprotectants on embryo development in a murine model. Korean J. Fertil. Steril. 31:59-65.