Cryopreserving turkey semen in straws and nitrogen vapor using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality

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Running Head: Turkey semen cryopreservation by straws

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Abstract

1. This study was designed to identify a suitable protocol for freezing turkey semen in straws exposed to nitrogen vapor by examining the effects of dimethylacetamide (DMA) or dimethylsulfoxide (DMSO) as cryoprotectant (CPA), CPA concentration, freezing rate and thawing rate on *in vitro* post-thaw semen quality.
2. Pooled semen samples were diluted 1:1 (v:v) with a freezing extender composed of Tselutin diluent containing DMA or DMSO to give final concentrations of 8% or 18% DMA and 4% or 10% DMSO. The semen was packaged in 0.25 ml plastic straws and frozen at different heights above the liquid nitrogen (LN_2) surface (1, 5 and 10 cm) for 10 min. Semen samples were thawed at 4°C for 5 min or at 50°C for 10 seconds. After thawing, sperm motility, viability and osmotic tolerance were determined.
3. Cryosurvival of turkey sperm was affected by DMSO concentration. Freezing rate affected the motility of sperm cryopreserved using both CPAs, while thawing rates showed a significant effect on the motility of sperm cryopreserved using DMA and on the viability of sperm cryopreserved using DMSO. Significant interactions between freezing rate × thawing rate on sperm viability in the DMA protocol were found.
4. The most effective freezing protocol was the use of 18% DMA or 10% DMSO with freezing 10 cm above the LN_2 surface and a thawing temperature of 50°C. An efficient protocol for turkey semen would improve prospects for sperm cryobanks and the commercial use of frozen turkey semen.

Introduction

The cryopreservation and storage of germplasm has long been valued for the indefinite preservation of genetic material, especially in cases of high-risk populations. An immediate need for this practice was identified for research using unique poultry lines (Long and Kularni, 2004). Today, however, semen cryopreservation seems to be the only effective method of storing reproductive cells for the *ex situ* management of genetic diversity in birds (Blesbois, 2011; Kowalczyk and Łukaszewicz, 2015). Successful semen cryopreservation has enabled the creation of semen banks for several wild and some domestic chicken species and breeds (Saint Jalme *et al.,* 2003; Blackburn, 2006; Woelders *et al.,* 2006; Blesbois, 2007; Blanco *et al.,* 2009; Kowalczyk *et al.,* 2012). However, research efforts have not yet served to create a turkey semen cryobank. The possibility of using turkey semen in frozen form for artificial insemination (AI), besides maintaining and ensuring the long-term conservation of this bird's genetic diversity, would have practical benefits for turkey production.

Turkeys are the only commercial livestock species that depend entirely upon AI for fertile egg production. Hence, the turkey industry would greatly benefit if semen could be cryopreserved soon after its collection and used for subsequent AI (Rosato *et al.,* 2012). Protocols for cryopreserving turkey semen are unsatisfactory, leading to poor post-thaw sperm quality with obvious consequences on fertility (Blesbois, 2007; Iaffaldano *et al.,* 2011). Due to their different biophysical and biological characteristics, turkey spermatozoa are much more sensitive to damage caused by cooling, freezing and thawing than chicken semen (Blanco *et al.,* 2000, 2008; Blesbois, 2007; Iaffaldano *et al.,* 2011). Thus the freezing and thawing procedures developed for chickens or other birds are inefficient for turkey spermatozoa.

Researchers have turned their attention to developing freezing protocols for the improved cryopreservation of turkey semen by reducing the cell damage caused by freezing and thawing. Among the procedures tested, the pellet method has shown some promise. Recently, we optimised the pellet procedure by examining the effects of different combinations of critical steps (Iaffaldano *et al*., 2011). However, unlike straws, as a packaging system, pellets do not ensure sperm traceability or the safe transport of semen for breeding and the identification of each sample, which is required in cryobanks. Each cryopreservation procedure has its own particular variables influencing sperm cryosurvival.

Numerous factors may affect the success of turkey semen cryopreservation although a decisive role is played by combinations of factors such as the cryoprotectant (CPA) used and its concentration, the speed of freezing and the packaging system, as mentioned by several authors (Tselutin *et al.,* 1995; Blanco *et al.,* 2011, 2012; Iaffaldano *et al.,* 2011; Long *et al.,* 2014).

Optimal freezing and thawing rates minimize the damage caused by intracellular ice formation, cell shrinkage and exposure to multiple osmotic gradients, and these factors are critical for developing successful semen cryopreservation protocols. The effects of freezing rates on the quality of cryopreserved chicken sperm have been established (Blanco *et al.,* 2000; Woelders *et al.,* 2006) though some of these data are still lacking for turkey sperm (Blanco *et al.,* 2012).

The most important factors for an effective freezing protocol are the choice of CPA and its concentration. The CPAs mainly involved in freezing protocols for turkey semen are glycerol, dimethylsulfoxide (DMSO), ethylene glycol, and dimethylacetamide (DMA) (Blesbois, 2007; Iaffaldano, 2015). DMA and DMSO have been used as alternative CPA to glycerol because of its contraceptive effect (Hammerstedt and Graham, 1992; Blanco *et al*., 2000). DMA was largely adopted as CPA for turkey semen cryopreservation using rapid or low freezing-thawing procedures and pellets or packaging in straws (Blanco et al., 2011, 2012; Iaffaldano et al., 2011; Long et al., 2014), whereas little is known about the use of DMSO.

There is a clear need to standardise the complete freezing and thawing process to improve the post-thaw quality of turkey semen and minimise variability in results. This study aimed to identify a suitable protocol for the in-straw freezing in nitrogen vapour of turkey semen using DMA or DMSO as CPA without any special freezing equipment.

We tested the effects of two concentrations of DMA or DMSO and different freezing and thawing rates on *in vitro* post-thaw semen quality.

Materials and methods

Experimental design

The model used for the cryopreservation of the turkey semen for both CPAs (DMA or DMSO) was a 2 × 3 × 2 design as follows: CPA concentration (8 and 18% DMA, 4 and 10% DMSO), freezing rate (three different heights, 1, 5 and 10 cm, above the liquid nitrogen level) and thawing rate (4°C for 5 min and 50°C for 10 s). Samples of pooled turkey semen were processed for freezing using the full combinations of these factors.

Chemicals

The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes, Inc. (Eugene, OR, USA). DMSO, DMA and all the other chemicals used in this study were purchased from Sigma Chemical Co. (Milan, Italy).

Birds

A total of 50 turkey males of the Hybrid Large White line supplied by Agricola Santo Stefano (Amadori Group, TE, Italy). Turkeys were reared in a poultry house in a controlled environment with artificial lighting (14 h light-10 h dark cycle) and given free access to a standard commercial feed and water. The 7-week trial was started when the birds were 45 weeks of age.

Semen processing

Semen was collected once a week by abdominal massage, yellow and abnormal semen samples were discarded. Ejaculates were pooled (1 ejaculate/male; 4–6 ejaculates/pool) to avoid the effects of individual differences among males.

Seven pools were used, each containing at least 4 ml of semen and an average concentration of $10.12 \pm 0.32 \times 10^9$ spz/ml.

The quality of the fresh semen was assessed in an aliquot taken from each pool as described below and the remaining undiluted semen pool was cooled at 4°C for 25 min before freezing.

After cooling, the pools of turkey semen were diluted 1:1 (v:v) with a pre-cooled freezing extender composed of Tselutin diluent (Tselutin *et al.*, 1995) containing DMA or DMSO (as permeable CPAs) to give final concentrations of 8% and 18% DMA, and 4% and 10%

DMSO. The extended semen was packaged in 0.25 ml plastic straws that were sealed with polyvinyl chloride powder (PVC). The straws grouped by treatment and equilibrated at 4°C for 20 min (equilibration time). Semen was frozen by exposure to liquid nitrogen vapor at different heights above the liquid nitrogen surface (1, 5 and 10 cm) for 10 min to give three different freezing rates. During these 10 min, the temperature of straws at 1 cm fell from +4°C to -140°C, at 5 cm from +4°C to -125°C and at 10 cm from +4°C to -90°C, indicating a slower freezing rate as the distance from the liquid nitrogen increases. Temperatures were monitored by a temperature sensor (Ascon M1). Subsequently, the straws were plunged into liquid nitrogen for storage at -196°C. Sperm samples were thawed by immersion of the straws in water bath: 1) at 4°C for 5 min; or 2) at 50°C for 10 s.

Spermatozoa quality

In both the fresh and thawed semen samples, spermatozoa motility, viability and osmotic tolerance were determined in duplicate. Spermatozoa motility was subjectively evaluated by visual estimation. A 5 µl-drop was diluted in 45 µl of Tselutin extender, and then 5 µl of extended semen was deposited on a clean glass slide prewarmed to 38°C and covered with a coverslip. The mounted slides were observed on a warm-plate at × 400 magnification using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany). Percentage motility was estimated in five microscopy fields.
Spermatozoa viability was determined as described previously by Rosato *et al.* (2012) using the fluorescent stains SYBR-14 and propidium iodide (PI). This procedure was performed on 5 µl of semen, which were added to 80 µl of extender containing 2 µl SYBR-14 (diluted 1:100 in DMSO). The extended semen was then incubated at 38°C for 10 min,

and 5 µl PI (diluted 1:100 in PBS) added followed by incubation at 38°C for a further 5 min. Next, 10 µl of the suspension were placed on microscope slides, covered with a coverslip and viable/non-viable spermatozoa were determined by fluorescence microscopy (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany; blue excitation filter λ = 488 nm; × 100 oil immersion objective; total magnification × 1000). SYBR-14 is a membrane-permeable DNA stain for live spermatozoa producing bright green fluorescence of nuclei. PI stains the nuclei of membrane-damaged cells red, so that spermatozoa showing green fluorescence are recorded as live and those fluorescing red as dead. After counting at least 200 spermatozoa, percentages of viable spermatozoa were calculated as the ratio: green cells/(green cells + red cells) × 100.

To determine the osmotic tolerance of the sperm membrane, a hypo-osmotic swelling test (HOST) was used (Iaffaldano *et al.,* 2011). Aliquots of 5 µl of diluted semen were added to 80 µl of distilled H_2O and then stained with SYBR and PI and counted as described above for sperm viability. This test is effective for assessing the percentage of viable spermatozoa that are capable of withstanding hypo-osmotic stress *in vitro*. Under hypo-osmotic conditions, viable thawed spermatozoa with intact membranes will fluoresce green (SYBR) and exclude PI. Conversely, damaged membranes permit the passage of PI, staining spermatozoa that have lost their functional integrity red.

Statistical analysis

To compare the different treatments, we used a randomized block design in a 2 × 3 × 2 factorial arrangement (2 CPA concentrations × 3 freezing rates × 2 thawing rates), with 7 replicates per treatment.

Sperm variables (motility, viability and osmotic tolerance) were compared among the treatments by ANOVA followed by Duncan's comparison test. A generalized linear model procedure was then used to determine the fixed effects of CPA concentration, freezing rate, thawing rate and their interactions on the sperm quality variables. Significance was set at $P \leq 0.05$. All statistical tests were performed using SPSS software (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA).

Results

Spermatozoa motility in fresh semen was 77.2 ± 2.0 %, sperm viability and sperm osmotic tolerance were 78.8 ± 1.3 % and 58.9 ± 2.2, respectively.

Semen quality variables assessed after thawing were motility, viability and osmotic tolerance (Tables 1 and 2). The fixed effects of CPA concentration, freezing rate, thawing rate and their interactions on sperm quality variables for the DMA freezing protocol are shown in Table 1. Effects of freezing and thawing rates ($P \leq 0.05$) were detected on sperm motility, while a significant effect interaction between freezing rate × thawing rate was produced on sperm viability.

Regarding the remaining treatments, the greatest motile sperm percentages ($P<0.05$) were recorded for semen frozen 10 cm above the liquid nitrogen level in the presence of 18% DMA and thawed at 50°C (18% DMA/10 cm/50°C) with the exception of the treatment combination 8% DMA/10 cm/50°C. Lowest motile sperm percentages were observed for the treatments 8% DMA/5 cm/4°C and 8% DMA/5 cm/50°C.

Higher sperm viability percentages were observed also for the combinations 18% DMA/10 cm/50°C or 4°C with no significant difference between the two or with respect to the other treatments except 8% DMA/5 cm/50°C. The fixed effects of CPA concentration, freezing rate, thawing rate and their interactions observed on the spermatozoa quality variables for the DMSO freezing protocols are provided in Table 2. An effect of CPA concentration was observed on all the sperm quality variables examined. In addition, the freezing rate affected sperm motility, while the thawing rate significantly affected sperm viability. Better post-thaw sperm motility was recorded for semen frozen using the treatment combination 10% DMSO/10 cm/50°C with respect to all the other treatment combinations ($P<0.05$). The best treatment combination in terms of effects on viability was also 10% DMSO/10 cm/50°C and showed significance with respect to all other treatment combinations with the exceptions 10% DMSO/10 cm/4°C and 10% DMSO/5 cm/50°C. Higher rates of sperm osmotic tolerance were observed for 10% DMSO/10 cm/50°C versus 4% DMSO/1 cm/4 and 50°C or 4% DMSO/5 cm/4 and 50°C.

Discussion

This study sought to identify effective freezing protocols for the cryopreservation of turkey semen using straws and nitrogen vapour, and DMA or DMSO as the CPA. The treatment combinations that were most effective for the DMA protocol were: a CPA concentration of 18% DMA, sample freezing 10 cm above the liquid nitrogen (LN_2) surface and a thawing temperature of 50°C. This combination (18% DMA/10 cm/50°C) returned recovery rates (value in frozen semen/value in the fresh semen × 100) of about 30.5% for sperm viability, 27.5% for sperm motility and 26% for sperm osmotic tolerance. For DMSO, the best treatment combination was 10% DMSO/10 cm/50°C which yielded recovery rates of 47% viability, 53% motility and 42% osmotic tolerance. As previously reported in the literature, many factors may affect the success of semen cryopreservation including the freezing medium, CPA and its concentration, along with the freezing and thawing conditions (Iaffaldano, 2015), all of which affect sperm structure and function of spermatozoa (Garner *et al.,* 1999; Bailey *et al.,* 2003). In particular, the combination of these factors plays an important role (Tselutin *et al.,* 1995; Blanco *et al.,* 2011, 2012; Iaffaldano *et al.,* 2011; Long *et al.,* 2014). The choice of CPA is among the most important factors for an effective turkey semen freezing protocol. In this study, DMA and DMSO as an alternative to glycerol because this compound has to be removed from the semen before insemination due to its contraceptive effect (Hammerstedt and Graham, 1992).

Both DMSO and DMA are penetrating CPAs. Such CPAs are membrane-permeable solutes that act intra- and extracellularly, causing the dehydration of spermatozoa because of an osmotically driven flow of water, which varies according to CPA composition (Purdy, 2006). Penetrating CPAs also cause membrane lipid and protein reorganization. This improves membrane fluidity causing greater dehydration at lower temperatures, and thus an increased ability to survive cryopreservation (Holt, 2000).

Permeable CPAs may paradoxically have a toxic effect on sperm, causing membrane destabilization and protein and enzyme denaturation. This toxicity is directly related to the CPA concentration used and the time of cell exposure (Swain and Smith, 2010; Iaffaldano *et al.,* 2014). In the present study, an effect of CPA concentration on post-thaw semen quality was observed although this was only significant for DMSO. Thus, concentrations of 18% DMA and 10% DMSO better protect the spermatozoa from cryodamage. It is assumed that these CPA concentrations were, on one hand, able to increase osmolarity to suitably dehydrate the cells avoiding ice crystal formation during cryopreservation and, on the other, produced no toxic effects. Blanco *et al*. (2011) using cryovials as the packaging system also reported 18% DMA out of different concentrations tested (6%, 10%, 18%, 24%, 26%) as the most effective in protecting in vitro post-thaw semen quality. Compared with the present results, Long *et al*. (2014) recorded higher intact sperm-membrane and similar motility rates using 6% DMA, although work conditions differed. Little is known about the use of DMSO as CPA for freezing turkey semen. Published results only exist for studies performed around the 1980's and the semen processing conditions, and *in vitro* sperm quality were not always specified (Bakst and Sexton, 1979; Sexton, 1981). The best DMSO freezing protocol in the present study gave rise to a better quality of semen than the DMA freezing protocol previously identified as best. Although both DMA and DMSO are permeable CPAs and share many physical-chemical properties, their different molecular structures confer different permeabilities in a given phospholipid bilayer. This could account for variations in the relative permeability of the turkey sperm membrane and thus explain their relative cryoprotection efficiencies as reported by the present authors for the cryopreservation of rabbit semen (Iaffaldano *et al.,* 2012). Although it was observed that DMSO performs better than DMA (data not shown), there is still a need to further improve post-thaw semen quality by also including non-permeable CPAs in semen freezing protocols and to test both DMSO and DMA *in vivo*.

Another step that emerged here as critical for semen cryopreservation was the freezing rate. The present results revealed an effect of freezing rate only on sperm motility for both CPAs tested. Loaded straws frozen 10 cm above the liquid nitrogen surface returned better post-thaw sperm motility results compared to other heights.

It is hypothesized that the slower freezing rate (10 cm) led to reduction of ice crystals formation owing to a better cellular dehydration and adequate cell shrinkage. This finding is consistent with previous reports that the cooling rate is crucial and that inaccurate cooling rates can negatively affect sperm survival, motility, plasma membrane integrity and mitochondrial function (Henry *et al.,* 1993). A sufficiently slow cooling rate means there is sufficient time for intracellular water efflux and balanced dehydration. However, if cooling is too slow, damage may occur due to exposure of cells to high concentrations of intracellular solutes. Extreme cellular dehydration leads to shrinkage of cells below the minimum cell volume necessary to maintain its cytoskeleton, genomic structures, and ultimately cell viability (Mazur, 1984). Conversely, if cooling rates are too fast, external ice can induce intracellular ice formation and potential rupture of the plasma membrane, thus damaging intracellular organelles. In addition, mechanical damage to cells is possible due to extracellular ice compression and a close proximity of frozen cells can lead to cellular deformation and membrane damage (Fujikawa and Miura, 1986). Blanco *et al.* (2012) also observed using cryovials and a programmable freezer better semen quality when turkey semen was frozen via a moderate (5°C/min from +4°C to −70°C) or slow (1°C/min from +4°C to −20°C) cooling rate compared to rapid cooling (plunging directly into liquid nitrogen). Conversely, the best results using 10 cm above liquid nitrogen (slower freezing rate) were poorer than those registered by Long *et al*. (2014) using a height above liquid nitrogen of 1.25 cm (faster freezing rate) though their experimental conditions differed from those of present study.

A further factor that emerged as critical was warming temperature; a higher warming temperature (50°C) over a shorter period (10) was better than longer exposure to a cooler temperature (4°C for 5 min).

During thawing, sperm cells suffer additional damage due to recrystallization. Recrystallization refers to the growth of large ice crystals from small crystals. This process exerts additional tension on entrapped proteins and causes further cell damage (Cao *et al.,* 2003). Rapid thawing improves survival (Farrant, 1980) by avoiding recrystallization, while slow thawing is more damaging because of a longer total exposure time to sub-zero ice temperatures (Mazur, 2004).

Many factors may affect the success of turkey semen cryopreservation, while a given combination of factors is important, such as the CPA used and its concentration, the freezing and thawing rate and freezing vehicle as reported by Tselutin *et al.* (1995) and Iaffaldano *et al.* (2011). In the present study, a significant interaction effect on sperm viability was only observed for freezing rate × thawing rate when DMA was present as the CPA.

In conclusion, these findings have identified an effective method for freezing semen from Hybrid Large White turkeys but it need to be further evaluated with fertility trials and tested on other turkey lines.

Acknowledgements

The authors thank Carmine Marini for help with semen collection, the Amadori group for allowing us to use their breeders, Luca Romagnoli for help with the statistical treatment of data, and Ana Burton for editorial assistance.

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Table captions and footnotes

Table 1. Sperm quality variable (mean ± SE) recorded for semen frozen using DMA as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).
[a-c]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).
CPA: cryoprotectant; DMA: dimethylacetamide; LN2: liquid nitrogen.

Table 2. Sperm quality variable (mean ± SE) recorded for semen frozen using DMSO as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).
[a-c]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).
CPA: cryoprotectant; DMSO: dimethylsulfoxide; LN_2: liquid nitrogen.

Table 1. *Sperm quality variable (mean ± SE) recorded for semen frozen using DMA as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).*

Semen treatment			Sperm variable (%)		
DMA concentration (%)	Freezing rate (cm above LN_2)	Thawing rate (°C × min or s)	Motility	Viability	Osmotic tolerance
8	1	4	10.71 ± 1.57^{bc}	22.28 ± 1.51^{a}	12.22 ± 1.22^{a}
8	1	50	15.07 ± 2.46^{bc}	24.65 ± 1.70^{a}	10.01 ± 1.64^{a}
8	5	4	10.21 ± 1.55^{c}	22.51 ± 1.91^{a}	12.31 ± 1.90^{a}
8	5	50	10.28 ± 1.49^{c}	17.08 ± 1.49^{b}	10.06 ± 0.96^{a}
8	10	4	13.14 ± 1.57^{bc}	22.58 ± 1.00^{a}	14.76 ± 2.08^{a}
8	10	50	16.93 ± 1.59^{ab}	20.97 ± 0.82^{ab}	13.24 ± 1.35^{a}
18	1	4	11.71 ± 1.13^{bc}	20.72 ± 1.03^{ab}	13.32 ± 2.19^{a}
18	1	50	13.57 ± 2.20^{bc}	24.09 ± 1.59^{a}	15.12 ± 2.21^{a}
18	5	4	10.50 ± 1.14^{bc}	22.22 ± 1.60^{a}	12.61 ± 1.34^{a}
18	5	50	12.92 ± 3.14^{bc}	22.26 ± 1.68^{a}	12.19 ± 1.53^{a}
18	10	4	14.78 ± 2.70^{bc}	25.48 ± 2.91^{a}	13.84 ± 1.11^{a}
18	10	50	21.28 ± 2.05^{a}	24.12 ± 1.17^{a}	15.40 ± 1.71^{a}
Concentration effect			P = 0.223	P = 0.122	P = 0.089
Freezing rate effect			P = 0.001	P = 0.111	P = 0.099
Thawing rate effect			P = 0.007	P = 0.642	P = 0.595
Concentration × freezing rate effect			P = 0.512	P = 0.165	P = 0.544
Concentration × thawing rate effect			P = 0.709	P = 0.236	P = 0.124
Freezing rate × thawing rate effect			P = 0.385	P = 0.045	P = 0.826
Concentration × freezing rate × thawing rate effect			P = 0.584	P = 0.474	P = 0.896

[a-c]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).

CPA: cryoprotectant; DMA: dimethylacetamide; LN_2: liquid nitrogen.

Table 2. *Sperm quality variable (mean ± SE) recorded for semen frozen using DMSO as cryoprotectant according to CPA concentration, freezing rate and thawing rate (N = 7).*

Semen treatment			Sperm variable (%)		
DMSO concentration (%)	Freezing rate (cm above LN_2)	Thawing rate (°C × min or s)	Motility	Viability	Osmotic tolerance
4	1	4	14.36 ± 1.63^{e}	25.16 ± 2.70^{d}	14.68 ± 1.97^{b}
4	1	50	17.93 ± 1.50^{de}	27.52 ± 2.41^{cd}	15.81 ± 2.41^{b}
4	5	4	20.14 ± 1.34^{cde}	25.85 ± 2.55^{cd}	16.34 ± 1.38^{b}
4	5	50	17.71 ± 2.25^{de}	30.90 ± 2.99^{bcd}	17.40 ± 2.78^{b}
4	10	4	22.00 ± 1.98^{bcd}	25.75 ± 3.01^{cd}	18.78 ± 1.45^{ab}
4	10	50	23.57 ± 3.60^{bcd}	31.09 ± 4.95^{bcd}	19.36 ± 2.61^{ab}
10	1	4	21.35 ± 2.38^{bcde}	30.52 ± 2.04^{bcd}	17.92 ± 2.11^{ab}
10	1	50	24.78 ± 2.83^{bcd}	33.31 ± 2.57^{bcd}	19.50 ± 1.66^{ab}
10	5	4	26.57 ± 3.12^{bc}	31.61 ± 2.16^{bcd}	20.31 ± 2.29^{ab}
10	5	50	28.50 ± 3.20^{b}	36.83 ± 3.40^{ab}	21.93 ± 2.66^{ab}
10	10	4	28.57 ± 2.58^{b}	34.91 ± 2.03^{abc}	20.21 ± 2.20^{ab}
10	10	50	36.57 ± 1.78^{a}	42.10 ± 1.50^{a}	25.15 ± 2.66^{a}
Concentration effect			P = 0.000	P = 0.000	P = 0.004
Freezing rate effect			P = 0.000	P = 0.102	P = 0.053
Thawing rate effect			P = 0.056	P = 0.006	P = 0.162
Concentration × freezing rate effect			P = 0.699	P = 0.452	P = 0.965
Concentration × thawing rate effect			P = 0.203	P = 0.804	P = 0.488
Freezing rate × thawing rate effect			P = 0.308	P = 0.640	P = 0.875
Concentration × freezing rate × thawing rate effect			P = 0.613	P = 0.975	P = 0.780

[a-e]Different superscript letter within the same column indicates a significant difference ($P < 0.05$).

CPA: cryoprotectant; DMSO: dimethylsulfoxide; LN_2: liquid nitrogen.