Effects of Orvus Es Paste (OEP) on the Viability of Bull Spermatozoa After Double Freezing and Thawing

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ABSTRACT

Refreezing of spermatozoa is necessary for flow cytometric sex sorting when using semen from proven bulls that have already been frozen-stored. This study evaluated the effects of Orvus ES Paste (OEP) on the post-thaw viability of refrozen bull spermatozoa. Semen samples that had been frozen by the standard procedures were thawed and then refrozen in freezing extender supplemented with 0%, 0.375%, 0.75%, 1.5%, or 3% of OEP. The post-thaw indicators (motility, viability, and plasma membrane integrity) were higher ($P < 0.05$) in the spermatozoa refrozen with 0.375% or 0.75% OEP than in the spermatozoa refrozen without OEP. Moreover, the addition of 0.375% OEP increased the percentages of viability and plasma membrane integrity of the post-refrozen spermatozoa compared with 0.75% OEP. When the effect of the 0.375% OEP supplementation on the viability of the refrozen-thawed spermatozoa was assessed

in six bulls, the presence of OEP significantly increased the percentages of the total and progressive motility in three bulls and the percentages of the viability and plasma membrane integrity in five bulls ($P < 0.05$). Our findings indicate that the addition of 0.375% OEP to freezing extender improves the motility, viability, and plasma membrane integrity of refrozen-thawed spermatozoa.

INTRODUCTION

Controlling the sex ratio permits faster genetic progress and higher productivity due to the elimination of the unwanted sex. The sex pre-selection of semen is beneficial for livestock production because inseminations can be planned to produce a specific sex. Sexed semen using a flow cytometer/sperm sorter is now widely available in dairy industries around the world, and has become a commercial application. Flow cytometric sex sorting is only one useable and reasonably accurate method for sexing sperm, in which the sperm are sorted by the equipment on the basis of a 4% difference in DNA content between bovine sperm containing X and Y chromosomes (Johnson et al., 1989; Butler et al., 2014). On the other hand, the re-freezing of spermatozoa is necessary for flow cytometric sex sorting when using semen from proven bulls that have been already frozen-stored or imported or when the sex-sorting facility is located at a long distance from where the semen are used. If the frozen-thawed semen is sorted and then refrozen and still keeps its functionality to be used for artificial insemination (AI) or other assisted reproductive technologies, it would enable shipping after sorting. To date, some studies in ram semen demonstrate that the double freezing of sex-sorted spermatozoa is possible (Hollinshead et al., 2004; de Graaf et al., 2007). It is suggested that dead or damaged cells are sorted out, and thus the sample used for the second freezing is rich in viable, motile cells (Saragusty et al., 2009). However, it remains unclear whether more than one freeze–thaw cycle affects the viability of bull spermatozoa and their functionality.

Freezing conditions interfere with semen quality, and the low fertility of frozen–thawed semen is associated with the damage that occurs during cryopreservation. It is generally assumed that 40–50% of the spermatozoa do not survive the freezing and thawing process, even with optimized protocols (Watson, 2000). Many factors involved in the cryopreservation of semen directly or indirectly interfere with spermatozoa viability after thawing, of which a semen extender plays a significant role in successful semen freezing (Layek et al., 2016). Currently, combinations of glycerol and detergents, such as Orvus ES Paste (OEP) and Equex STM Paste, are reported to benefit successful freezing in several species; e.g., pigs (Pursel et al., 1978), cats (Axner et al., 2004), and dogs (Rota et al., 1997; Mizutani et al., 2010). OEP and Equex STM Paste contain a water-soluble anionic detergent that solubilizes active molecules. The supplementation of the detergents to a freezing extender is demonstrated to improve the post-thaw motility of bovine and Yak spermatozoa (Arriola and Foote, 1987; Shimazaki et al., 2015). Thus, the present study was conducted to evaluate the potential effects of OEP on the refreezing of bull spermatozoa.

MATERIALS AND METHODS

Semen

All the semen samples used in the present study were frozen according to the standard procedures that use a modified Tris-egg yolk extender, which consists of 13.63 g Tris [hydroxymethyl] aminomethane (Tris; Sigma-Aldrich, St. Louis, MO, USA), 7.62 g citric acid monohydrate (Wako Pure Chemical Industries Ltd., Osaka, Japan), 3.75 g fructose (Sigma-Aldrich), 15.0 g lactose (Wako Pure Chemical Industries Ltd.), 27.0 g raffinose (Wako Pure Chemical Industries Ltd.), 0.09 g theophylline (Sigma-Aldrich), 200 ml egg-yolk, 1,000,000 IU penicillin (Meiji Seika Co., Tokyo, Japan), and 1 g streptomycin (Meiji Seika Co.), and was adjusted to 1,000 ml with distilled water (Takahashi, 2015). The ejaculated samples were collected from

six Japanese Black bulls (3-9 years old) raised in the Yamaguchi Prefectural Agriculture & Forestry General Technology Center (Yamaguchi, Japan) that were frozen in the Tris-egg yolk extender, containing a final concentration of 6.5% glycerol (spermatozoa concentration, 5 × 107 cells/ml) and then were packed in 0.5-ml plastic straws.

Semen Refreezing

After thawing the frozen straws (the same batches made from one ejaculate from each bull), the semen was expelled into a polystyrene conical tube containing modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) and then washed by centrifugation at 650 × g for 5 min. The pellet of the spermatozoa was gently re-suspended, first, in Tris-egg yolk extender. After the suspension, the spermatozoa were diluted to final concentrations of 6 × 107 cells/ml with the first Tris-egg yolk extender. A polystyrene conical tube containing the spermatozoa suspension was transferred to a 500-ml glass beaker containing 350 ml of water at room temperature, which was then maintained at approximately 5°C for 2 h. The cooled spermatozoa suspensions were then mixed with a one-half volume of the second extender (the first Tris-egg yolk extender with 13% [v/v] glycerol) supplemented with or without the Orvus ES Paste (OEP; Miyazaki-kagaku, Tokyo, Japan). After 5 min at approximately 5°C, an additional one-half volume of the second extender was added to the spermatozoa suspensions to achieve a final concentration of 6.5% glycerol. The concentrations of the spermatozoa were adjusted to one-half (3 × 107 cells/ml). Aliquots of the spermatozoa suspensions were then immediately loaded into 0.25-ml plastic straws (Fujihira Co., Tokyo, Japan), which were placed on a Styrofoam plate in liquid nitrogen (LN2) vapour (4 cm above the surface of the LN2) and frozen. The straws were kept on the plate for 20 min and then were plunged into the LN2 for storage.

Sperm Quality Assessments

A 300 µl aliquot of each frozen-thawed semen sample was mixed by pipetting and was warmed at 37°C for 10 min before evaluation. Motility analyses were performed using the computer-assisted sperm analysis (CASA) system. Analyses of the viability, acrosome integrity, and plasma membrane integrity were conducted according to the methods described by Wittayarat et al. (2012).

Each sample was diluted 10-fold with PBS supplemented with 0.3% BSA and was placed in a warm glass chamber to assess motility. Briefly, approximately 5 µl of the sperm suspension was transferred to a warm chamber (2-chamber slide, 20 microns in depth; Leja Products B.V., Nieuw-Vennep, The Netherlands) and then was placed on a warm plate at 37°C. Sperm motility was evaluated using the CASA system (Sperm Class Analyzer®: SCA® v.4.2 Microptic, Barcelona, Spain). The analysis was based on the examination of 25 consecutive, digitised images obtained from 3 fields using a ×10 phase contrast objective, and at least 300 spermatozoa per sample were analysed. The analysis time was 1 sec per field, and the images were taken with a time lapse of 1 sec. Therefore, the image capture speed was one every 40 msec. After acquiring the representative fields, the total motile spermatozoa and progressive motile spermatozoa (> 45% of straightness coefficient) were recorded.

The sperm viability was assessed using a live/dead stain combination (SYBR-14/ propidium iodide [PI], LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol, with minor modifications. Briefly, an aliquot of semen (5 µl) was mixed with 50 µl of a solution containing 3 µl of PI (diluted 1:10 in distilled water) and 3 µl of SYBR-14 (diluted 1:500 in DMSO) and was incubated according to the manufacturer's instructions. One hundred spermatozoa were assessed in each of two duplicate aliquots of each sample and was evaluated using a fluorescence microscope (200×, Nikon Eclipse 80i; Nikon Corp.)

with a 488-nm filter. The live sperm nuclei were stained with SYBR-14 and were bright fluorescent green, whereas the dead sperm nuclei exhibited a red (PI) fluorescence.

The plasma membrane integrity of the sperm was assessed using the hypo-osmotic swelling test. Briefly, an aliquot of semen (20 µl) was mixed with 80 µl of a hypo-osmotic solution (150 mOsm/kg) containing 13.5 mg/ml D-fructose and 7.35 mg/ml trisodium citrate dehydrate in distilled water. The samples were incubated at 37°C for 10 min. Then, 10 µl of each sample was placed on a slide and was overlaid with a coverslip. One hundred spermatozoa in three fields from one aliquot of each sample were assessed using a phase-contrast microscope (400×, Nikon TE300; Nikon Corp.). The plasma membrane integrity of the sperm was expressed as the percentage of sperm with curled tails (intact plasma membrane) out of the total number of spermatozoa.

The acrosomal integrity of the spermatozoa was measured using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA). The samples were spread on slides, air-dried at room temperature, and fixed with absolute ethanol for 10 min at room temperature. After drying, the slides were spread with 30 µl of FITC-PNA (100 µg/ml) in PBS and were incubated in a dark, moist chamber for 30 min at 37°C. The slides were then rinsed with PBS, air-dried, and overlaid with a coverslip. The acrosomal status (intact or reacted) was determined from the FITC-PNA staining pattern observed using fluorescence microscopy (400x, Nikon Eclipse 80i; Nikon Corp.) with a 488-nm filter. In total, 100 spermatozoa were counted over at least three different fields. The spermatozoa were considered acrosome intact if the acrosome stained green, while those with no staining or a single band of green staining at the equatorial segment were considered to have non-intact acrosomes (a damaged acrosome). The acrosomal integrity was expressed as the mean percentage of spermatozoa with intact acrosomes.

Experimental Design

In the first experiment, we examined the effect of the OEP concentration on the quality of the refrozen spermatozoa. The cooled spermatozoa suspensions, derived from a bull, were mixed with the second extender supplemented with OEP and were frozen with final concentrations of 0%, 0.375%, 0.75%, 1.5% or 3% OEP. After thawing, the sperm quality (motility, viability, plasma membrane integrity, and acrosomal integrity) was assessed as described above.

In the second experiment, we tested the effect of OEP supplementation on the quality of refrozen spermatozoa derived from different bulls. The final concentration of OEP (0.375%) that was found to be most suitable for the quality of refrozen spermatozoa in the first experiment was used in this experiment. The cooled spermatozoa suspensions, derived from six bulls, were frozen in the freezing extender supplemented with or without 0.375% OEP.

Statistical Analysis

All the experiments were repeated 5 times. For the analysis of the sperm characteristics in Experiment 1, the statistical significance was inferred based on the analyses of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) tests that were performed with STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All the percentage data were subjected to arc sin transformation prior to the statistical analysis. For the analysis of the sperm characteristics in Experiment 2, the differences in each bull were evaluated using an independent Student's t-test. The data were expressed as the means ± the SEMs. The differences with probability values (P) of 0.05 or less were regarded as significant.

RESULTS

Effect of OEP Concentration

The mean percentages of total and progressive motility of the spermatozoa refrozen with 0.375% and 0.75% OEP were significantly higher ($P < 0.05$) than those of the

Table 1. *Quality of the bull spermatozoa after refreezing with various concentrations of Orvus Es Paste (OEP)**

Concentration of OEP (%)	Percentages of spermatozoa				
	Total motility	Progressive motility	Viability	Plasma membrane integrity	Acrosomal integrity
Pre-freezing**	67.7 ± 2.7[a]	38.1 ± 5.2[a]	52.1 ± 2.1[a]	45.7 ± 0.7[a]	96.6 ± 0.3[a]
0	14.1 ± 3.7[b,d]	3.3 ± 0.9[b]	9.0 ± 2.6[b]	15.6 ± 0.8[b]	97.5 ± 0.2[b]
0.375	29.3 ± 3.2[c]	14.1 ± 1.6[c]	37.3 ± 1.7[c]	32.6 ± 0.7[c]	97.5 ± 0.2[b]
0.75	23.4 ± 3.1[c,e]	11.7 ± 1.8[c]	25.0 ± 1.1[d]	22.9 ± 1.7[d]	97.2 ± 0.3[a,b]
1.5	17.4 ± 1.1[b,e]	7.0 ± 0.4[b,c]	17.9 ± 2.3[e]	15.7 ± 2.1[b]	97.6 ± 0.1[b]
3	6.0 ± 1.7[d]	0.2 ± 0.2[b]	1.0 ± 0.4[f]	3.4 ± 0.3[e]	96.9 ± 0.3[a,b]

**Five replicate trials were carried out. The data are expressed as the mean ± SEM.*
***The quality of spermatozoa was examined before refreezing.*
a-fValues with different superscripts in the same column are significantly different (P < 0.05).

control spermatozoa refrozen without OEP (Table 1). Moreover, the percentages of viability and the plasma membrane integrity of spermatozoa refrozen with 0.375% OEP were significantly higher ($P < 0.05$) than those of the spermatozoa refrozen with the other concentrations of OEP. The percentages of the motility, viability, and plasma membrane integrity of the refrozen spermatozoa decreased compared with those of the pre-frozen spermatozoa, irrespective of the OEP concentration ($P < 0.05$). However, the acrosomal integrity of the spermatozoa were unaffected by the refreezing-thawing and the OEP concentration.

Effect of OEP Supplementation

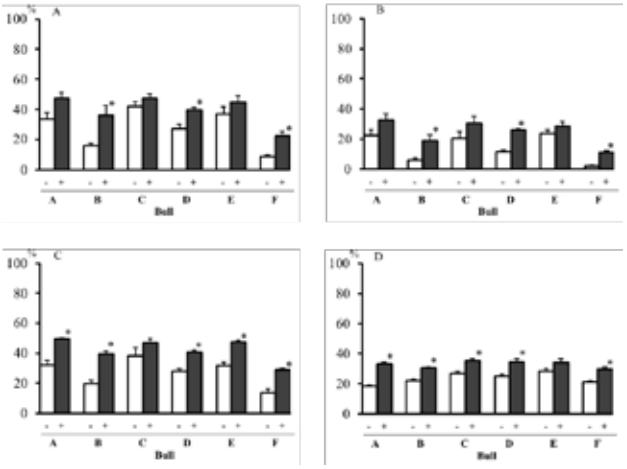
The effect of 0.375% OEP supplementation on the quality of the refrozen-thawed spermatozoa was assessed in six bulls, and of which, the spermatozoa from three bulls had significantly higher percentages of total and progressive motility ($P < 0.05$) (Fig. 2, A and B). Moreover, the spermatozoa from five bulls significantly increased the percentages of viability and plasma membrane integrity by the OEP supplementation ($P < 0.05$) (Fig. 2, C and D). However, the acrosomal integrity of the spermatozoa was unaffected by the OEP supplementation in all the bulls.

DISCUSSION

In the present study, we showed that the addition of 0.375% OEP to the freezing extender significantly improved the motility, viability, and plasma membrane integrity of refrozen-thawed spermatozoa. It is demonstrated that 0.5-1.5% OEP is the optimum concentration for the preservation of fresh spermatozoa in other species during the freeze-thaw process (Pursel et al., 1978; Mizutani et al., 2010; Shimazaki et al., 2015). In this study, however, we found that the addition of OEP with a lower concentration to the freezing extender was effective for the preservation of the refrozen-thawed spermatozoa. In general, about half of the spermatozoa cannot survive cryopreservation even with optimized freezing protocols (Watson, 2000). Our results also showed that the post-thaw viability of the fresh spermatozoa was 52%. Moreover, the percentages of motility, viability, and plasma membrane integrity of the spermatozoa that were refrozen without OEP decreased by less than 20% after thawing.

The reduction is suggested to arise from factors affecting the proportion of survivors (e.g., cold shock susceptibility, cooling rate, diluent composition and osmotic stress) and factors influencing the functional status of the survivors (e.g., membrane stability, oxidative damage, membrane receptor integrity, and nuclear structure) (Holt,

Figure 1. *Total motility (A), progressive motility (B), viability (C), and plasma membrane integrity (V) of the spermatozoa after refreezing with (+) or without (-) 0.375% Orvus ES Paste (OEP) in six bulls. Each bar represents the mean ± SEM. *denotes a significant difference in each bull (P < 0.05).*



2000; Watson, 2000). Alterations in the cell membrane properties that occur during the freeze-thaw process change the stability and water permeability of the cells. Therefore, the optimum concentration of OEP for the refreezing of bull spermatozoa might be different from that for the fresh spermatozoa.

It has been reported that OEP has beneficial effects on sperm function in the presence of egg yolk (Hofmo and Almlid, 1991). OEP contains sodium dodecyl sulfate (SDS), which may act by modifying the structure of the egg yolk lipoproteins in the freezing extender (Arriola and Foote, 1987). Arriola and Foote (1987) demonstrated that when bull spermatozoa were frozen, the presence of SDS was less susceptible to the osmotic shock induced by the addition of glycerol. It is suggested that the SDS in OEP stabilizes the sperm membrane and delays capacitation-like changes that eventually lead to the acrosome reaction and subsequent cell death (Pena et al., 2003). Therefore, the precise mechanism by which OEP improves sperm cryosurvival is indirect. In the present study, we observed that the post-thaw indicators (the motility, viability, and plasma membrane integrity) were higher in the spermatozoa refrozen with 0.375% or 0.75% OEP than in the spermatozoa refrozen without OEP (Table 1). Moreover, the addition of 0.375% OEP increased the percentages of viability and plasma membrane integrity of the post-refrozen spermatozoa compared with 0.75% OEP. The indicators examined in the different bull spermatozoa consistently showed that the protective effect was more pronounced when the spermatozoa were refrozen with 0.375% OEP. Therefore, our findings indicate that the addition of 0.375% OEP exhibited the beneficial effects on the refreezing of bull spermatozoa.

On the other hand, previous studies suggest that the use of OEP and glycerol for the cryopreservation of semen in pig, dog, and cat protects the acrosome caps of the sperm and, thereby, increases and maintains post-thaw sperm motility (Pursel et al., 1978; Tsutsui et al., 2000; Mizutani et al., 2010). However, our results showed that acrosome integrity of the post-refrozen spermatozoa was unaffected, irrespective of the OEP concentration and the different bulls. These results are in agreement with the experiment of Morton et al. (2010) who reported that post-thaw motility of epididymal alpaca spermatozoa was higher when the spermatozoa were frozen and thawed in the presence of SDS, while the acrosome integrity was unaffected. In the present study, the acrosomal status was determined by the FITC-PNA staining pattern. This discrepancy about the effect of OEP on the acrosome integrity remains to be explained, but it might result from different classification of the acrosome status or other factors (e.g., species, and freezing protocol).

In conclusion, the results presented herein demonstrated that the optimum con-

centration of OEP for the refreezing of bull spermatozoa is 0.375%, and the addition of OEP to the freezing extender may improve the motility, viability, and plasma membrane integrity of refrozen-thawed spermatozoa. However, almost 70% of the population did not survive after refreezing and thawing. Further studies are necessary to improve the viability of post-refrozen spermatozoa for successful AI using sex-sorted semen that have already been frozen-stored.

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