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Dog Sperm Cryopreservation Using Glucose in Glycerol-free TRIS: Glucose Concentration, Exposure Time

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Abstract : The aim of the present study was to develop glycerol-free TRIS extender using glucose for dog sperm cryopreservation. We determined the appropriate concentration of glucose in glycerol-free TRIS and the exposure time in glycerol-free TRIS containing 0.3 M glucose at 4°C. Ejaculates of six dog sperm were cooled in glycerol-free TRIS through 4°C for 100 min, cooled at 4°C in TRIS with different glucose concentrations 0 M, 0.04 M, 0.1 M, 0.2 M and 0.3 M, respectively for 30 min followed by cryopreservation. After thawing at 37°C for 25 sec, membrane and acrosome integrities of dog sperm were evaluated. In addition, the effect of exposure time (10, 30, 50 and 70 min) of sperm to glycerol-free TRIS containing 0.3 M glucose at 4°C on progressive motility, viability, and DNA integrity following sperm cryopreservation was studied. Membrane integrity and acrosome integrity were assessed by 6-carboxyfluoresceindiacetate (6-CFDA)/propidium iodide (PI) fluorescent staining and *Pisum sativum agglutinin* conjugated to fluorescein isothiocyanate, respectively. DNA integrity was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling, using flow cytometry. Sperm frozen in glycerol-free TRIS supplemented with 0.2 M or 0.3 M glucose have an intact plasma membrane (CFDA+/PI-) after cryopreservation than sperm frozen in the extenders with lower glucose concentrations ($p < 0.05$). Acrosome integrity was significantly higher in the 0.3 M group than less than 0.1 M groups ($p < 0.05$). The sperm DNA fragmentation index did not differ according to exposure time, although progressive motility was significantly higher in the 50 min exposure group than the other groups ($p < 0.05$). These results indicate that cryopreservation of dog sperm is feasible and yields more motile sperm following freezing and thawing in glycerol-free TRIS containing 0.3 M glucose with the exposure time for 50 min at 4°C.

Key words **:** Dog, sperm, cryopreservation, Glycerol-free TRIS, glucose.

Introduction

Glycerol is generally used cryoprotectant to freeze dog spermatozoa (7,18). However, glycerol has toxicity, which has been shown to have a deleterious effect on boar and stallion sperm (3,12). Therefore, Sánchez *et al*. (20) developed a vitrification method using only sucrose without a permeating cryoprotectant. Vitrification, however, involves the use of highly concentrated cryoprotectants and is not suitable for spermatozoa due to the lethal osmotic and cytotoxic effects of highly concentrated cryoprotectants (13).

Although sugars has been used as an additive to cryopreservation extenders containing permeating cryoprotectants (20), they improve post-thaw sperm viability by providing an energy source, promoting the excretion of water out of the cell and thereby decreasing intracellular ice crystal formation, and maintaining the osmotic pressure of the extender (11,21). Glucose is commonly used as a component of dog sperm freezing extenders (19). Therefore, we have been developing glycerol-free extender using glucose for dog sperm cryopreservation.

On the other hand, the sperm plasma membrane is the primary site of cold-induced damage (4), and it is likely that these membrane stresses are related to phase changes in lipids and an altered functional state of the membrane (26). Sperm DNA integrity is an important parameter of sperm quality in the prognosis of infertility and in the outcome of assisted reproductive procedures (22).

Our first aim was to determine the most effective glucose concentration in glycerol-free TRIS to maintain membrane integrity as well as acrosome integrity. Our second aim was to investigate the appropriate exposure time of dog sperm to glycerol-free TRIS containing glucose at 4°C by examining DNA fragmentation.

Materials and Methods

Chemicals

Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and solutions were prepared with high purity water (6114VF, Sartorius AG,

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Goettingen, Germany). Equex STM paste was obtained from Nova Chemical Sales Inc. (Scituate, MA, USA).

Collection of semen

Semen was collected from six dogs (two mixed breed, one poodle, two Shih-Tzu, and one Welsh corgi), ranging between 2 and 4 years of age. The dogs used in this study were treated and received care under the Guiding Principles for the Care and Use of Research Animals, as established by Chonbuk National University. The dogs were caged individually, were provided water ad libitum and formulated dog food (Jindo®, Purina) twice daily, and exercised daily throughout the experiment.

One ejaculate from each of six dogs was obtained by digital manipulation and the sperm-rich second fraction of the ejaculates was collected. Only normal ejaculates (sperm concentration ≥ 200×10^6 spermatozoa/mL, motility ≥ 70%, and normal morphology ≥ 80%) were used in this study.

Sperm freezing and thawing

Extenders which are modifications of TRIS extender (19) were used in experiments. The extenders used in experiments were composed of extender 1 and extender 2. For experiment 1, extender 1 was composed of basal TRIS extender and 0.04 M glucose. The components of basal TRIS were 2.42 g Tris (Tris [hydroxymethyl]aminomethane), 1.4 g citric acid, 0.06 g penicillin G, 0.1 g streptomycin, and 20% egg yolk in 100 mL sterile non-pyrogenic water. Extender 2 consisted of basal TRIS extender supplemented with glucose concentrations of 0 M (249 mOsm), 0.04 M (308 mOsm), 0.1 M (370 mOsm), 0.2 M (511 mOsm), or 0.3 M (626 mOsm). For experiment 2, the components of extender 1 used in the experiment were the same as for the extender 1 in experiment 1. Extender 2 was composed of 0.3 M glucose in TRIS extender. Osmolality was measured with a vapor pressure osmometer (VAPRO 5520, Logan, UT, USA). Extenders were prepared in single batches and stored frozen to avoid variation among batches.

Semen was processed according to the straw freezing procedure. Briefly, semen was centrifuged at 300 × g for 3 min at room temperature. The supernatant was then removed, and the semen pellet was resuspended with extender 1 to a concentration of 1×10^8/mL. Sperm suspensions were cooled down gradually from 25°C to 4°C for 100 min. At this temperature, a second extension step to 5×10^7/mL was performed with extender 2 and the sperm were maintained at 4°C for 30 min. A Styrofoam box (outside dimensions [l, w, h]: $33.5 \times 22.7 \times 24$ cm^3; inside dimensions: $29.5 \times 18.7 \times 24$ cm^3) and rack were used to achieve a cooling rate of 63.4°C/min from room temperature to −36.4°C and a cooling rate of 7.8°C/min from −36.4°C to −86.7°C. The cooling rate was measured five times using a thermocouple (Model 91100, Cole-Parmer, USA) inserted into an empty straw. The Styrofoam box was filled with liquid nitrogen (LN_2) to a depth of 5 cm and the rack was set 7 cm from the surface of the LN_2. For sperm cryopreservation, a 300 μL aliquot of diluted spermatozoa was loaded in a 0.5 mL straw. Straws were aligned horizontally for 20 min on the rack and plunged into LN_2. The frozen spermatozoa were thawed for 25 sec in a water bath at 37°C.

Motility

Ten microliters of semen were placed on a slide and coverslipped. The percentage of progressive motile sperm was estimated under microscopic examination at 400 × magnification. Sperm were considered to be motile only if they exhibited progressive motility of a score of at least 3 on a scale of 0-4, as described by Yu & Leibo (25). The mean of six successive estimations was recorded as the final motility score.

Viability

Viability was measured (25) using the fluorescent double stain Fertilight® (Molecular Probes Inc., Eugene, OR, USA). For each replicate sample, two slides were prepared, and approximately 200 spermatozoa were counted per slide. The number of green or red fluorescent spermatozoa was counted

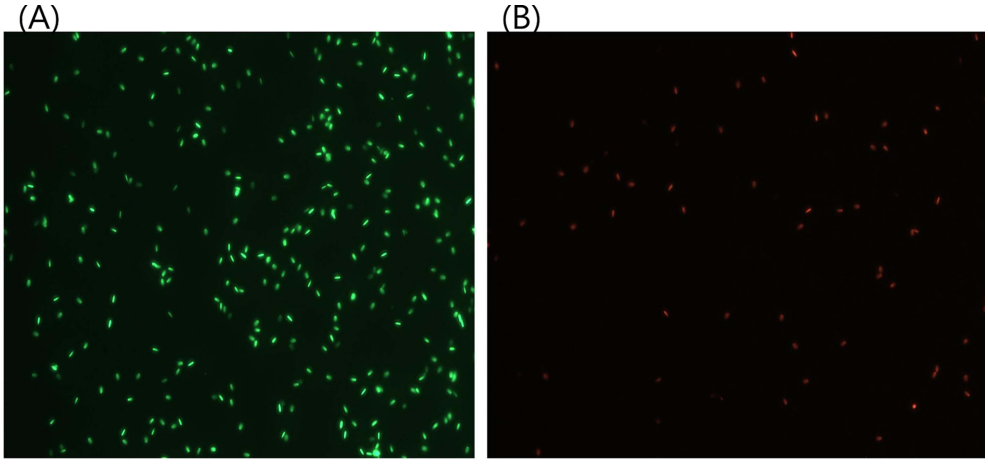

Fig 1. Viability of dog spermatozoa stained with Fertilight® at a magnification of 200 ×. Green fluorescence (A) and red fluorescence (B) in sperm head were considered as viable and dead spermatozoa, respectively.

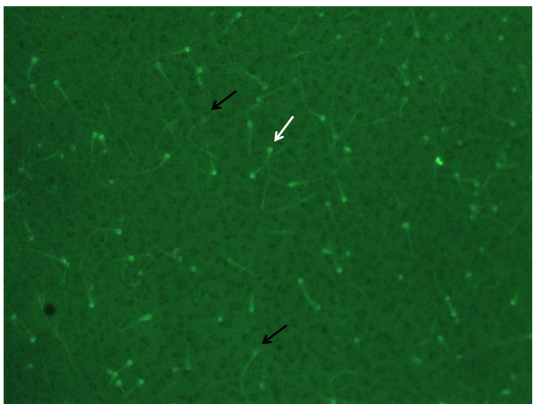

Fig 2. Acrosome integrity of dog spermatozoa stained with PSA conjugated to FITC at a magnification of 200 ×. Green fluorescence on sperm anterior acrosomal region (a white arrow) was considered to have an intact acrosome. Either slight fluorescence or no fluorescence (black arrows) was considered as having damaged acrosome.

under a fluorescence microscope (Axio, Carl Zeiss, Germany) fitted with a 488 nm excitation filter, and the percentage of membrane-intact spermatozoa (green fluorescence in sperm head) was calculated (Fig 1).

Acrosome integrity

Acrosome integrity was determined using the method described by Yu and Leibo (25). Spermatozoa were stained with *Pisum sativum agglutinin* (PSA) conjugated to fluorescein isothiocyanate (FITC). For each replicate sample, two slides were examined under a fluorescence microscope (Axio, Carl Zeiss, Germany), and approximately 200 spermatozoa were counted per slide. The percentage of spermatozoa with an intact acrosome (green fluorescence on sperm anterior acrosomal region) was calculated (Fig 2).

Membrane integrity

Sperm plasma membrane integrity was assessed using a 6-carboxyfluoresceindiacetate (6-CFDA)/propidium iodide (PI) fluorescent staining technique (17). Five hundred microliter aliquots of sperm suspension (5×10^5/mL spermatozoa) were mixed with 5 μL 6-CFDA (1 μg/mL) and 5 μL PI (0.1 mg/mL) stock solution. Samples were analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson, USA). Spermatozoa were classified as having an intact plasma membrane (CFDA+/PI-), a damaged plasma-membrane (CFDA+/PI+), or as dead sperm (CFDA-/PI+).

DNA integrity

For DNA analysis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on post-thawed sperm. TUNEL labeling was carried out using the In Situ Cell Death Detection Kit Fluorescein (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, samples were centrifuged (500 × g for 6 min), and the pellet was resuspended in PBS with 0.1% (w/v) polyvinyl alcohol (PVA) to a final concentration of 1.5×10^6 cells/100 μL. One hundred microliters of 1% paraformaldehyde were added, and samples were shaken for 1 h at 15-25°C. The fixed cells were resuspended in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 20 min at 4°C. After centrifugation, samples were resuspended in 50 μL reaction mix containing 45 μL labeling solution and 5 μL terminal deoxynucleotidyl transferase (TdT). A positive control (incubation of fixed cells with 200 U/mL DNase I) and a negative control (replacing the DNA labeling mixture with distilled water) were used to standardize the assay. Negative controls were suspended in labeling solution without TdT. Samples were incubated for 1 h at 37°C in the dark. At the end of this incubation, cells were washed twice and resuspended in PBS containing 5 μg/mL PI (stock 1.603 mg/mL). Cells were analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson, USA). The different labeling patterns in the TUNEL/PI analysis were classified as follows: viable (TUNEL-/PI-); viable but with DNA fragmentation (TUNEL+/PI-); nonviable with DNA fragmentation (TUNEL+/PI+); and nonviable and late necrotic sperm (TUNEL-/PI+). We defined the ratio between the TUNEL+/PI- sperm and the TUNEL+/PI+ sperm as the DNA fragmentation index.

Experimental design

Effect of glucose concentration in glycerol-free TRIS on dog sperm cryopreservation

To determine the most effective glucose concentration in glycerol-free TRIS to successfully freeze dog sperm, dog sperm were cooled in extender 1 through 4°C for 100 min, then cooled at 4°C in extender 2 with various glucose concentrations (0 M, 0.04 M, 0.1 M, 0.2 M and 0.3 M, respectively) for 30 min followed by cryopreservation. After thawing at 37°C for 25 sec, sperm membrane integrity and acrosome integrity were evaluated as described above.

Effect of exposure time to glycerol-free TRIS containing 0.3 M glucose on dog sperm cryopreservation

To determine the most effective exposure time of dog sperm to glycerol-free containing 0.3 M glucose for successful cryopreservation, dog sperm were cooled in extender 1 through 4°C for 100 min, exposed to glycerol-free TRIS with 0.3 M glucose at 4°C for 10, 30, 50, or 70 min, and then cryopreserved. After thawing at 37°C for 25 sec, sperm motility, viability and DNA integrity were evaluated.

Statistical analysis

Seven replicates were conducted for each experiment. Percentage data were subjected to arcsine transformation before analysis. All data are presented as means ± SE and were analyzed by analyses of variance (ANOVA) followed by Duncan's multiple range test using Statistical Analysis System ver. 8x software (SAS, Cary, NC, USA). $p < 0.05$ was con-

sidered to indicate statistical significance.

Results

Effect of glucose concentration in glycerol-free TRIS on dog sperm cryopreservation

Sperm membrane integrity according to glucose concentrations following freezing and thawing are shown in Table 1. The percentage of sperm with an intact plasma membrane (CFDA+/PI-) was significantly higher ($p < 0.05$) in the 0.2 M and 0.3 M groups than the 0 M, 0.04 M and 0.1 M groups (Fig 3). The number of dead sperm with an intact membrane was significantly lower in the 0.3 M group than the other groups with the exception of the 0.2 M group ($p < 0.05$), while the number of dead sperm with damaged membranes (CFDA-/PI+) was significantly higher in the 0.3 M group than the 0 M and 0.04 M groups ($p < 0.05$).

Acrosome integrity of sperm cryopreserved in glycerol-free TRIS with 0.3 M glucose was significantly higher ($p < 0.05$) than those of sperm cryopreserved in 0 M, 0.04 M or 0.1 M glucose-supplemented glycerol-free TRIS (Table 2).

Table 2. The effect of glucose concentration in glycerol-free TRIS on the acrosome integrity of dog frozen spermatozoa

	Glucose concentration (M)				
	0	0.04	0.1	0.2	0.3
Acrosome integrity (%)	46.1 ± 6.7^{b}	45.6 ± 5.0^{b}	47.1 ± 6.0^{b}	$59.2 \pm 5.2^{a,b}$	68.4 ± 4.8^{a}

Values are expressed as means ± SE. ^{a,b}Different superscripts within the same rows indicate significant differences ($p < 0.05$).

Table 1. The effect of glucose concentration in glycerol-free TRIS on the plasma membrane integrity of dog frozen spermatozoa

6-CFDA/PI stain (%)	Glucose concentration (M)				
	0	0.04	0.1	0.2	0.3
6-CFDA+/PI-	9.83 ± 1.3^{b}	$11.2 \pm 2.21.8^{b}$	11.3 ± 2.9^{b}	21.4 ± 2.1^{a}	23.8 ± 1.3^{a}
6-CFDA+/PI+	77.5 ± 2.0^{a}	$72.6 \pm 1.9^{a,b}$	$73.3 \pm 4.4^{a,b}$	$66.8 \pm 3.1^{b,c}$	61.3 ± 3.0^{c}
6-CFDA-/PI+	3.4 ± 0.7^{b}	2.6 ± 0.5^{b}	$4.0 \pm 1.4^{a,b}$	$4.4 \pm 0.4^{a,b}$	6.2 ± 0.9^{a}

Spermatozoa were classified as having an intact plasma membrane (CFDA+/PI-), a damaged plasma-membrane (CFDA+/PI+), or as dead sperm (CFDA-/PI+). Values are expressed as means ± SE. ^{a,b,c}Different superscripts within the same rows indicate significant differences ($p < 0.05$).

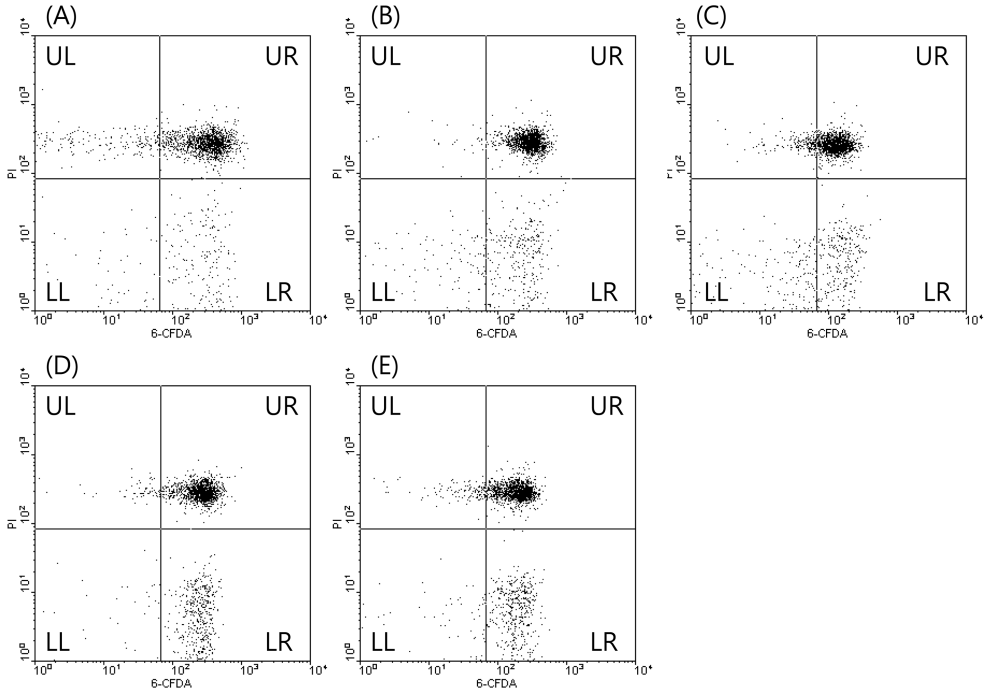

Fig 3. Plasma membrane integrity of spermatozoa following freezing in glycerol-free TRIS containing different glucose concentration. Flow cytometry analysis of a sperm population sample. (A) 0 M glucose (B) 0.04 M glucose (C) 0.1 M glucose (D) 0.2 M glucose and (E) 0.3 M glucose. UL: dead spermatozoa with a damaged membrane, UR: dead spermatozoa with an intact membrane (moribund spermatozoa), LL: cellular debris or fragments of cells, LR: viable spermatozoa with an intact membrane.

Table 3. Effect of exposure time to 0.3 M glucose supplemented glycerol-free TRIS on the motility, viability and DNA fragmentation of dog frozen spermatozoa

Exposure time (min)	Progressive motility (%)	Viability (%)	DNA fragmentation index
10	20.7 ± 2.3^{c}	35.1 ± 4.9^{b}	8.7 ± 1.8
30	40.0 ± 2.4^{b}	45.4 ± 2.1^{a}	8.8 ± 2.1
50	53.6 ± 3.6^{a}	46.4 ± 2.7^{a}	9.2 ± 2.5
70	41.4 ± 2.6^{b}	$40.7 \pm 3.1^{a,b}$	7.5 ± 2.0

Values are expressed as means ± SE. ^{a,b,c}Different superscripts within the same columns indicate significant differences ($p < 0.05$).

Effect of exposure time to glycerol-free TRIS containing 0.3 M glucose on dog sperm cryopreservation

Sperm characteristics according to exposure time following freezing and thawing are shown in Table 3. Progressive motility was significantly higher in sperm exposed to 0.3 M glucose supplemented glycerol-free TRIS for 50 min than those exposed to this solution for 10, 30, or 70 min ($p < 0.05$). The viability of sperm exposed for 10 min was significantly lower than those of sperm exposed for 30 or 50 min at 4°C ($p < 0.05$). The sperm DNA fragmentation index was not affected by the exposure time (Table 3; Fig 4).

Discussion

In the present study, dog sperm cryopreserved in 0.2 M or 0.3 M glucose in gylcerol-free TRIS had significantly better plasma membrane integrity than sperm preserved in extenders supplemented with lower concentrations of glucose. Koshimoto and Mazur (11) proposed that sugars protect cells extracellularly, as mammalian cells are generally impermeable to sugars. Glucose addition to the extender conferred better improvement in forward motility percentage than the other disaccharides addition after cryopreservation (14). The possibility that glucose was the monosaccharide providing higher cryoprotective effect was observed (5). In addition, these authors observed that disaccharides that have glucose as a subunit (lactose, sucrose, melobiose and trehalose) had a greater cryoprotectant effect than those sugars that do not (lactulose). It is known that sugars stabilize membranes by interacting with polar head groups of phospholipids (9,10). This interaction may render the membrane less vulnerable to morphological changes that occur during the rapid reflux of water, and prevent deleterious alterations to the membrane during times of reduced water, such as freezing (1,2). Sugars are thereby able to maintain the structural integrity and permeability of lipidic bilayers (5,23).

It has been widely reported that sperm freezing and thawing not only alter sperm motility and viability, but also increase sperm DNA damage (24). In the present study, time of exposure of sperm to glycerol-free TRIS containing 0.3 M glucose before freezing affected sperm viability and motility. Exposure to glycerol-free TRIS containing 0.3 M glucose for 50 min at 4°C yielded sperm with the best motility and viability upon thawing. That is, sperm motility and viability after freezing and thawing was dependent on exposure time to glycerol-free TRIS containing 0.3 M glucose. Sugars are able to create osmotic pressure, inducing cell dehydration and a reduction in intracellular ice formation (6). We infer that sufficient exposure time to a glycerol-free TRIS containing 0.3 M glucose at 4°C to remove intracellular water decreases ice formation in sperm during freezing, thereby resulting in improved sperm viability and motility after thawing. DNA integrity has also become an important indicator of fertile spermatozoa (21). Because the quality of sperm DNA is an important parameter for the correct transmission of genetic material from one generation to another, additional damage caused by the freezing-thawing process has important implications (15). In the present study, DNA fragmentation according to time of exposure to glycerol-free TRIS containing 0.3 M glucose did not differ significantly among groups. The DNA instability reported in cryopreserved sperm is thought to be related to oxidative stresses that arise during freezing and thawing (16). Further studies are required to determine how the formation of reactive oxygen species differs between glycerol-free TRIS containing 0.3 M glucose and conventional freezing extenders that contain glycerol. Additionally, we ought to investigate if sperm frozen using this extender can be useful for artificial insemination.

Our results suggest that glucose can be used as the main cryoprotectant without glycerol to improve the efficiency of dog sperm cryopreservation, although sugars such as raffi-

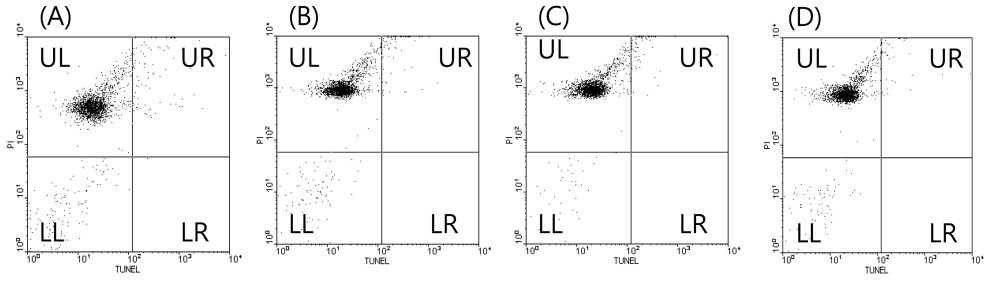

Fig 4. DNA integrity of spermatozoa according to exposure time following freezing in glycerol-free TRIS containing 0.3 M glucose. Flow cytometry analysis of a sperm population sample. (A) 10 min (B) 30 min (C) 50 min and (D) 70 min. UL: dead spermatozoa, UR: dead spermatozoa with fragmented DNA, LL: viable spermatozoa, LR: viable spermatozoa with fragmented DNA.

nose, sucrose, and lactose have been identified as potential cryoprotectants (8).

Conclusion

We demonstrated that dog sperm exposed to glycerol-free TRIS containing 0.3 M glucose for 50 min at 4°C resulted in efficient sperm cryopreservation as reflected by membrane integrity, acrosome integrity and DNA integrity of sperm cryopreserved in this solution.

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Glycerol-free TRIS 배지내 glucose를 이용한 개 정자 동결: 포도당 농도, 노출시간

유일정[1]

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요 약 : 개 정액 동결을 위한 glucose가 첨가된 glycerol-free TRIS 희석액을 개발하기 위해 glycerol-free TRIS내 알맞은 glucose의 양과 0.3 M glucose가 첨가된 glycerol-free TRIS내 정자의 적정 노출시간을 조사하였다. 여섯 마리의 수캐의 사출액을 0.04 M glucose가 첨가된 glycerol-free TRIS내에서 4°C까지 100분 동안 냉각한 후 서로 다른 glucose농도 (0 M, 0,04 M, 0.1 M, 0.2 M, 0.3 M)의 glycerol-free TRIS에서 30분 동안 냉각하여 동결하였다. 37°C에서 25 초 동안 융해한 후 정자의 막 고유성과 첨단체 고유성을 검사하였다. 부가적으로 0.3 M glucose가 첨가된 glycerol-free TRIS내 정자의 적정 노출시간에 따른 정자의 동결 후 운동성, 생존성, DNA 고유성을 확인하였다. 막 고유성과 첨단체 고유성은 각각 6-carboxyfluoresceindiacetate(6-CFDA)/propidium iodide(PI) fluorescent staining와 *Pisum sativum agglutinin* conjugated-fluorescein isothiocyanate 방법에 의해 검사하였다. DNA 고유성은 terminal deoxynucleotidyl transferase dUTP nick end labeling로 염색하여 flow cytometry로 검사하였다. 0.2 M 또는 0.3 M glucose가 첨가된 glycerol-free TRIS에서 동결된 정자가 낮은 농도의 glucose가 첨가된 희석액에서 동결된 정자보다 막 고유성이 높게 나타났으며($p < 0.05$), 첨단체 고유성은 0.3 M 군에서 높게 나타났다($p < 0.05$). 운동성은 50 분 군에서 높게 나타났으나($p < 0.05$), DNA fragmentation index는 노출시간에 따라 차이가 없었다. 본 연구 결과 개정자가 0.3 M glucose가 첨가된 glycerol-free TRIS에서 4°C, 50 분간 냉각 후 동결과 융해 후 더 높은 생존성을 나타냈다.

주요어 : 개, 정자, 동결, Glycerol-free TRIS, 포도당.