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Effect of Thawing Rate on the Function of Cryopreserved Canine Sperm

Suhee Kim, Do-Hyeon Yu*, Tae-Woon Kang and Yong-Jun Kim[1]

Department of Veterinary Theriogenology, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea
**Department of Veterinary Internal Medicine, College of Veterinary Medicine, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea*

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Abstract : Sperm cryopreservation methods have been improved over the last few decades. However, an optimized thawing rate has not yet been established. Therefore, we investigated the effect of thawing rate on sperm function after cryopreservation. The ejaculates collected from beagle dogs were cryopreserved and then thawed at two different thawing rates (37°C for 1 min or 70°C for 15 sec). The thawed sperm were evaluated for motility, viability, morphology, plasma membrane integrity, phosphatidylserine (PS) translocation, and intracellular H_2O_2 level. The sperm thawed rapidly at 70°C showed improved motility, viability, normal morphology, plasma-membrane integrity and non-PS translocation compared to the sperm thawed slowly at 37°C ($P < 0.05$). However, the intracellular H_2O_2 levels were not significantly different between the rapid- and slow-thawed sperm ($P > 0.05$). In conclusion, sperm rapid thawing at 70°C could improve the function of cryopreserved canine sperm, and the appropriate thawing rate would enhance the quality of the cryopreserved sperm.

Key words **:** canine sperm, cryopreservation, motility, thawing rate.

Introduction

During the freezing-thawing processes, sperm undergo changes due to physical and chemical stresses (22), including alterations in the lipid composition of the plasma membrane (7), reduced head size (11), and externalization of phosphatidylserine (10). The factors that contribute to these changes include the choice of extender, cryoprotectant, packaging system, freezing rate, and thawing rate, which have all been shown to affect the quality of frozen-thawed canine semen (16). Recent investigations have led to the hypothesis that cryopreserved sperm are more susceptible to damage during thawing rather than during freezing since there is a marked tonicity change from hypertonic conditions during freezing to isosmotic conditions during thawing (18). However, frozen semen is frequently thawed at 37°C to reduce the risk of cell damage above 37°C and increase the practical use, despite importance of thawing rate and a higher post-thaw sperm viability in semen thawed rapidly at higher temperature than 37°C (15,23).

The mammalian sperm plasma membrane with its high polyunsaturated fatty acid content is highly susceptible to lipid peroxidation, a risk factor that may contribute to sperm cryodamage. The sperm are particularly susceptible to reactive oxygen species (ROS)-induced lipid peroxidation and there may be a greater risk of peroxidative damage at higher thawing temperatures rather than lower thawing temperatures (2). Therefore, the aim of this study was to compare the effects of standard (37°C for 1 min) and rapid thawing rates (70°C for 15 sec) on various canine sperm functions after cryopreservation, including ROS. This study performed to determine whether thawing cryopreserved canine sperm at 70°C results in better sperm quality than thawing at 37°C.

Materials and Methods

Semen collection

Five healthy and sexually mature beagle dogs were used in this study and ranged from two to five years of age. The animals used in this study were treated and received care under the Guiding Principles for the Care and Use of Research Animals, as established by the Chonbuk National University of Veterinary Medicine. Ejaculates from each dog were obtained by digital manipulation once a week and the sperm-rich second fractions of the ejaculates were collected. Only normal ejaculates with $\geq 200 \times 10^6$ sperm/mL, ≥ 70% motility, ≥ and 80% normal morphology were included in this study.

Sperm freezing and thawing process

Canine sperm freezing was performed as previously described (1,21) with modifications. The collected ejaculates were washed with Tris diluent (24 mg/mL Tris [hydroxymethyl] aminomethane, 14 mg/mL citric acid, 8 mg/mL glucose, 0.6 mg/mL Na-benzylpenicillin, and 1 mg/mL streptomycin

[1]Corresponding author.
E-mail : yjk@jbnu.ac.kr

sulphate in distilled water [pH 6.60, 253 mOsm]) by centrifugation at 300 × *g* for 5 min. The supernatant was discarded and the pellets were diluted with Extender 1 (20% [v/v] egg yolk and 3% [v/v] glycerol in a Tris diluent [pH 6.53, 740 mOsm]) to obtain a sperm concentration of 10^8/mL at room temperature. The diluted sperm was equilibrated to 4°C for 1 hour. One hour after beginning the cooling procedure, the diluted sperm was re-diluted (1:1, v/v) in Extender 2 (20% [v/v] egg yolk, 7% [v/v] glycerol, and 1% [v/v] Equex STM paste [Nova Chemical Sales, Scituate Inc., MA, USA] in a Tris diluent [pH 6.48, 1370 mOsm]). The extended sperm suspension was filled into 0.5-mL French straws (IMV, L'Aigle, France). Seventy-five min from the onset of cooling, the straws were placed horizontally in liquid nitrogen (LN_2) vapor in a rack 4 cm above the surface of the LN_2 in a closed styrene foam box (17 cm × 17 cm × 17 cm) for 10 min and then plunged into the LN_2. The straws were kept frozen in the LN_2 container for at least 1 week before being thawed for evaluation. The straws were thawed at different thawing rates as follows: 1) 37°C for 1 min (slow thawing) and 2) 70°C for 15 sec (rapid thawing). Sperm suspensions were diluted with Tris diluent (1:2 [v/v]) immediately after thawing. After holding the thawed sperm suspension at 37°C for 10 min, the thawed sperm were evaluated.

Sperm evaluation

Conventional sperm parameters

The percentages of total motile sperm and progressively motile sperm (sperm showing rapid steady forward [RSF] movement) were estimated using microscopic examination (5,20). The percentages of motile and progressively motile sperm were determined by observing a minimum of 300 sperm, in at least six different fields under a bright field microscope at 400 × magnification. The mean of six successive estimations was recorded as the final motility score. The viability of sperm was assessed by means of the eosin-nigrosin staining method (6). Viability was assessed by counting 200 sperm under a bright field microscope at 400 × magnification. Sperm displaying partial or complete purple staining were considered non-viable; only sperm showing strict exclusion of stain were counted as viable. The morphology of sperm was evaluated using the Diff-Quik kit (International Reagents Corp., Kobe, Japan). Briefly, a drop of sperm suspension was smeared on a glass slide, allowed to air-dry and fix. The slide was then stained with the Diff-Quik kit. A minimum of 200 sperm were evaluated under light microscopy at 1000 × magnification.

Sperm plasma membrane integrity

Sperm plasma membrane integrity was assessed using 6-carboxyfluoresceindiacetate (6-CFDA; Sigma-Aldrich, St. Louis, MO, USA)/propidium iodide (PI; Sigma-Aldrich) fluorescent staining (19). Briefly, 500 μL of the sperm sample (5×10^5 sperm/mL) was stained with 6-CFDA (5 μL of a 1 μg/mL) and PI (5 μL of a 0.1 mg/mL). Samples were then incubated at 37°C for 15 min and analyzed using flow cytometry. Analyzed sperm were classified as follows: intact acrosome and plasmalemma (CFDA+/PI−), intact acrosome but damaged plasmalemma (CFDA+/PI+), and damaged acrosome and plasmalemma (CFDA−/PI+).

Annexin V/PI binding assay

An Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instructions. The sperm suspension was centrifuged at 300 × *g* for 5 min and the supernatant was removed. The sperm pellet was resuspended in 1 × Annexin V binding buffer (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$) at room temperature to a concentration of 1×10^6 sperm/mL. Aliquots (100 μL each [1×10^5 cells]) of sperm suspension were transferred to 5 ml culture tubes. Five microliters of Annexin (AN) V-FITC and 5 μL of PI were added to the samples. The tubes were gently mixed and incubated at room temperature for 15 min in the dark. After incubation, an additional 1 × binding buffer (400 μl) was added to each tube. The flow cytometric analysis was conducted within 1 hour. The different labeling patterns in the Annexin V/PI analysis were classified as follows: viable and non-PS translocated (AN−/PI−); viable but PS translocated (AN+/PI−), non-viable and PS translocated (AN+/PI+), and non-viable and late necrotic (AN−/PI+). We defined the ratio between the AN+/PI− sperm and the total living (PI−) sperm as the PS translocation index (19).

Sperm intracellular H_2O_2

The present study used 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; Molecular Probes Inc., Eugene, OR, USA) fluorescent dye to estimate the intracellular H_2O_2 levels in the sperm with flow cytometry (12,14). Briefly, DCFDA (final concentration of 20 μM) and PI (final concentration of 1 μg/ml) were added to the sperm suspension (5×10^5/mL sperm). The mixture was incubated at 25°C for 60 min in the dark and the labeled sperm were analyzed by flow cytometry. Dead sperm were differentiated from viable sperm using a counter stain dye (PI). The Mean DCF Fluorescent Intensity (MFI) was measured to evaluate the intracellular mean H_2O_2 level per the total and the viable (PI−) sperm.

Flow cytometric analysis

All flow cytometry analyses were performed using a FACScalibur flow cytometer (Becton Dickinson, San José, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser and Cell Quest Pro software (Becton Dickinson). A total of 10,000 individual sperm-sized events were selected based on forward and side scatter and were collected at a flow rate < 200 events/second. FL1 signals (6-CFDA, AN V-FITC and DCF) were detected between a 500 and 530 nm band pass filter, and FL3 signals (PI) were detected through a > 630 nm band pass filter.

Table 1. Conventional sperm parameters according to thawing rate in cryopreserved sperm

Thawing rate	Motility, %		Viability, %	Abnormality, %				
	Total	Pro-gressive		Head	Acro-some	Mid-piece	Tail	Total
Slow	31.54 ± 3.94[a]	27.31 ± 3.70[a]	40.05 ± 3.89[a]	6.78 ± 1.02[a]	7.22 ± 1.96[a]	3.33 ± 1.01[a]	16.33 ± 4.03[a]	33.44 ± 3.93[a]
Rapid	38.46 ± 4.06[b]	34.23 ± 3.88[b]	44.60 ± 5.07[b]	5.22 ± 0.89[a]	6.00 ± 1.51[a]	3.00 ± 0.41[a]	14.56 ± 3.61[a]	28.33 ± 3.63[b]

Within a column, values with different superscripts differ significantly at $P < 0.05$ (n = 13, 10, and 9 for motility, viability, and abnormality, respectively).

Table 2. Sperm plasma membrane integrity detected by 6-CFDA/PI according to thawing rate in cryopreserved sperm

Thawing rate	CFDA/PI stain, %		
	CFDA+/PI– (Intact acrosome and plasmalemma)	CFDA+/PI+ (Intact acrosome but damaged plasmalemma)	CFDA–/PI+ (Damaged acrosome and plasmalemma)
Slow	26.05 ± 1.97[a]	5.26 ± 1.56[a]	69.95 ± 2.47[a]
Rapid	33.69 ± 3.11[b]	6.13 ± 2.08[a]	60.18 ± 4.33[b]

Within a column, values with different superscripts differ significantly at $P < 0.05$ (n = 8).

Statistical analysis

Statistical analysis of the data was performed using SPSS software (version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was utilized to evaluate normality. The Wilcoxon signed ranks test or compared *t*-test was used for analysis following determination of data normality. Statistical significance was set at $P < 0.05$ and all data were presented as the mean ± standard error of the mean (SEM).

Results

The percentages of the total motility, progressive motility, and viability were increased in the rapid-thawed group compared with those in the slow-thawed group ($P < 0.05$; Table 1). Likewise, the rapid-thawed group showed a decrease in the percentage of total abnormalities compared with that in the slow-thawed group ($P < 0.05$), although there were no significant differences between the rapid- and slow-thawed groups in the percentages of head, acrosome, midpiece and tail abnormalities (Table 1).

The percentage of sperm with intact plasma membranes was also increased in the rapid-thawed group compared with that in the slow-thawed group ($P < 0.05$; Table 2). The rapid-thawed group had an increased percentage of AN–/PI– (viable and non-PS translocated) sperm ($P < 0.05$), while the other PS-translocated parameters of rapid-thawed group were not significantly different from those of the slow-thawed group ($P < 0.05$; Table 3).

The percentages of total sperm with a high intracellular H_2O_2 level and MFI value per total sperm were not significantly different between the rapid- and slow-thawed groups ($P > 0.05$; Table 4). Furthermore, there were no significant differences between the rapid- and slow-thawed groups in the percentage of viable sperm with a high intracellular H_2O_2 level and the MFI value of viable sperm ($P > 0.05$; Table 4).

Table 3. Phosphatidylserine (PS) translocation detected by Annexin V/PI binding assay according to thawing rate in cryopreserved sperm

AN/PI staining patterns, %	Slow thawing	Rapid thawing
Annexin V–/PI–, (Live and non-PS translocated)	18.81 ± 2.85[a]	26.37 ± 2.56[b]
Annexin V+/PI– (Live and PS translocated)	2.79 ± 0.82[a]	3.33 ± 0.78[a]
Annexin V+/PI+ (Dead and PS translocated)	58.94 ± 12.14[a]	48.16 ± 11.53[a]
Annexin V–/PI+ (Dead and late necrotic)	19.47 ± 14.47[a]	22.14 ± 12.89[a]
PS translocation index	11.39 ± 2.74[a]	10.97 ± 2.08[a]

Different superscripts within a row indicate significant differences at $P < 0.05$ (n = 6).

Table 4. Measurement of intracellular hydrogen peroxide by 6-DCFDA/PI according to thawing rate in cryopreserved sperm

	Slow thawing	Rapid thawing
Including non-viable cells (Total [viable + dead] sperm)		
Sperm with high H_2O_2 level (%)	32.64 ± 13.54[a]	35.70 ± 12.10[a]
MFI value/total sperm	120.15 ± 19.42[a]	135.14 ± 28.24[a]
Excluding non-viable cells (Viable sperm)		
Sperm with high H_2O_2 level (%)	53.74 ± 13.64[a]	55.52 ± 13.02[a]
MFI value/viable sperm	211.17 ± 42.56[a]	216.81 ± 50.16[a]

Different superscripts within a row indicate significant differences at $P < 0.05$ (n = 5).

Discussion

Various authors have shown that a rapid thawing rate, achieved by submerging straws in water at 70-75°C resulted in a better post-thaw sperm quality compared to that thawed more slowly in water at 35-38°C (16-18). However, the evaluation methods have been limited to conventional sperm parameters when determining the effects of thawing rate. The main finding of this study is the improvement in membrane integrity as well as conventional sperm parameters in rapid-thawed sperm. A decrease in PS translocation with no increase in ROS level (intracellular H_2O_2) was observed in group thawed rapidly at 70°C compared to group thawed slowly at 37°C. These results indicate that the function of cryopreserved sperm could be improved with a simple change in thawing temperature. Thawing temperature is considered an important factor that may directly affect sperm quality in cryopreserved canine sperm.

Rapid thawing of sperm restricts the recrystallization of intracellular ice (24). The sperm that are thawed at a faster rate are exposed to the concentrated solute and cryoprotectant for a shorter time, and the restoration of the intra and extracellular equilibriums is more rapid than with slower thawing (4,9). However, rapid thawing may damage the sperm due to the rapid influx of water (24). The results of our study suggest that 70°C is a proper thawing temperature that does not produce recrystallization or rapid water influx into canine sperm.

Increasing the thawing temperature above 37°C could have detrimental effects on sperm viability due to an increase in the production and reactivity of ROS (3,8). However, in current study, an increase in the ROS level was not significantly observed between thawing at 70°C and thawing at 37°C. Thawing at 70°C improved sperm viability without an increase in ROS level compared thawing at 37°C. There were no significant differences in ROS level between the slow- and rapid-thawed sperm. However, the frozen-thawed canine sperm had a high ROS level compared to fresh sperm in our previous report (13), suggesting that the ROS level may not be controlled with change in thawing temperature or thawing temperature may not affect the ROS level. The reduction in ROS level could be tied to other mechanisms or induced from thawing itself regardless of thawing rate.

In conclusion, thawing at 70°C improved sperm function with no increase in ROS level compared to thawing at 37°C. The rapid thawing rate of 70°C was suitable for enhancement of sperm quality in frozen canine sperm. Further studies on thawing rate are necessary to produce adequate functioning of frozen sperm.

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융해 속도가 동결·융해된 개 정자의 기능에 미치는 영향

김수희 · 유도현 · 강태운 · 김용준[1]

전북대학교 수의과대학

요 약 : 정액 동결 방법들은 지속적으로 향상되어 왔지만 융해 속도에 대한 연구는 여전히 정립되어 있지 않다. 따라서, 본 연구의 목적은 동결 후 융해 속도가 정액의 기능에 미치는 영향을 알아보고자 하였다. 비글견으로부터 채취된 정액은 동결 보존 후 다른 융해 속도 (37°C/1분 or 70°C/15초)에서 융해 되었다. 융해 후, 운동성, 생존성, 정상 형태율, 형질막 온전성, phosphatidylserine (PS) translocation, 세포내 H_2O_2 수준을 평가하였다. 70°C에서 융해된 정자는 37°C에서 융해된 정자에 비해 향상된 정자 운동성, 생존성, 정상 형태율, 세포막 온전성, non-PS translocation을 보였으나($P < 0.05$), 70°C와 37°C에서 융해된 실험군간 세포내 H_2O_2 수준에서 유의적인 차이는 나타나지 않았다($P > 0.05$). 결론으로, 70°C에서의 융해는 개 정자 동결 후 정자 기능을 증진시켰으며, 적절한 융해 온도는 동결 정자의 기능을 향상시킬 수 있을 것으로 판단된다.

주요어 : 개 정자, 동결, 운동성, 융해 속도