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Original Article

Development of a new mini straw for cryopreservation of boar semen

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ABSTRACT Sperm cryopreservation is a fundamental process for the long-term conservation of livestock genetic resources. Yet, the packaging method has been shown, among other factors, to affect the frozen-thawed (FT) sperm quality. This study aimed to develop a new mini-straw for sperm cryopreservation. In addition, the kinematic patterns, viability, acrosome integrity, and mitochondrial membrane potential (MMP) of boar spermatozoa frozen in the developed 0.25 mL straw, 0.25 mL (minitube, Germany), or 0.5 mL (IMV technologies, France) straws were assessed. Post-thaw kinematic parameters were not different (experiment 1: total motility (33.89%, 32.42%), progressive motility (19.13%, 19.09%), curvilinear velocity (42.32, 42.86), and average path velocity (33.40, 33.62) for minitube and the developed straws, respectively. Further, the viability (38.56%, 34.03%), acrosome integrity (53.38%, 48.88%), MMP (42.32%, 36.71%) of spermatozoa frozen using both straw were not differ statistically ($p > 0.05$). In experiment two, the quality parameters for semen frozen in the developed straw were compared with the 0.5 mL IMV straw. The total motility (41.26%, 39.1%), progressive motility (24.62%, 23.25%), curvilinear velocity (46.44, 48.25), and average path velocity (37.98, 39.12), respectively, for IMV and the developed straw, did not differ statistically. Additionally, there was no significant difference in the viability (39.60%, 33.17%), acrosome integrity (46.23%, 43.23%), and MMP (39.66, 32.51) for IMV and the developed straw, respectively. These results validate the safety and efficiency of the developed straw and highlight its great potential for clinical application. Moreover, both 0.25 mL and 0.5 mL straws fit the present protocol for cryopreservation of boar spermatozoa.

Keywords: boar, cryoinjury, freezing-thawing, mini-straw, semen packaging

INTRODUCTION

Sperm cryopreservation is considered an essential tool of assisted reproductive technology, for the conservation of endangered species, breeding and distribution of superior genetic material throughout the animal industry (Grötter et al., 2019; Kumar et al., 2019). However, frozen thawed (FT) sperm has a short lifespan and reduced fertility (Sancho et al., 2007; Jovičić et al., 2020). It has been indicated that this is due to both a loss of sperm viability and impairment of function among survivors (Watson, 2000; Ozkavukcu et al., 2008; Khan et al., 2021). Notably,

cryopreserved boar sperm, which is particularly sensitive to the cellular stress imposed by cryopreservation, emerges from this process with poor viability (Rath et al., 2009; Choi et al., 2014), and subsequently, the FT boar semen has not been integrated into artificial insemination centers (AI) at a rate comparable to that of other species (Yeste, 2015).

Numerous factors have been shown to affect semen quality during the freezing-thawing process. In this sense, the type of extender, cryoprotectants, packaging system, freezing and thawing rate (Buhr et al., 2001; Athurupana and Funahashi, 2014; Ravagnani et al., 2018; Grötter et al., 2019) have been emphasized. Considering the cost-effectiveness and saving storage space without compromising the post-thaw quality and fertility of semen, the sperm cryopreservation industry continuously modified semen packaging methods from bottles, bags, tubes and ampules to straws of different sizes (Sharma et al., 2015; Karan et al., 2018; Kaneko et al., 2021). Presently, sperm cryopreservation is routinely performed using 0.5 mL and 0.25 mL straws, with similar or varying results among species and freezing-thawing methods (Pesch and Hoffmann, 2007; Buranaamnuay et al., 2009; Kang et al., 2020).

It is important to note that the cryopreservation laboratories in Korea use imported straws of different sizes for the sperm FT process. However, it's well-recognized that the COVID-19 pandemic has posed significant challenges for supply chains globally (Raj et al., 2022). Inconsistency of supply has been identified as the most prominent challenge and is associated with the uncertainty of supply from upstream vendors, irregular and indefinite lead times and price volatility (Okorie et al., 2020; Paul and Chowdhury, 2021). Yet, manufacturing and supply chains have experienced large-scale disruption due to natural disasters and political risks.

It has been indicated that the cryopreservation of semen in mini-straws (0.25 mL) increases the number of doses and storage efficiency in liquid nitrogen and decreases extender and antibiotics costs (Johnson et al., 1995; Stevenson et al., 2009). However, because of the much higher surface-to-volume ratio in 0.25 mL straws heat dissipation or accumulation occurs much faster than with 0.5 mL straws. Consequently, greater caution is necessary with 0.25 mL straws (Diskin, 2018). Information on the impact of mini vs. medium size straws on post-thaw semen quality of boar semen is variable (Jun-Feng et al., 2008; Buranaamnuay et al., 2009) and thus needs to be investigated. Therefore, this study was aimed to develop a new 0.25 mL straw (made of Pellethane 75D) and to examine the post-thaw kinematic patterns, viability, acrosomal membrane integrity and mitochondrial membrane potential (MMP) of boar spermatozoa frozen using the developed straw and commercially available straws (0.25 mL, minitube, Germany) and (0.5 mL, IMV Technologies, France).

MATERIALS AND METHODS

Experimental design

Chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise. The procedures and media used for sperm cryopreservation and sperm thawing were essentially the same as those described previously (Almubarak et al., 2021) with some modifications described below. To validate the developed 0.25 mL straw (they were high-quality, non-toxic straws made of Pellethane 2363-75D with a length of 140 mm and an inner diameter of 1.55 mm; sungwon medical, Korea), two experiments (two trials in each) were conducted and each trial was repeated at least four times. In experiment one, porcine semen was extended, cooled and transferred to the developed 0.25 mL and 0.25 mL straw (reference no. 13407/3144; minitube, Germany). The straws were then cryopreserved as described subsequently. After thawing, the kinematic patterns of sperm frozen in the developed straw were compared with the commercial 0.25 mL straw group. Then, the effect of the FT process on the viability, acrosome integrity and MMP of both straws was studied. In experiment two: the post-thaw kinematic patterns, viability, acrosome integrity and MMP of boar spermatozoa frozen in the developed 0.25 mL straw and 0.5 mL straw (reference no. 028285; IMV technologies, France) were examined.

Semen collection and sperm cryopreservation

Semen was collected from duroc boars belonging to a local AI center by the gloved-hand method. Ejaculates were diluted in Beltsville thawing solution (BTS) and transferred to the laboratory within one hour. Sperm motility was assessed upon arrival at the laboratory and only samples with total motility ≥ 80% were considered for the further experiment (Park et al., 2017). Qualified samples were diluted (2×10^8 cell/mL) with the extender consisted

of TES 12 g/L, Trizma Base 2 g/L, D (+) Glucose 32 g/L, OEP (Equex) 0.7% (v/v), Gentamycin Sulfate 0.02 g/L and 20% (v/v) egg yolk. the extended semen was cooled to 4℃ in 1 h, after which, Extender 2 (composed of extender 1 plus 4% (v/v) glycerol) was added 1:1 (v/v). Semen was packed in straws according to the experimental design, straws were then sealed and incubated for 25 min at 4℃. Then, kept 4 cm above liquid nitrogen vapors for 20 min. Straws were plunged into liquid nitrogen (-196℃) for storage. After at least 24 h, straws were thawed at 38℃ for 25 sec and the FT semen was used for assessment of different sperm quality parameters.

Evaluation of post-thaw motility and kinematic parameters

FT sperm were diluted 1:4 (v/v) in BTS. Sperm motility was evaluated by phase-contrast microscopy (Nikon, Model Eclipse E200) at 10x magnification using computer-assisted sperm analysis (Sperm Class Analyzer, Microptic, Spain). Briefly, two μL of semen was placed in a counting chamber (Leja, Nieuw Vennep, Netherlands) on a heated stage of 38℃. For each analysis, four fields were evaluated and at least 500 cells were counted. Kinematic patterns including total sperm motility (TM, %), progressive motility (PM, %), rapid progressive motility (RPM, %), medium progressive motility (MPM, %), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) were measured.

Evaluation of post-thaw viability

The evaluation of FT sperm viability was carried out using the LIVE/DEAD® Sperm Viability Kit (ThermoFisher, USA). A total of 200 spermatozoa were evaluated for each sample; with live and dead sperm, respectively stained green (SYBR-14+), and red (PI+). The results were expressed as live sperm mean percentage.

Evaluation of post-thaw acrosomal integrity

Acrosome status was determined according to a staining procedure described previously (Yu and Leibo, 2002) using FITC-PSA stain. Samples were examined using a fluorescence microscope (Axio, Carl Zeiss). Two hundred spermatozoa were evaluated and classified into intact or reacted acrosome. Only the percentage of sperm with intact acrosome was presented in the result.

Evaluation of post-thaw mitochondrial membrane potential (MMP)

Rhodamine 123 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) were used to determine mitochondrial activity. For this assay, 5 μL of R123 and 5 μL PI solution were added to 250 μL aliquot of diluted semen samples and incubated for 15 min at 37℃ in the dark. The stained samples were analyzed under a fluorescence microscope. Sperm displaying only green fluorescence at the mid-piece region were considered viable sperm with functional mitochondria (Najafi et al., 2018). The results were denoted as the proportion of spermatozoa with functional MMP.

Statistical analysis

The results are shown as the means ± standard error. Data were analyzed by independent *t*-test using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). Values of ($p < 0.05$) were considered to indicate the significant difference.

RESULTS

Experiment one

The post-thaw kinematic patterns of spermatozoa determined by TM, PM, VCL, VAP and other parameters did not differ significantly ($p > 0.05$) between the developed and 0.25 mL Straws (Fig. 1 and Table 1). Furthermore, no statistically significant difference in the viability, acrosome integrity, or MMP between the developed and 0.25 mL straws ($p > .05$) (Fig. 2 and 3).

Experiment two

Examination of the effects of the FT process on the different semen quality parameters revealed that straw size had no statistically significant influence on kinematics patterns, viability, acrosome integrity and the mitochondrial membrane potential. As shown in (Fig. 4 and Table 2), the kinematics patterns of sperm frozen in the developed 0.25 mL straw did not differ from the 0.5 mL straw. Similar results ($p > 0.05$) were observed for the percentage of live spermatozoa, intact acrosomal membrane, and MMP, after cryopreservation using both straws (Fig. 5 and 6).

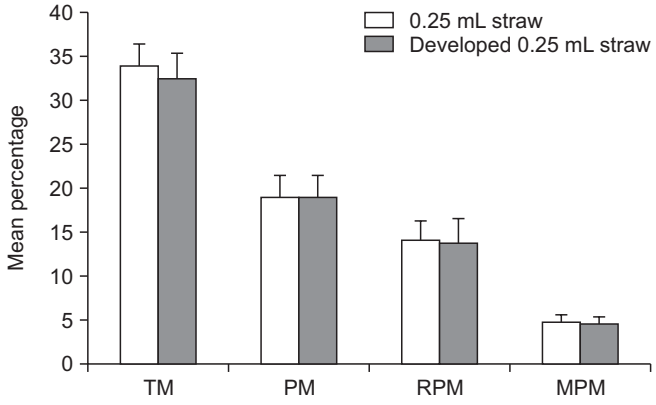

Fig. 1. Motility patterns of boar spermatozoa after freezing-thawing with 0.25 mL straw (minitube, Germany) and the developed 0.25 mL straw. TM, Total motility; PM, progressive motility; RPM, rapid progressive motility; MPM, Medium progressive motility ($p > 0.05$). Results are presented as the mean percentage ± SE. Error bars represent the standard error of the mean.

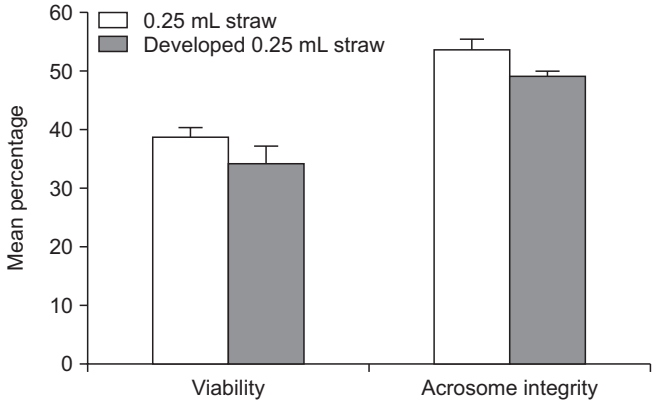

Fig. 2. Viability and acrosome integrity of boar spermatozoa after freezing-thawing using 0.25 mL straw (minitube, Germany) and the developed 0.25 mL straw ($p > 0.05$). Results are presented as the mean percentage ± SE. Error bars indicate the standard error of the mean.

Table 1. Kinematic patterns of boar spermatozoa after freezing-thawing using 0.25 mL straw (minitube, Germany) and the developed 0.25 mL straw

Parameter*	0.25 mL straw	Developed 0.25 mL straw
VCL (μm/s)	42.32±4.34	42.86±5.86
VAP (μm/s)	33.40±3.35	33.62±4.61
VSL (μm/s)	26.89±2.94	27.08±4.10
STR (%)	71.11±1.78	70.33±1.90
LIN (%)	56.81±1.95	55.01±1.79
WOB (%)	75.80±1.06	73.47±0.58
ALH (μm)	1.58±0.13	1.59±0.17
BCF (Hz)	5.71±0.26	5.61±0.25

Values represent the mean ± standard error of the mean (SEM).
*Experiment was repeated 4 times, $p > 0.05$.
VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

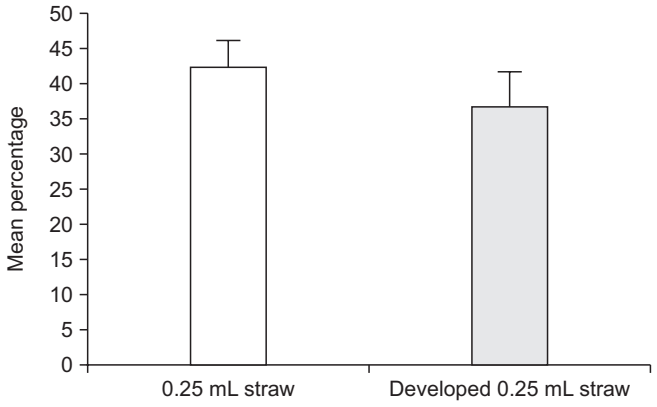

Fig. 3. Mitochondrial membrane potential of boar spermatozoa after freezing-thawing using 0.25 mL straw (minitube, Germany) and the developed 0.25 mL straw ($p > 0.05$). Results are presented as the mean percentage ± SE. Error bars indicate the standard error of the mean.

DISCUSSION

Several methods have been studied to find the most suitable container for sperm cryopreservation. Most carriers described to date have demonstrated reasonable success, with varying degrees of technical difficulty (Duplaix and Sexton, 1984; Rodriguez-Martinez and Wallgren, 2011). Of note, the packaging system is a critical aspect to keep in mind in the development of sperm cryopreservation protocols. Semen packaging is also necessary for practical purposes and when using FT sperm for AI, as it determines both the means of identification of each semen dose and how it could be arranged in the liquid nitrogen container for storage (Johnson et al., 2000; Pesch and Hoffmann, 2007). Currently, 0.25 mL and 0.5 mL straws are the most commonly used with results varying with species and freezing-thawing protocol (Stuart et al., 2019; Lone et al., 2020). In the current research, we developed a new 0.25 mL straw for freezing spermatozoa. Further, the kinematic patterns, viability, acrosome integrity, and MMP of boar spermatozoa frozen in the developed straw, 0.25 mL (minitube) or 0.5 mL (IMV technologies) straws were regarded as indices for assessing the effect of the freezing-thawing process. These parameters are crucial markers for semen assessment. Indeed, fertility is correlated to semen characteristics, such as kinematic variables

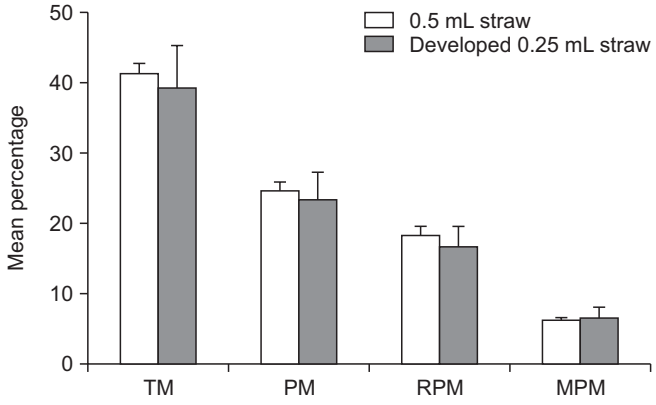

Fig. 4. Motility patterns of boar spermatozoa after freezing-thawing using 0.5 mL straw (IMV, France) and the developed 0.25 mL straw. TM, Total motility; PM, progressive motility; RPM, rapid progressive motility; MPM, Medium progressive motility. Results are presented as the mean percentage ± SE. Error bars indicate the standard error of the mean ($p > 0.05$).

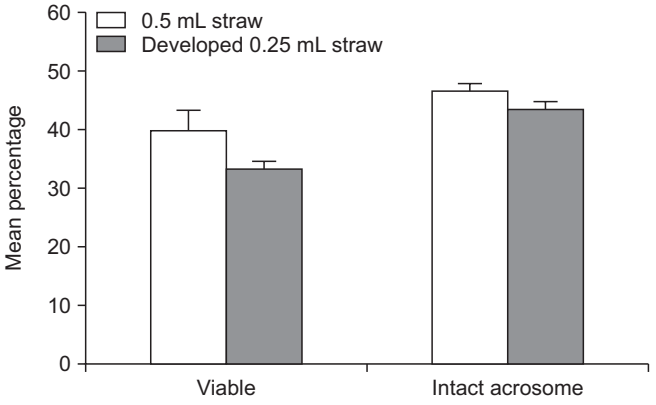

Fig. 5. Viability and acrosome integrity of boar spermatozoa after freezing-thawing using 0.5 mL straw (IMV, France) and the developed 0.25 mL straw. Results are given as the mean percentage ± SE. Error bars indicate the standard error of the mean ($p > 0.05$).

Table 2. Kinematic patterns of boar spermatozoa after freezing-thawing using 0.5 mL straw (IMV, France) and the developed 0.25 mL straw

Parameter*	0.5 mL straw	Developed 0.25 mL straw
VCL (μm/s)	46.44±1.79	48.25±2.85
VAP (μm/s)	37.98±2.56	39.12±2.65
VSL (μm/s)	31.82±3.04	33.18±2.45
STR (%)	68.64±3.09	70.47±1.70
LIN (%)	55.43±4.36	56.25±3.18
WOB (%)	74.30±2.84	73.97±2.78
ALH (μm)	1.53±0.08	1.59±0.13
BCF (Hz)	5.39±0.22	5.70±0.21

Values represent the mean ± standard error of the mean (SEM).
*Experiment was repeated 4 times, $p > 0.05$.
VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

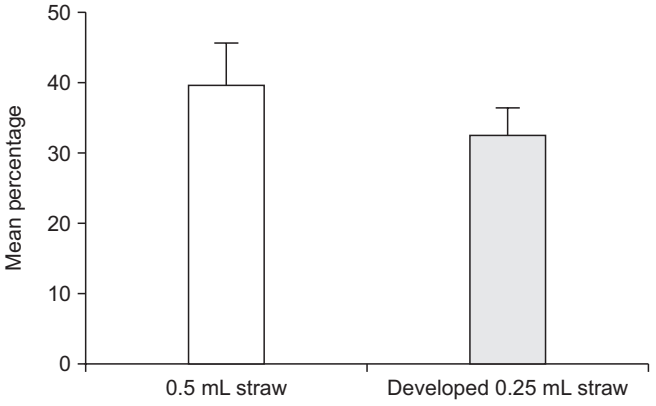

Fig. 6. Mitochondrial membrane potential of boar spermatozoa after freezing-thawing with 0.5 mL straw (IMV, France) and the developed 0.25 mL straw. Results are presented as the mean percentage ± SE. Error bars indicate the standard error of the mean ($p > 0.05$).

(Broekhuijse et al., 2012; Tremoen et al., 2018), viability, plasma and acrosomal membrane integrity (Flesch and Gadella, 2000; Sutkeviciene et al., 2009) and MMP (Amaral, et al., 2013; Park and Pang, 2021).

In the present study, the assessment of the FT semen samples revealed comparable results ($p > 0.05$) in the kinematics parameters, viability, acrosome integrity and MMP between the developed 0.25 mL straw and commercial straws of different sizes. In agreement with these findings, (Stuart et al., 2019; Zong et al., 2022) reported no difference in sperm motility between 0.25 mL and 0.5 mL straws of FT spermatozoa in alpaca and rooster, respectively. Similarly, (Nordstoga et al., 2009) indicated that straw type has no significant effect on the fertility of FT ram semen. On the other hand, (Ansari et al., 2011) observed that bovine semen cryopreserved with 0.25 mL straw resulted in a higher post-thaw quality than that of 0.5 mL straw. Also, (Kang et al., 2020) demonstrated that straw size had a substantial effect on sperm quality parameters, with 0.25 mL straw improving the motility, viability, and acrosomal, MMP, and plasma membrane integrity of bull spermatozoa compared to 0.5 mL straw. On the contrary, equine sperm cryopreserved in 0.5 mL straws has better sperm kinematics than in 0.25 mL straws (Dias et al., 2013). Higher motility and acrosome integrity were also obtained using 0.5 mL straw rather than 0.25

mL straw after freezing-thawing dog spermatozoa (Nöthling and Shuttleworth, 2005). These contradictory results, however, might be due to species and cooling-freezing-thawing protocol differences. Moreover, the cooling pattern and ice crystallization initiation depend, among other factors, on the size of the straw. The 0.25 mL straw has a higher surface-to-volume ratio than the 0.5 mL straw, which increases the probability of semen temperature fluctuations within the straw. Indeed, such changes can compromise the recovery of live spermatozoa after thawing and consequently decrease fertility. Thus, particular attention must be paid when handling 0.25 mL straws (Johnson et al., 1995; Diskin, 2018). Taken together, both 0.25 mL and 0.5 mL straws fit the present protocol for FT boar spermatozoa. However, further research is needed concerning the assessment of fertilization capacity and using different freezing-thawing techniques.

CONCLUSION

In conclusion, the results of this study indicated the efficiency and safety of the developed 0.25 mL straw and highlight its great potential for clinical application. Additionally, straw size had no substantial effect on the kinematic parameters, viability, acrosome integrity, and the mitochondrial membrane potential of FT boar semen under the current experimental condition.

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