J Anim Reprod Biotechnol 2020;35:307-314
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.35.4.307

Original Article

Spermatozoa motility, viability, acrosome integrity, mitochondrial membrane potential and plasma membrane integrity in 0.25 mL and 0.5 mL straw after frozen-thawing in Hanwoo bull

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Received October 20, 2020
Revised November 17, 2020
Accepted November 30, 2020

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ABSTRACT In the present study, we examined the effect of straw size on spermatozoa motility, viability, acrosome integrity, mitochondrial membrane potential, and plasma membrane integrity after freezing-thawing. Hanwoo semen was collected from three bulls and diluted with an animal protein-free extender, divided into two groups, namely, 10 million spermatozoa in 0.25 mL and 20 million spermatozoa in 0.5 mL straw, and cryopreserved. In Experiment 1, the motility and motility parameters of the frozen-thawed spermatozoa were evaluated. After freezing-thawing, the spermatozoa motility parameters fast progressive, straight line velocity, and average path velocity were compared between the 0.25 mL straw and 0.5 mL straw groups. They were 35.2 ± 1.0 and 32.3 ± 0.7%, 34.6 ± 0.7 and 31.8 ± 0.5 μm/s, 51.4 ± 1.3 and 47.1 ± 1.1 μm/s, 0.25 mL straw and 0.5 mL straw groups, respectively. In Experiment 2, the viability, acrosome membrane integrity, and mitochondrial membrane potential of the frozen-thawed spermatozoa were assessed. After freezing-thawing, the percentages of spermatozoa with live, intact acrosomes and high mitochondrial membrane potential were compared between the in 0.25 mL straw and 0.5 mL straw groups. They were 48.0 ± 2.6% and 35.6 ± 2.8% between the 0.25 mL straw and 0.5 mL straw groups. In Experiment 3, the plasma membrane integrity of frozen-thawed spermatozoa was compared. After freezing-thawing, the plasma membrane integrity was higher for the in 0.25 mL straw group than the 0.5 mL straw group. They were 62.0 ± 2.2 and 54.1 ± 1.3% between the 0.25 mL straw and 0.5 mL straw groups. In conclusion, our results suggest that freezing semen in 0.25 mL straw improves the relative motility, viability, and acrosomal, mitochondrial membrane potential, and plasma membrane integrity of Hanwoo bull spermatozoa.

Keywords: bull, freezing-thawing, spermatozoa, straw size

INTRODUCTION

Natural mating with superior bulls and artificial insemination (AI) using semen collected from elite bulls have been globally applied in cattle breeding (Lima et al., 2009). However, natural mating may spread diseases borne by bulls. The adoption of AI in cattle reproduction mitigates the risk of disease transmission via bulls and lowers breeding costs (Valergakis et al., 2007). Elite bull semen may be transported to other states within the same

country and to other countries (López-Gatius, 2012). The desired phenotypes and genes may be transferred to the offspring (Berry et al., 2020). In AI, semen is collected from elite bulls, diluted with suitable semen extenders, and stored in liquid or frozen solid form (Vishwanath and Shannon, 2000). Liquid semen is used in dairy cow reproduction with short (2-mo) short breeding seasons. In Ireland and New Zealand, it is adjusted according to pasture growth (Butler, 2014) and grass nutrient levels (Yang et al., 2018). However, liquid semen in extender can only provide conception rates comparable to those of frozen-thawed semen within the first 3 d (Vishwanath and Shannon, 2000). In contrast, frozen semen is semi-permanently cryopreserved in liquid nitrogen (LN_2) and is generally used more often than liquid semen in AI (Layek et al., 2016). In general, semen collected from elite sires is diluted with semen extenders, filled in 0.25 mL or 0.5 mL straw, and cryopreserved in LN_2 before use (Diskin, 2018). About 10-30 million spermatozoa are filled in 0.25 mL or 0.5 mL straw, distributed to farms, and used for AI worldwide (Stevenson et al., 2009). The 0.25 mL straw has half the volume of the 0.5 mL straw and more than two 0.25 mL straws can be preserved in the LN_2 tank (Johnson et al., 1995). AI technicians and dairy and beef farm owners are often concerned that the low spermatozoa may reduce the conception rate. Nevertheless, there was no reduction in the conception rate after the introduction of 10-30 million spermatozoa for AI (Stevenson et al., 2009).

The Hanwoo Bull Center (Hanwoo Improvement Center, NH, Seosan, Korea) selected elite Hanwoo bulls and produced ~10 million frozen straws per Hanwoo bull lifespan. The Hanwoo Bull Center reported that it packed 18 million spermatozoa per 0.5 mL straw and distributed the frozen straws to all parts of Korea. Asia, Central and South America, and the United States produced 0.5 mL frozen straw. Canada and Europe commercially produced 0.25 mL frozen straw (Diskin, 2018). If 0.25 mL straw is substituted for 0.5 mL straw in semen freezing, the space in the LN_2 cryopreservation tank can be doubled. Moreover, frozen straw production time and extender consumption can be reduced. However, the 0.25 mL straw has a larger exposure space than the 0.5 mL straw. Furthermore, the former is more sensitive than the latter to the external LN_2 tank temperature and air currents. Therefore, short application time and minimum temperature change are required when 0.25 mL frozen straw is used in AI (Johnson et al., 1995). Despite these precautions, 0.25 mL straw has several advantages over 0.5 mL straw especially when the semen is diluted with *tris*-citric acid extender supplemented with 20% egg yolk (Anzar et al., 2011). For dairy bulls, frozen-thawed spermatozoa in 0.25 mL straw showed high motility and viability, low incidence of damaged acrosomal membranes (Senger et al., 1983), and high mitochondrial membrane potential compared to frozen-thawed spermatozoa in 0.5 mL straw (Anzar et al., 2011). Until recently, Hanwoo bull semen was only packed in 0.5 mL straw and to the best of our knowledge there were no reports comparing the properties of frozen-thawed spermatozoa in 0.25 mL and 0.5 mL straw.

In the present study, then, we investigated the effects of packing semen in 0.25 mL and 0.5 mL straw after freezing-thawing and compared their relative preservation efficacy. In Experiment 1, we compared the motility and motility parameters of frozen-thawed spermatozoa packed in 0.25 mL and 0.5 mL straw. To this end, we used a computer sperm analysis system. In Experiment 2, we examined the viability, acrosomal membrane integrity, and mitochondrial membrane potential of spermatozoa in 0.25 mL and 0.5 mL straw. For this purpose, we used fluorescent staining after freezing-thawing. In Experiment 3, we applied the hypoosmotic swelling test to study the plasma membrane integrity of spermatozoa in 0.25 mL and 0.5 mL straw after freezing-thawing.

MATERIALS AND METHODS

Bull semen collection and cryopreservation

Bull semen was collected by an electro-ejaculator machine (Electro Jac 6; Neogen Corp., Lansing, MI, USA) between February and March 2020 at the Hanwoo Research Institute, NIAS, RDA, Pyeongchang, Korea. Three bulls aged 15 mo with mean weight 461.0 ± 47.8 kg were used for semen collection. Bulls A and B had two ejaculates each and bull C had one ejaculate. Semen volume, spermatozoa concentration and pH of raw semen were examined before dilution. Spermatozoa motility and motility parameters of raw semen were evaluated after dilution with semen extender and presented in Table 1. Spermatozoa motility was evaluated with a computer-assisted analytical system (CASA; sperm class analyzer; Microoptic SL, Barcelona, Spain). Over 90% of the motile spermatozoa sample was introduced for semen freezing. In brief, the

Table 1. The basic characteristics of raw semen, spermatozoa motility and motility parameters after dilution

Raw semen information	Mean value (5 replicates)
Volume (mL)	3.8 ± 0.7
Sperm concentration (× 10^6 cells/mL)	828.5 ± 252.6
pH	7.9 ± 0.2
Total motile (%)	99.7 ± 0.1
Fast progressive (%)	27.0 ± 3.7
Slow progressive (%)	65.7 ± 4.3
Non-progressive (%)	7.1 ± 1.2
Immotile (%)	0.3 ± 0.1
VCL (μm/s)	140.3 ± 1.1
VSL (μm/s)	39.7 ± 3.2
VAP (μm/s)	79.5 ± 5.5
LIN (%)	28.6 ± 2.1
STR (%)	50.0 ± 2.6
ALH (μm/s)	4.0 ± 0.5
BCF (Hz)	15.3 ± 0.7

Mean ± SE. Volume, sperm concentration and pH of raw semen were examined after semen collection. Spermatozoa motility and motility parameters were examined after dilution with semen extender. Values indicate mean of 5 replicates from each ejaculates.

semen was diluted with animal protein-free diluent (Optixcell; IMV Technologies, L'Aigle, France). The spermatozoa concentration was adjusted to 40 × 10^6 cells/mL and the dilution was cooled in a refrigerator at 4℃ for 3-4 h. The diluted semen was then introduced into 0.25 mL straw (10 × 10^6 spermatozoa/straw) and 0.5 mL straw (20 × 10^6 spermatozoa/straw). Spermatozoa density was identical for both the 0.25 mL and 0.5 mL straw. Straws were preserved by immersion for 14 min at 3 cm above the surface of LN_2 in a styrofoam box. The straws were then dropped in LN_2. The frozen straws were cryopreserved in LN_2 tanks until spermatozoa motility and characteristics were evaluated.

Spermatozoa motility and motility parameters after freezing-thawing

Spermatozoa motility was examined as previously described (Yang et al., 2015; Kang et al., 2016), with slight modifications. In brief, frozen semen was immersed in water at 37.5℃ for 40 s and mixed in a 1.5 mL tube. Then 3 μL frozen-thawed semen was introduced to the chamber of a microscope slide (SC 20-01-04-B; Leja, Nieuw-Vennep, Netherlands). At least 800 spermatozoa in 4-5 fields per slide chamber were counted and spermatozoa motility and motility parameters were evaluated by CASA. Spermatozoa motility and the percentages of total motile, fast progressive, slow progressive, non-progressive, and immotile spermatozoa were evaluated. The other spermatozoa motility parameters assessed included curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), amplitude of lateral head (ALH, μm/s), and flagellar beat cross frequency (BCF, Hz).

Spermatozoa viability, acrosomal membrane integrity, and mitochondrial membrane potential of after freezing-thawing

Spermatozoa characteristics were measured according to a previous report (Celeghini et al., 2007), with a modification. Frozen-thawed semen in 0.25 mL and 0.5 mL straw was transferred to a 1.5 mL tube and vortexed for 3 s. One hundred microliters frozen-thawed semen was diluted with 900 μL pre-warmed DPBS (-) at 37℃. One hundred microliters diluted semen was mixed with 150 μL of 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide (JC-1; mitochondrial membrane potential detection kit; Cell Technology Inc., Danvers, MA, USA) working solution and incubated for 30 min at 37℃ in the dark. After incubation, 1 μL Hoechst 33342 (H1339; Molecular Probes, Eugene, OR, USA) stock solution was mixed with diluted semen and incubated for 10 min at 37℃ in the dark. Then 1 μL propidium iodide (PI; P-4172; Sigma-Aldrich Crop., St. Louis, MO, USA) stock solution and 1 μL fluorescein peanut agglutinin FITC conjugate (FITC-PNA; FL-1071; Vector Laboratories, Piedmont, Italy) were mixed with diluted semen and incubated for 8 min at 37℃ in the dark. Two microliters of 10% (v/v) formaldehyde was added to the stained semen mixture to impede spermatozoa movement (Harrison and Vickers, 1990). Five microliters stained semen was mounted on a slide glass and covered with a cover slip. More than 200 spermatozoa per microscope slide were counted at × 400 magnification under a fluorescent microscope (Eclipse Ti; Nikon, Tokyo, Japan). Live and dead spermatozoa, acrosomal membrane integrity, and mitochondrial membrane potential were assessed with a triple band filter (DAPI/FITC/TRITC; Nikon, Tokyo, Japan). The heads of live spermatozoa were stained with Hoechst 33342 (blue) while those of dead spermatozoa were stained with PI (red). High mitochondrial membrane potential was indicated by

orange JC-1 staining of the spermatozoa midpiece. Low mitochondrial membrane potential was indicated by faint orange or no JC-1 staining of the spermatozoa midpiece. Damaged acrosomal membranes were stained with FITC-PNA (green) at the anterior spermatozoa head while intact acrosomal membranes were not stained at the anterior spermatozoa head.

Fluorescence preparation

One milligram Hoechst 33342 was diluted with 960 μL DPBS (-) and 40 μL dimethyl sulfoxide (DMSO) and cryopreserved at -20℃. The JC-1 stock solution contained 1 mg/mL DMSO in a mitochondrial membrane potential detection kit. To prepare the JC-1 working solution, 5 μL JC-1 stock solution at -20℃ and 500 μL DPBS (-) were mixed and cryopreserved at -20℃. PI stock solution was prepared by mixing 25 mg PI and 1 mL DMSO, diluting this mixture to 2 mg/mL with DPBS (-), and cryopreserving the dilution at -20℃. All fluorescent probe stock solutions except FITC-PNA were stored at -20℃ in the dark. The latter was preserved at 4℃. A 10% (v/v) formaldehyde working solution was prepared by mixing 2.9 mL of 35% (v/v) formaldehyde (Daejung Chemical & Metals Co., Gyeonggi-do, Korea) with 7.1 mL DPBS (-) and storing the dilution at 4℃.

Spermatozoa plasma membrane integrity after freezing–thawing

Spermatozoa plasma membrane integrity was determined as previously described (Kang et al., 2019). After freezing-thawing semen in 0.25 mL and 0.5 mL straw, the thawed semen was transferred to a 1.5 mL tube. Thirty microliters frozen-thawed semen was diluted with 300 μL hypoosmotic swelling test solution (Correa and Zavos, 1994) and incubated for 40 min at 37℃. Five microliters incubated semen was mounted on a glass microscope slide and covered with a cover slip. More than 200 spermatozoa per microscope slide were examined at × 400 magnification and scored as swelling or non-swelling. Swelling spermatozoa were considered to have intact plasma membranes.

Statistical analysis

Motility, motility parameters, viability, acrosomal membrane integrity, mitochondrial membrane potential, and plasma membrane integrity were analyzed by one-way ANOVA followed by Duncan's test as a post hoc analysis. All analyses were performed in SAS v. 9.2 (SAS Institute, Cary, NC, USA). Spermatozoa viability, acrosomal membrane integrity, and mitochondrial membrane potential were calculated as %. Spermatozoa stained blue on head and orange on midpiece considered to have live, intact acrosomes and high mitochondrial membrane potential (LIAH). Spermatozoa stained blue on head and orange or colorless on midpiece considered to have live, intact acrosomes and low mitochondrial membrane potential (LIAL). Spermatozoa stained red on head considered to have dead, intact acrosomes and low mitochondrial membrane potential (DIAL). Spermatozoa stained red on postal head, green on arterial head, and colorless on midpiece considered to have dead, damaged acrosomes and low mitochondrial membrane potential (DDAL).

RESULTS

Experiment 1

To examine the effects of 0.25 and 0.5 mL straw, we compared frozen-thawed spermatozoa motility in 0.25 mL and 0.5 mL straw. Table 2 shows that the % of fast-progressive spermatozoa in the 0.25 mL straw group was

Table 2. Spermatozoa motility and motility parameters after freezing-thawing

	Straw size (replicates)	
	0.25 mL (25)	0.5 mL (25)
Total motile (%)	92.1 ± 1.3	88.7 ± 1.3
Fast progressive (%)	35.2 ± 1.0^{a}	32.3 ± 0.7^{b}
Slow progressive (%)	34.0 ± 1.6	30.4 ± 1.7
Non-progressive (%)	22.9 ± 0.7^{d}	26.0 ± 0.6^{c}
Immotile (%)	7.9 ± 1.3	11.3 ± 1.3
VCL (μm/s)	85.5 ± 2.8	79.8 ± 2.4
VSL (μm/s)	34.6 ± 0.7^{c}	31.8 ± 0.5^{d}
VAP (μm/s)	51.4 ± 1.3^{a}	47.1 ± 1.1^{b}
LIN (%)	41.0 ± 0.8	40.4 ± 0.8
STR (%)	67.6 ± 0.8	68.0 ± 0.9
ALH (μm/s)	3.1 ± 0.1	3.1 ± 0.1
BCF (Hz)	15.3 ± 0.3	14.6 ± 0.1

^{a,b}Values (mean ± SE) with different superscripts in the same row are significantly different ($p < 0.05$). ^{c,d}Values (mean ± SE) with different superscripts in the same row are significantly different ($p < 0.01$). Five straws of frozen thawed semen were used to evaluate sperm motility per ejaculation group. Values indicate mean of 25 replicates (5 replicates × 5 ejaculates).

significantly higher than that in the 0.5 mL straw group (35.2 ± 1.0 vs. 32.3 ± 0.7%, respectively; $p < 0.05$). The % of slow progressive in the 0.25 mL straw group was significantly lower than that in the 0.5 mL straw group (Table 2) (22.9 ± 0.7 vs. 26.0 ± 0.6%. respectively; $p < 0.01$). The % of VSL in the 0.25 mL straw group was significantly higher than that in 0.5 mL straw group (34.6 ± 0.7 vs. 31.8 ± 0.5%, respectively; $p < 0.01$). The % of VAP in the 0.25 mL straw group was significantly higher than that in 0.5 mL straw group (51.4 ± 1.3 vs. 47.1 ± 1.1%, respectively; $p < 0.05$).

Experiment 2

Spermatozoa viability, acrosomal membrane integrity, and mitochondrial membrane potential after freezing-thawing were examined by the quadruple staining method. Table 3 shows that the % of LIAH in the 0.25 mL straw group was significantly higher than that in the 0.5 mL straw group (48.0 ± 2.6% vs. 35.6 ± 2.8%, respectively; $p < 0.01$). The % of LIAL in the 0.25 mL straw group was significantly lower than that in 0.5 mL straw group (8.4 ± 0.7 vs. 16.6 ± 1.8%, respectively; $p < 0.001$).

Experiment 3

The differences in plasma membrane integrity between the 0.25 mL and 0.5 mL straw groups are shown in Table 4. The % of intact plasma membranes in the 0.25 mL straw group was significantly higher than that in the 0.5 mL straw (62.0 ± 2.2 vs. 54.1 ± 1.3%, respectively, $p < 0.01$).

DISCUSSION

The sizes of the straws used to freeze-thaw bull semen vary with continent and country. Canada and Europe have adopted 0.25 mL straw while the United States and South America have adopted 0.5 mL straw. The optimal straw size and fertility rate are disputed among herdsman and technicians (Stevenson et al., 2009). In Korea, bull semen has been cryopreserved in 0.5 mL straw with semen extender based on *tris*-citric acid supplemented with 20% egg yolk. The latter has been used as a cryoprotectant in semen extender (Pace and Graham, 1974). However, animal proteins derived from egg yolk has disadvantages such as bacterial and mycoplasmal contamination (Bousseau et al., 1998). Recently, animal protein-free semen extenders have been investigated (Anzar et al., 2019). Here, we used an animal protein-free extender for Hanwoo bull semen. To the best of our knowledge, this work is the first to compare Hanwoo spermatozoa characteristics after freezing-thawing in 0.25 mL and 0.5 mL straw containing animal protein-free extender. We determined the relative effects of two different straw sizes on spermatozoa motility, viability, acrosomal membrane integrity, mitochondrial membrane potential, and plasma membrane integrity after freezing-thawing.

Spermatozoa motility and motility parameters have been used to predict fertility in livestock animals (Jepson et al., 2019). The percentages of fast progressive, VSL, and VAP for spermatozoa after thawing in 0.25 mL straw were significantly higher than those for spermatozoa af-

Table 4. Plasma membrane integrity of spermatozoa after freezing-thawing

Straw size (mL)	Replicates	Intact plasma membrane (%)*
0.25	25	62.0 ± 2.2[c]
0.5	25	54.1 ± 1.3[d]

[c,d]Values (mean ± SE) with different superscripts in the same column are significantly different ($p < 0.01$). Asterisk indicates % intact plasma membrane spermatozoa. Five straws of frozen–thawed semen were used to evaluate intact plasma membrane spermatozoa per ejaculation group. Swollen spermatozoa were scored as spermatozoa with intact plasma membranes.

Table 3. Viability, acrosomal membrane integrity, and mitochondrial membrane potential of spermatozoa after freezing-thawing

Straw size (mL)	Replicates	LIAH	LIAL	DIAL	DDAL
0.25	25	48.0 ± 2.6[c]	8.4 ± 0.7[f]	23.1 ± 2.0	18.6 ± 1.6
0.5	25	35.6 ± 2.8[d]	16.6 ± 1.8[e]	25.7 ± 2.1	20.4 ± 1.2

[c,d]Values (mean ± SE) with different superscripts in the same column are significantly different ($p < 0.01$). [e,f]Values (mean ± SE) with different superscripts in the same column are significantly different ($p < 0.001$). Values indicate mean of 25 replicates (5 replicates × 5 ejaculates). Spermatozoa with live intact acrosome and high mitochondrial membrane integrity, LIAH; spermatozoa with live intact acrosome and low mitochondrial membrane integrity, LIAL; spermatozoa with dead intact acrosome and low mitochondrial membrane integrity, DIAL; spermatozoa with dead damaged acrosome and low mitochondrial membrane integrity, DDAL; five straws of frozen–thawed semen were used to evaluate spermatozoa characteristics per ejaculation group.

ter thawing in 0.5 mL straw (Table 2). These findings are consistent with a previous report showing that the fast progressive, VSL, and VAP of spermatozoa in 0.25 mL straw were higher than those of spermatozoa in 0.5 mL straw after 2 h thawing (Anzar et al., 2011). Enhanced fast progressive sperm motility in frozen-thawed bull semen increased its relative fertility (Morrell et al., 2017). The VSL and VAP were highly correlated with fertility *in vitro* (Kathiravan et al., 2008) and with fertility prediction *in vivo* (Farrell and Brockett, 1998). These earlier studies demonstrated that spermatozoa quality in 0.25 mL straw after freezing-thawing was superior to that of spermatozoa after freezing-thawing in 0.5 mL straw. Semen freezing in 0.25 mL straw augments post-AI fertility compared to that for semen freezing in 0.5 mL straw.

The proportion of spermatozoa with live, intact acrosome membranes, and high mitochondrial membrane potential (LIAH) was ~12% higher in 0.25 mL straw than it was in 0.5 mL straw (Table 3). Previous reports indicated higher LIAH for spermatozoa in 0.25 mL straw than those in 0.5 mL straw. An elevated proportion of LIAH may improve relative fertility (Anzar et al., 2011). Ansari et al. (2011) reported that the 0.25 mL straw group had 14% more viable spermatozoa than the 0.5 mL straw. The acrosome reaction is essential for fertilization. Cryopreservation and thawing induce the acrosome reaction in spermatozoa (Birck et al., 2010). The reduction of the acrosome reaction in spermatozoa during freezing and thawing is critical for increasing the relative proportion of spermatozoa with intact acrosomes (Nagata et al., 2019). Mitochondrial membrane potential is a predictor of in spermatozoa fertility potential in boars and bulls (Hu et al., 2017), stallions (Meyers et al., 2019), and humans (Kasai et al., 2002). The plasma membrane integrity of spermatozoa in 0.25 mL straw was 7.9% higher that of spermatozoa in 0.5 mL straw after freezing-thawing (Table 4). Elevated plasma membrane integrity of spermatozoa is an important indicator of bull fertility (Correa et al., 1997; Januskauskas et al., 2003). Hence, we propose that freezing bull semen in 0.25 mL straw increases spermatozoa motility, viability, acrosome integrity, mitochondrial membrane potential, and plasma membrane integrity.

In the present study, we assessed spermatozoa motility by CASA and spermatozoa characteristics by fluorescent staining and HOST. Reductions in spermatozoa quality by semen dilution, freezing, and thawing processes are inevitable in frozen semen fabrication (Chaveiro, 2006). As it has a large surface-to-volume, the 0.25 mL straw induces faster cooling and thawing than the 0.5 mL straw (Mocé et al., 2010). Cooling acceleration in 0.25 mL straw causes spermatozoa to pass through the fracture temperature and minimizes intracellular crystallization (Morris, 2006). However, the relatively improved motility, viability, acrosome membrane integrity, mitochondrial membrane potential, and plasma membrane integrity of the spermatozoa in 0.25 mL straw *in vitro* does not necessarily guarantee enhanced conception rates *in vivo* after AI. In fact, AI with 0.25 mL straw conferred only a 0.74% improvement in the expected fertility relative to AI using 0.5 mL straw (Stevenson et al., 2009). The use of 0.25 mL and 0.5 mL straw varies with country and continent and fertility rates vary with AI technician skills and straw handling (Diskin, 2018). The 0.25 mL straw is sensitive to exposure time, thawing conditions, and transportation after freezing-thawing (Stevenson et al., 2009). Future studies should evaluate the post-AI fertility rates using Hanwoo frozen-thawed spermatozoa in 0.25 mL straw. Moreover, the effects of freezing and thawing in 0.25 mL straw on spermatozoa characteristics should also be examined.

CONCLUSION

The present study demonstrated that semen freezing in 0.25 mL straw improved relative spermatozoa motility, viability, acrosome integrity, mitochondrial membrane potential, and plasma membrane integrity after freezing-thawing. The use of 0.25 mL straw doubles the number of straws that can be processed simultaneously in the LN_2 tank compared to the 0.5 mL straws. Nevertheless, the *in vivo* post-AI fertility rates obtained with 0.25 mL straw should be investigated.

CONFLICTS OF INTEREST

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

ACKNOWLEDGEMENTS

This study received support from the Cooperative Research Program for Agriculture Science & Technology Development project: "Development of improved tech-

nologies for preservation of Hanwoo bull semen and technology application", PJ0143252020, RDA, Korea, and the 2020 Postdoctoral Fellowship Program of the Hanwoo Research Institute, NIAS, RDA, Korea.

We thank Mr. In-Rak Yoon, Mr. Woo-Heon Choi, Mr. Jong-Hwan Jang, Mr. Kyu-Myung Kim who care for bulls, supporting of semen collection and semen cryopreservation in Hanwoo Research Institute.

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