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Original Article

Washing solution and centrifugation affect kinematics of cryopreserved boar semen

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ABSTRACT Cryopreservation is a widely-used efficient means of long-term sperm preservation. However, unlike other types of semen, cryopreserved boar semen has reduced fertility and the efforts continue to optimize post-thawing sperm recovery. In this study, we evaluated the effects of various washing solutions (Hulsen solution, lab-made DPBS and commercial DPBS) on post-thawing porcine sperm kinematics (CASA system), viability (SYBR-14/PI) and acrosome integrity (PSA/FITC). We also examined the effect of washing-centrifugation on frozen-thawed semen kinematics. The results indicate that type of washing solution and post-thawing centrifugation alters parameters linked to sperm quality (total motility, progressive motility, viability and acrosome integrity). Significantly higher ($p < 0.05$) motility and progressive motility were obtained when cryopreserved semen was processed with Hulsen solution. The post-thaw percentage of live and intact acrosomal sperm was significantly higher in group 1 (Hulsen solution) as compared to other groups. Following thawing-centrifugation, the results showed significantly higher motility and progressive motility in group 1 than other groups. However, the latter two DPBS groups did not differ statistically. Taken together, Frozen-thawed spermatozoa motility, acrosome integrity and viability can be affected by the type of washing solution used. Moreover, centrifugation of frozen-thawed semen has an unfavorable effect on total motility and progressive motility.

Keywords: boar, centrifugation, cryopreservation, freezing-thawing, washing solution

INTRODUCTION

Cryopreservation is the most effective method for the long-term storage of semen. Furthermore, cryopreservation enhances genetic utilization by facilitating the distribution of desirable genes and control the transmission of certain diseases (Grossfeld et al., 2008). However, the cryopreservation process exposes semen to physical and chemical stress that impairs sperm quality (Hezavehei et al., 2018). Each step of the cryopreservation including dilution, cooling, freezing, and thawing, diminishes the fertilization potential, with approximately 40% to 50% of spermatozoa not even surviving the process (Rath et al., 2009; Watson, 2000). In comparison with fresh semen, insemination with frozen-thawed semen results in lower fertility and farrowing rates by 20% to 30% (Knox, 2015; Yeste et al., 2017). Boar spermatozoa in particular are very susceptible to peroxidative damage because of their high polyunsaturated fatty acid content and low concentrations of cholesterol in their plasma membrane (Mandal

et al., 2014; Yeste, 2015). Although the commercial use of cryopreserved semen for artificial insemination (AI) has become common for many farm animal species worldwide, cryopreserved boar semen has not been integrated into AI centers at a rate comparable to that of other species (DeJarnette et al., 2004; Rodriguez-Martinez and Wallgren, 2010).

There have been numerous attempts to reduce the detrimental functional and structural alterations the cryopreservation process induces in sperm cells. Factors like quality of fresh semen (Parrilla et al., 2012), type and the composition of extender (Park et al., 2012; Blanch et al., 2014), antioxidants supplementation, type and concentration of cryoprotectant (Buranaamnuay et al., 2011; Malo et al., 2010b), and the freezing and thawing rate (Eriksson and Rodriguez-Martinez, 2000) have been tested to improve the freezing-thawing process. Furthermore, different diluents (Noguchi et al., 2015) and centrifugation regimes (Martinez-Alborcia et al., 2012) have been shown to influence post-thaw sperm survival. However, considering the sensitivity of boar semen, the results are still sub-optimal. In this regard, the present study aimed to examine the effects of different washing solutions during cryopreservation of boar semen on post-thawing motility, progressive motility, viability and acrosome integrity. Also, the effect of washing-centrifugation on post-thawing sperm kinematics was evaluated.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and prepared using ultra-pure water (ProGen, Genetrone Biotech, Korea). Bovine serum albumin (BSA, REF 10738328) was from Roche Diagnostics (New Zealand). Commercial DPBS was purchased from BYLABS (Lu Science, Korea). Equex-Paste STM was obtained from bestandteil von TG-Verdünner für Eber und Hund (Minitube, Germany).

Compositions of washing solutions and freezing extenders

The components of washing solution used in the present study were shown in Table 1. Hulsen solution (Group 1) was composed of D(+) Glucose 57.5 g/L, α-lactose 2.5 g/L, Sodium citrate 4.5 g/L, Na_2-EDTA 3.5 g/L, $NaHCO_3$ 1.2 g/L, KCl 0.4 g/L and Gentamycin Sulfate 0.02 g/L. Lab-made DPBS (Group 2) was composed of 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 1.174 g/L Na_2HPO_4, 1 g/L D(+) Glucose, 0.036 g/L Na-pyruvate and 1 g/L BSA. Commercial DPBS (Group 3) consists of 8.12 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.20 g/L KH_2PO_4.

The freezing extenders used in the experiments were extender (1) Tris-egg yolk based extender (TEY), which was composed of TES 12 g/L, Trizma Base 2 g/L, D(+) Glucose 32 g/L, OEP (Equex) 0.5% (v/v), Gentamycin Sulfate 0.02 g/L and 20% (v/v) egg yolk. Extender (2) was composed of extender 1 plus 2% (v/v) glycerol.

Semen collection and transportation

Porcine semen used in the current study was from duroc boars of proven fertility belonged to a local livestock center (KPG, Korea). Samples were collected by the gloved-hand method and were diluted ($2.5 \times 10^9 \pm 0.5$ spermatozoa /90 mL) in Beltsville thawing solution (BTS). The diluted semen samples were cooled and maintained at 17℃ for shipment to the laboratory within 1 h.

Semen cryopreservation

Spermatozoa were cryopreserved using a modified two-step freezing protocol described by (Córdova et al., 1997). Semen was analyzed upon arrival to ensure its quality and only samples with greater than 80% motility were used in this study. The diluted semen was transferred to a 15 mL conical tube and equilibrated at 17℃. After 2 h semen was centrifuged at 2,000 rpm for 5 min at 15℃, whereupon the supernatant was discarded and the pellet

Table 1. Composition of various washing solutions

Component (g/L)	Hulsen solution	Lab DPBS	Commercial DPBS
D(+) Glucose	57.50	1.00	–
α-lactose	2.50	–	–
Sodium citrate · 2 H_2O	4.50	–	–
Na_2EDTA	3.50	–	–
$NaHCO_3$	1.20	–	–
KCl	0.40	0.20	0.20
Gentamycin Sulfate	0.02	–	–
NaCl	–	8.00	8.12
KH_2PO_4	–	0.20	0.20
Na_2HPO_4	–	1.174	1.44
Na-pyruvate	–	0.036	–
BSA	–	1.00	–

was resuspended in each washing solution according to experimental design. Each solution was then centrifuged at 2,000 rpm for 5 min at 15℃. The supernatant was then discarded and the sperm pellet (2×10^8 spermatozoa/mL) was resuspended in extender 1. The extended semen was cooled at 5℃ for 60 min, after which the cooled-extended semen was mixed 1:1 (v/v) with extender 2. The extended semen was then loaded into 0.5 mL straws (Fujihira, Japan), sealed and incubated at 5℃ for 20 min. Straws were placed in a polystyrene box 4 cm above liquid nitrogen vapor for 20 min before being plunged into liquid nitrogen for storage.

Assessment of frozen-thawed semen kinematics

The frozen semen was thawed in a water bath at 38℃ for 25 sec. Then, diluted according to the experiment design in each washing solution 1:4 (v/v). Frozen-thawed (FT) sperm motility was measured using computer-assisted sperm analysis (Sperm Class Analyzer, Microptic, Spain). Briefly, 5 μL of semen was placed in a counting chamber (GoldCyto, Microptic, Spain) on a heated stage 38℃. For each analysis, three fields were evaluated and at least 1,000 cells were counted. Motility patterns including total sperm motility (TM, %), progressive motility (PM, %), rapid progressive motility (RPM, %) and medium progressive motility (MPM, %) were measured.

Assessment of sperm viability

The determination of FT sperm viability was carried out using LIVE/DEAD® Sperm Viability Kit (ThermoFisher, USA) following the method of (Yu and Leibo, 2002). In brief, 5 μL SYBR-14 was added to 50 μL spermatozoa and incubated for 5 min in the dark. Then, 5 μL PI was added, and the mixture was incubated again for 5 min. Two smears from each group were made using the semen-stain mixture and dried in the air, then examined under a fluorescence microscope (Axio, Carl Zeiss). Appropriately 200 spermatozoa were counted per slide and classified as live (green fluorescent) or dead spermatozoa (red fluorescent).

Assessment of acrosome integrity

Sperm acrosome integrity was evaluated using the fluorescent stain method described by (Yu and Leibo, 2002). Briefly, thin smears were made from FT semen of each group and dried in the air. Smears were fixed with absolute methanol and stained with Pisum sativum agglutinin (PSA) labeled with fluorescein isothiocyanate (FITC). Stained smears were covered with parafilm for 20 min, dipped in distilled water for 15 min and then allowed to dry. For each replicate, two slides were examined using a fluorescence microscope (Axio, Carl Zeiss). The percentage of acrosome-intact sperm (spermatozoa with strong green fluorescence on acrosomal region) was counted in a minimum of 200 sperms per slide.

Experimental design

In experiment one, the effect of using different washing solutions during the cryopreservation process was assessed. Spermatozoa were washed (using either Hulsen solution, lab-made DPBS or commercial DPBS), extended in TEY extender, cooled and cryopreserved as described above. After thawing, each sample was diluted with the same washing solution, then sperm motility, progressive motility, viability and acrosome integrity for each group were evaluated. In experiment two, the effect of dilution with each solution and centrifugation on post-thawing sperm parameters were assessed. FT semen was diluted and centrifuged at 2,000 rpm for 2 min (using either Hulsen solution, lab-made DPBS or commercial DPBS), the supernatant was discarded and pellets were resuspended in each washing solution, then motility parameters were assessed as described above using CASA system.

Statistical analyses

Each experiment was repeated a minimum of four times. Data were analyzed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA). Percentage of data were compared by one-way ANOVA, followed by Duncan's multiple range test. The results are expressed as mean ± standard error and values of $p < 0.05$ were considered significant.

RESULTS

Effects of washing solution on post-thawing sperm motility

The percentage of motile spermatozoa and spermatozoa exhibited progressive motility was significantly higher in the group processed with Hulsen solution in comparison to other groups ($p < 0.05$). There was no statistically significant difference in the percentage of FT motile and progressive motile sperm between the two DPBS groups (Fig. 1).

Effects of washing solution on post-thawing sperm viability

As shown in Fig. 2, the post-thaw percentage of live sperm was higher ($p < 0.05$) in Group 1 as compared to other groups (40.25 ± 1.91, 33.03 ± 4.47 and 28.10 ± 2.97) for Hulsen solution, lab and commercial DPBS, respectively.

Effects of washing solution on acrosome integrity

The results of the post-thaw semen analysis shown in Fig. 3, indicated that the percentage of sperm with the normal acrosomal region was significantly higher ($p < 0.05$) in group 1 than other groups (42.50 % ± 2.34 %, 35.02 % ± 1.56 % and 37.18 % ± 2.15 %) for Hulsen solution, lab and commercial DPBS, respectively.

Effect of post-thawing centrifugation on sperm kinematics

The effect of washing-centrifugation on frozen-thawed semen shown in Fig. 4. The percentage of FT motile and progressive motile sperm after centrifugation was higher in Group 1 as compared to other groups ($p < 0.05$). There

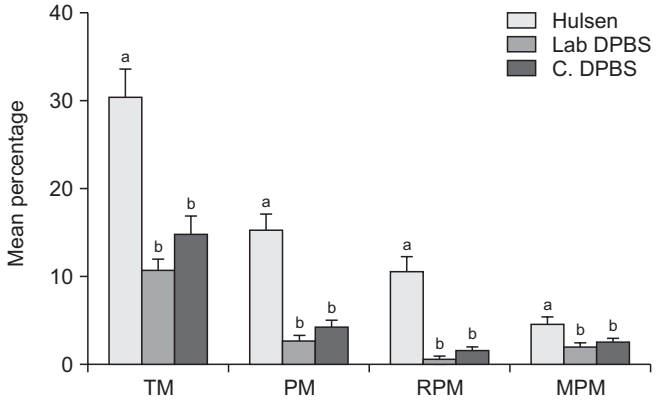

Fig. 1. Effect of different washing solutions used during cryopreservation of boar semen on post-thawing sperm motility. TM: total motility, PM: progressive motility, RPM: rapid progressive motility, MPM: medium progressive motility. Letters a and b represent significant differences between groups ($p < 0.05$). Error bars show the standard error of the mean.

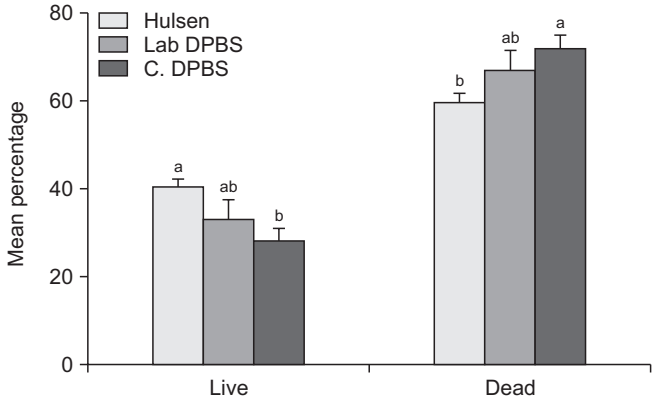

Fig. 2. Effect of different washing solutions used during cryopreservation of boar semen on post-thawing sperm viability. Frozen-thawed spermatozoa were stained with Sybr-14/PI and examined under green and red fluorescence. Stained spermatozoa were classified as live (Sybr-14+) or dead (PI+). Values are expressed as the mean ± SE. Letters a and b represent significant differences between groups ($p < 0.05$). Error bars show the standard error of the mean.

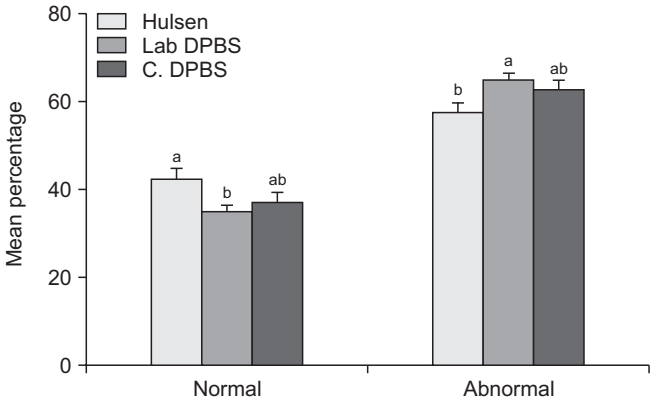

Fig. 3. Effect of different washing solution used during cryopreservation of boar semen on post-thawing acrosome integrity. Frozen-thawed spermatozoa were stained with PSA/FITC and examined under green fluorescence. Stained spermatozoa were classified as normal (intact acrosome) or abnormal (un-intact acrosome). Values are expressed as the mean ± SE. Letters a and b represent significant differences between groups ($p < 0.05$). Error bars show the standard error of the mean.

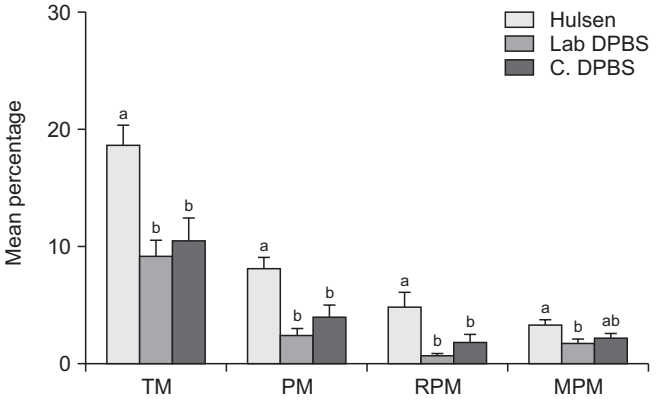

Fig. 4. Effect of post-thawing washing-centrifugation of cryopreserved boar semen on sperm motility. TM: total motility, PM: progressive motility, RPM: rapid progressive motility, MPM: medium progressive motility. Letters a and b represent significant differences between groups ($p < 0.05$). Error bars show the standard error of the mean.

was no statistically significant difference in the percentage of FT motile and progressive motile sperm between the two other groups.

DISCUSSION

Cryopreservation is an efficient method for semen storage that allows for long-term preservation and distribution of superior genetic material throughout the animal industry. However, sperm subjected to the cooling-freezing-thawing process has a short lifespan and reduced fertility (Jovičič et al., 2020). It has been shown that this is due to both a loss of sperm viability and impairment of function among survivors (Watson, 2000). 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). However, increase understanding of this process may lead to improved cryopreservation methods and methodological refinements that enhance post-thaw viability and fertility.

Considering the aforementioned, more work is required for the cryopreservation protocols to be fully optimized. Several studies have attempted to improve cryopreserved semen quality by supplementing extenders with antioxidants (Luño et al., 2014; Lee and Kim, 2017) and proteins (Park et al., 2018; Robles et al., 2019), and adding saccharides (Pezo et al., 2020). Other researchers have reported that freezing-thawing protocol can affect with diluent type and centrifugation regime (Carvajal et al., 2004; Stuart et al., 2019). In the present study, we determined that the type of washing solution used affects the quality of the frozen-thawed boar semen. Significantly higher motility and progressive motility were obtained when cryopreserved semen was processed with Hulsen solution. Concerning post-thawing sperm viability and acrosome integrity, the best results were also observed in the group processed with Hulsen solution. These parameters are an important indicator of semen quality and have been correlated with fertility (Foote, 2003).

The presence of sugar in the Hulsen solution might explain the better results obtained from this group when compared to other DPBS groups. The exact mechanism by which sugars improve semen's freezability is not fully understood, though it has been suggested that a hypertonic solution of sugars generates osmotic pressure, reducing the amount of intracellular water and intracellular ice formation, thereby preserving the sperm during the freezing process and rendering the membrane less vulnerable to the morphological changes that occur during the rapid reflux of water (Jain and Roy, 2009; Malo et al., 2010a). Furthermore, The beneficial effects of using sugar during semen freezing-thawing process in this study concur with previous reports in the literature that demonstrated glucose (Ciereszko et al., 2014), trehalose (Athurupana et al., 2015), and raffinose plus fructose (Yildiz et al., 2007) delivered high motility and plasma membrane integrity rates after sperm freezing and thawing.

Alternatively, the presence of sodium EDTA as a component of Hulsen solution might have stimulated total and progressive motility in the frozen-thawed boar spermatozoa. It has been reported elsewhere that supplementing sperm preparation medium with EDTA significantly improved forward motility and reduced DNA fragmentation in human sperm (Chi et al., 2008) and buffalo sperm (Hussain et al., 2019).

Centrifugation, which is part of semen freezing-thawing protocol, is essential for the preparation of sperm for further uses in *in-vitro* fertilization and other assisted reproduction technologies. In the present study, we examined the combined effect of washing-centrifugation of frozen-thawed boar semen. Post-thaw semen analysis showed significantly higher motility and progressive motility in Group 1 (Hulsen solution) than other groups. Notably, we also observed lower total motility and progressive motility after centrifugation of FT semen. This finding consistent with that of (Shekarriz et al., 1995), who demonstrated that centrifugation imparts stress to spermatozoa and may increase lipid peroxidation. Previous studies concerning the impact of various centrifugation regimes on boar spermatozoa have reported that the use of short-term centrifugation with a relatively high force has a positive effect on boar sperm's cryosurvival (Jovičič et al., 2020). Accordingly, the results presented in the literature regarding the washing of spermatozoa are quite variable. Further studies are needed to clarify this issue certainly in terms of post-thawing centrifugation.

CONCLUSION

In conclusion, the present study demonstrated that the type of washing solution used during the freezing-thawing

process affects semen quality, FT semen processed with Hulsen solution showed enhanced motion parameters, higher rate of viability and acrosome integrity than other groups. Furthermore, centrifugation of frozen-thawed semen has an unfavorable effect on total motility and progressive motility.

CONFLICTS OF INTEREST

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

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