Original article

Supplementation of different concentrations of Orvus Es Paste (OEP) to ostrich egg yolk lipoprotein extender improves post-thaw boar semen quality

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Abstract

This study aimed to compare post-thaw quality of boar semen following freezing in an ostrich egg yolk lipoprotein (LPFo) extender supplemented with 0%, 0.25% and 0.50% Orvus Es Paste (OEP). Sperm assessments included total motility (TMOT), mitochondrial function (MF), plasma membrane integrity (PMI) and acrosome integrity (normal apical ridge, NAR). Considerable variations among boars and OEP treatments had a significant effect (P < 0.001) on post-thaw sperm characteristics. It was observed that post-thaw sperm characteristics were significantly compromised in semen samples frozen in the absence of OEP. By contrast, lactose-LPFo-glycerol extender supplemented with either 0.25% OEP or 0.50% OEP markedly enhanced post-thaw sperm characteristics. In all boars, there were no marked differences in post-thaw sperm TMOT between the freezing extenders supplemented with 0.25% and 0.50% OEP. However, a decline in the percentage of post-thaw motile spermatozoa was more pronounced in the extender supplemented with 0.50% OEP following a 120-min incubation period. Furthermore, the proportions of frozen-thawed spermatozoa with MF, PMI and NAR acrosomes varied significantly among the boars in the OEP-supplemented extenders. The findings of this study indicate that different OEP concentrations, in the presence of ostrich egg yolk lipoproteins, could have varying effects on post-thaw sperm survival.

Key words: boar, spermatozoa, cryopreservation, Orvus Es Paste, sperm characteristics

Introduction

Cryopreservation of boar semen is not used on a large scale due to the reduced fertility of frozen-thawed spermatozoa compared with liquid-stored semen (Waberski et al. 1994). There is increasing evidence indicating that the quality of post-thaw boar semen is influenced by many factors, such as packaging materials, individual differences and freezing extender composition (Pursel et al. 1972, Johnson et al. 2000, Fraser and Strzeżek 2007). Among these factors, the composition of the freezing extender has been shown to significantly limit sperm damage incurred during the freezing-thawing process (Pettitt and Buhr 1998, Cerolini et al. 2001).

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Hen egg yolk (HEY) or egg yolk from other avian species has been commonly used as a cryoprotective additive in freezing extender because of the presence of phospholipids, which protect sperm membrane structures during the cooling and freezing processes (Pursel et al. 1972, Johnson et al. 2000). Boar spermatozoa are exposed immediately before freezing to a synthetic detergent, Orvus Es Paste, OEP (known as Equex-STM), which is routinely used at a final concentration of 0.50% (v/v) in HEY-based media (Pettitt and Buhr 1998, Johnson et al. 2000). Little information is available about the precise composition of OEP. However, it has been reported that the active compound in OEP is sodium dodecyl sulphate (SDS), a water-soluble anionic detergent, which disintegrates aggregates of lipid-based egg yolk extender so that phospholipids can be more accessible to spermatozoa (Pursel et al. 1978, Pettit and Buhr 1998). Even though the optimal concentration of SDS in OEP is unclear, its concentrations in boar freezing extender ranged from 1.2 to 1.6 mg/ml (Kato et al. 1990). Several authors have demonstrated that prolonged exposure of sperm cells to SDS-treated egg yolk could have a negative effect on sperm function (Peña and Linde-Forsberg 2000, Axner et al. 2004, Niżański et al. 2009).

It is well recognized that low-density lipoproteins (LDL) of HEY protect spermatozoa from different animal species against cryoinduced damage when used in the absence of OEP (Moussa et al. 2002, Pillet et al. 2011). We have shown that lipoprotein fractions, isolated from ostrich egg yolk (LPFo), can be effectively used as a cryoprotective additive in the freezing extender of boar semen (Strzeżek et al. 2005, Fraser and Strzeżek 2007). Using a fluorescent membrane probe, 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), we have demonstrated that LPFo coat spermatozoa at different domains of the membrane architecture (Fraser et al. 2010). This study investigated the effects of different OEP concentrations in a LPFo-based extender on the post-thaw quality characteristics of boar spermatozoa. The analyzed sperm quality characteristics included total motility (TMOT), mitochondrial function (MF), plasma membrane integrity (PMI) and normal apical ridge (NAR) acrosome.

Materials and Methods

Animals and semen cryopreservation

Sperm-rich fractions (SRFs) were collected from 3 Polish Landrace, (aged 1.5-2 years), using the gloved-hand technique. The boars were designated as A, B and C, and a total of 15 ejaculates, 5 each from the boars, were collected. We used semen samples with at least 70% motile spermatozoa and more than 85% morphologically normal spermatozoa. The LPFo was lyophilized (Strzeżek et al. 2005) and stored until required. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

The SRFs were processed according to a standard protocol, with some modifications (Fraser and Strzeżek 2007). The SRFs were extended (1:4) in Beltsville Thawing Solution (BTS) prior to storage for 1h at 17°C. The cooled extended semen was centrifuged at $800 \times g$ for 10 min and the sperm pellets (750 $\times$ 106 spermatozoa/ml) were re-suspended in a cooling extender containing 11% lactose (w/v) with LPFo (lactose-LPFo extender). The diluted semen was gently mixed and cooled to 5°C for 3h. Following cooling, the semen was diluted 2:1 with a lactose-LPFo-glycerol extender containing 0%, 0.75% (v/v) or 1.50% (v/v) OEP (v/v), resulting in final OEP concentrations of 0%, 0.25%, or 0.50%, respectively. The final concentrations of LPFo (w/v) and glycerol (v/v) in each freezing extender were 5% and 3%, respectively. The cooled samples were packaged in 10-ml sterilized aluminum tubes and frozen in a controlled programmable freezer (Ice Cube 1810, SY-LAB, Austria), using a freezing protocol. The frozen samples were stored in liquid nitrogen prior to thawing in a water bath for 60 sec at 50°C. All thawed samples were diluted (1:10) with BTS and incubated for 15 min at 37°C prior to evaluation of sperm characteristics.

Evaluation of sperm characteristics

Sperm characteristics were analyzed in fresh semen and post-thaw semen samples. For analysis of post-thaw semen sperm TMOT samples were analyzed at different incubation periods: 0 min (15 min pre-incubation), 60 min and 120 min, whereas post-thaw MF, PMI and NAR were monitored only after the 15 min pre-incubation period (0 min).

Total motility (MOT) evaluations

Aliquots (6 µl) of semen samples were examined at 200× magnification under a bright light microscope (Olympus BX 40, Tokyo, Japan), equipped with an attached heated stage (37°C). Sperm TMOT was defined as the percentage of spermatozoa that had any form of motility by estimating the proportion of motile cells in at least 5 fields, and was subjectively assessed by a trained technician throughout this study.

Table 1. Characteristics of fresh boar spermatozoa prior to freezing-thawing. Values represent the means (± SD) of 5 ejaculates, each from 3 boars.

Boars	Total motility (TMOT)	Mitochondrial function (MF)	Plasma membrane integrity (PMI)	Normal apical ridge (NAR) acrosome
A	73.0 ± 4.5	79.6 ± 4.1	81.8 ± 7.3	87.4 ± 2.3
B	71.5 ± 2.2	77.3 ± 2.6	80.7 ± 8.0	86.2 ± 4.3
C	73.0 ± 2.7	80.2 ± 3.3	81.1 ± 7.5	88.2 ± 3.5

Table 2. ANOVA results for parameters of post-thaw sperm quality characteristics.

Sources of variation	d.f.	Total motility (TMOT)		Mitochondrial function (MF)		Plasma membrane integrity (PMI)		Normal apical ridge (NAR) acrosome	
		F-test	p	F-test	p	F-test	p	F-test	p
Boar	2	15.684	<0.001	33.728	<0.001	16.353	<0.001	19.364	<0.001
Treatment	2	66.227	<0.001	129.662	<0.001	112.427	<0.001	119.113	<0.001
Boar × treatment	4	1.069	n.s	1.830	n.s	0.611	n.s	0.415	n.s

ANOVA; boar; OEP treatment (0% OEP, 0.25% and 0.50% OEP); d.f. – degree of freedom; F – Fisher test; n.s – not significant

Mitochondrial function (MF)

Assessment of sperm MF was performed using fluorescent probes, JC-1 with propidium iodide, PI (Molecular Probes, Eugene, USA), according to a previously described method (Thomas et al. 1998). Briefly, semen samples (30×10^6 spermatozoa/ml) were incubated with 3 µl JC-1 solution (1 mg JC-1/ml anhydrous dimethyl sulfoxide) for 15 minutes at 37ºC and then counterstained with PI (10 µl of PI solution in 0.5 mg/ml phosphate buffered solution, PBS). Aliquots (10 µl) of stained sperm samples were examined at 600× magnification under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). Sperm cells displaying only orange-red fluorescence at the mid-piece region were considered viable spermatozoa with functional mitochondrial. A minimum of 100 cells per slide was examined in each aliquot.

Plasma membrane integrity (PMI)

Sperm PMI was assessed with dual fluorescent probes, SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA), according to a previously described method (Garner et al. 1995). Briefly, semen samples (30×10^6 spermatozoa/ml) were incubated with 10 µl of 1 mM SYBR-14 solution in HEPES-BSA solution (pH 7.4) and 10 µl of 2.4 µM PI in Tyrode's salt solution for 10 min at 37ºC. Aliquots of stained sperm cells were examined at 600× magnification under epifluorescence microscopy (Olympus CH 30, Tokyo, Japan). Approximately 100 cells per slide were examined in each aliquot, and were classified as membrane-intact and membrane--damaged spermatozoa.

Normal apical ridge (NAR) acrosome

The percentage of spermatozoa with NAR acrosomes was evaluated in semen samples as described by Watson (1975), using the Giemsa staining technique. Aliquots of stained cells were examined under a bright light microscope, equipped with oil-immersion lens, at 1000× magnification. A minimum of 100 cells per slide was examined and were classified as spermatozoa with NAR acrosome or damaged apical ridge acrosome

Statistical analysis

All results are expressed as the mean ± SD. The main effects of boar (A, B and C) and OEP treatments (0%, 0.25% and 0.50%), and their interactions

on post-thaw sperm characteristics were analyzed by repeated measures ANOVA, using the general linear model (GLM) procedure from Statistica software package, version 5 (StatSoft Incorporation, Tulsa OK., USA). Multiple comparisons were performed using the Neuman-Keuls *post hoc* test, and values were considered significant at P < 0.05

Results

Evaluation of fresh semen

In this study no significant differences in sperm characteristics among boars prior to freezing-thawing were observed (Table 1).

Post-thaw sperm characteristics

There were significant (P < 0.001) effects of boar and OEP treatment (0%, 0.25% and 0.50% OEP) on post-thaw sperm TMOT, MF, PMI and NAR acrosome (Table 2). However, no significant differences (P > 0.05) were found between boar × OEP treatment interaction.

In all boars spermatozoa frozen in the presence of OEP exhibited higher (P < 0.05) post-thaw TMOT than those frozen in the absence of OEP (Fig. 1). No significant differences (P > 0.05) in post-thaw sperm TMOT were observed between extenders supplemented with 0.25% OEP and 0.50% OEP in all boars at the onset of the incubation period (Fig. 1A). There was a marked reduction in post-thaw sperm TMOT following 60 and 120 min incubation periods (Fig. 1B and 1C, respectively), regardless of OEP treatments. However, the deterioration in post-thaw sperm TMOT was more pronounced in the extender supplemented with 0.50% OEP after the 120-min incubation period (Fig. 1C).

Post-thaw sperm MF did not differ significantly (P > 0.05) between extenders supplemented with 0% OEP and 0.25% OEP for Boars A and B (Fig. 2A). Furthermore, no marked significant differences in post-thaw sperm MF were observed between extenders supplemented with 0.25% OEP and 0.50% OEP for Boar A (Fig. 2A). Frozen-thawed spermatozoa of Boar A exhibited significantly lower (P < 0.05) MF for the extenders supplemented with 0.50% and 0.25% OEP compared with Boars B and C, respectively (Fig. 2A). In all boars, extender supplemented with 0.50% OEP gave better protection to sperm PMI following freezing-thawing (Fig. 2B). It was observed that the deterioration in post-thaw sperm PMI was more marked in Boar A compared with the other

boars, particularly in the OEP-supplemented extenders (Fig. 2B). Semen frozen in the absence of OEP showed significantly lower (P < 0.05) proportions of spermatozoa with NAR acrosomes (Fig. 2C). There were considerable variations among boars with respect to the percentage of frozen-thawed spermatozoa with NAR acrosomes, being significantly lower (P < 0.05) in Boar A for extenders supplemented without OEP and with 0.25% OEP (Fig. 2C). The percentage of spermatozoa with NAR acrosomes did not differ significantly (P > 0.05) between extenders supplemented with 0.25% OEP and 0.50% OEP for Boar B (Fig. 2C).
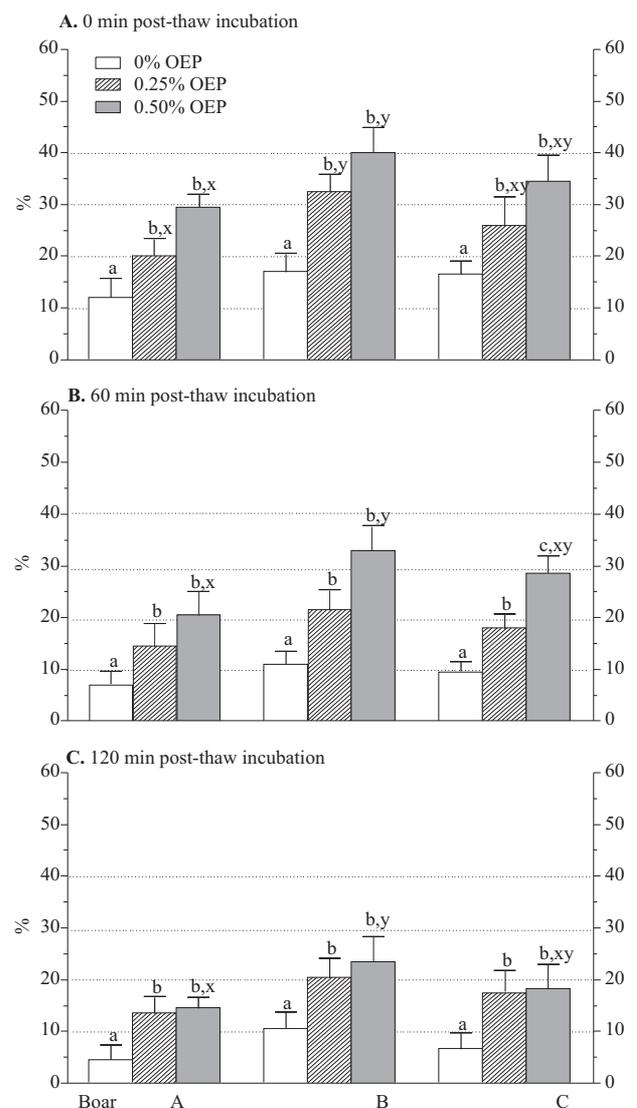

Fig. 1. Percentage of total motile (TMOT) frozen-thawed boar spermatozoa following supplementation of different Orvus Es Paste (OEP) concentrations to ostrich egg yolk lipoprotein extender. Values represent the means (+ SD) of 5 ejaculates, each from 3 boars. Within treatments, values with different superscripts (a,b,c) are significant at P < 0.05. Among boars, values with different superscripts (x,y) are significant at P < 0.05.
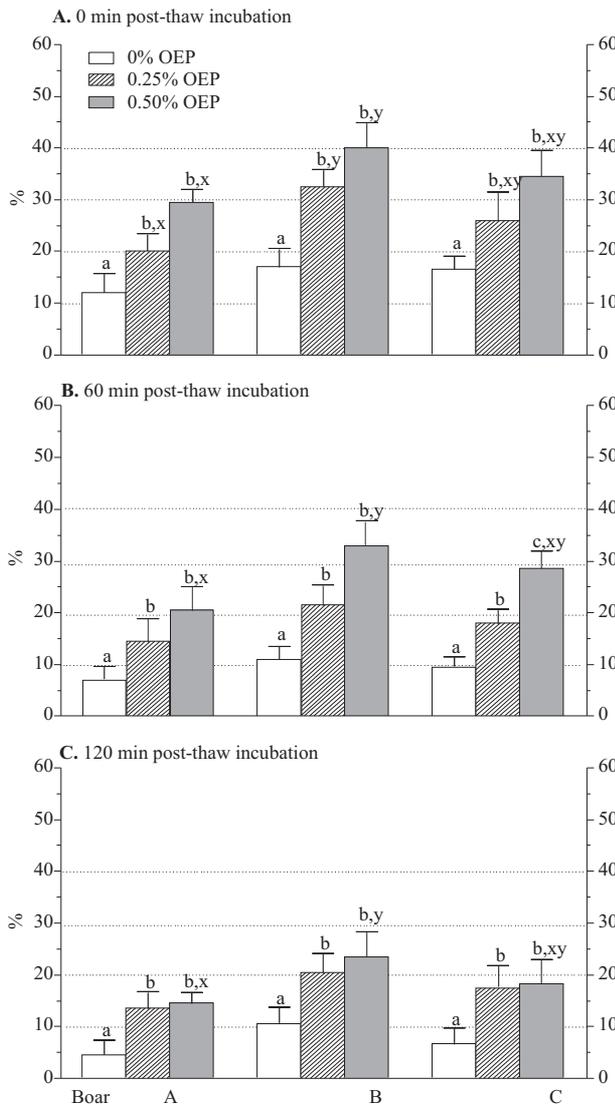
Fig. 2. Percentage of boar spermatozoa with (A) functional mitochondria (MF), (B) plasma membrane integrity (PMI) and (C) normal apical ridge (NAR) acrosome following supplementation of different Orvus Es Paste (OEP) concentrations to ostrich egg yolk lipoprotein extender. Values represent the means (+ SD) of 5 ejaculates, each from 3 boars. Within treatments, values with different superscripts (a,b,c) are significant at P < 0.05. Among boars, values with different superscripts (x,y) are significant at P < 0.05.

Discussion

The effectiveness of OEP in the cryopreservation of semen of different animal species has been reported by several authors (Arriola and Foote 1987, Aboagla and Terada 2004, Axner et al. 2004, Niżański et al. 2009). The findings of the current study showed that OEP, used at a concentration of 0.25%, gave comparable results to extender supplemented with 0.50% OEP, in terms of post-thaw sperm TMOT. Furthermore, the marked deterioration in post-thaw

sperm TMOT in extender supplemented with 0.50% OEP after a 120-min post-incubation period might suggest that excess OEP components exerted a negative effect on the sperm motility apparatus. In this study 0.25% OEP was the optimal concentration in the LPFo-based extender to protect sperm motility apparatus after freezing-thawing. Several studies have reported about the toxic effects of OEP components on sperm function (Pursel et al. 1978, Arriola and Foote 1987, Kato et al. 1990, Niżański et al. 2009). It is worth noting that the beneficial effect of OEP on post-thaw semen quality was time dependent (Peña and Linde-Forsberg 2000, Axnér et al. 2004).

It was found that there were wide variations in post-thaw sperm MF, PMI and NAR acrosome among the boars when the extender was supplemented either with 0.25% OEP or 0.50% OEP. It has been suggested that 0.50% OEP is the optimal concentration used in HEY-based extender to improve post-thaw boar sperm viability (Pettit and Buhr 1998, Johnson et al. 2000). Pursel et al. (1978) suggested that OEP concentration ranging from 0.50 to 1.5% was optimal for post-thaw sperm motility and acrosome. However, in a recent study it has been postulated that OEP concentration ranging from 1 to 1.5% could still have a beneficial effect on post-thaw sperm function (Wu et al. 2013). The findings of the current study showed that, OEP used at different concentrations in the LPFo-based extender, had varying effects on post-thaw sperm survival, which differed significantly among the boars.

The mechanism responsible for the action of OEP on post-thaw sperm function has not been fully elucidated as yet. It was suggested that bull spermatozoa frozen in HEY-based extender supplemented with SDS were less susceptible to osmotic shock (Arriola and Foote 1987). Previous studies demonstrated that OEP improved post-thaw viability of boar spermatozoa only in the presence of egg yolk components, presumably by modifying the structure of egg yolk lipids, making them more easily accessible to spermatozoa, probably by allowing integrations of the lipids into the membrane bilayer (Pursel et al. 1978, Pettit and Buhr 1998). The results of this study confirmed that the post-thaw characteristics of boar spermatozoa were only improved when semen was frozen in OEP-treated egg yolk lipoproteins, regardless of OEP concentration. It is possible that OEP is involved in the disruption of LPFo, resulting in the release of phospholipids that are absorbed by the spermatozoa during the cooling and freezing processes. A previous study has demonstrated that the components of egg yolk bind to sperm plasma membrane and can replace membrane phospholipids that are lost or damaged during cryopreservation (Cerolini et al. 2001). Ac-

cording to Pettit and Buhr (1998), lipid modifications occurring during the freezing-thawing processes indicate that the domains of the sperm head plasma membrane react differently to components of the cryopreservation extender. It has been suggested that the replacement of egg yolk-derived phospholipids protects spermatozoa by decreasing the temperature at which lipids change from the fluid to the crystalline state, resulting in reduced susceptibility to cryoinduced damage to spermatozoa (Pillet at al. 2010). Due to differences in the biochemical composition of LDL (Moussa et al. 2002) and LPFo (Strzeżek et al. 2005, Fraser and Strzeżek 2007), the former does not require SDS to sustain post-thaw sperm function, whereas the latter improves post-thaw sperm survival only in the presence of OEP, probably by enhancing the effectiveness of phospholipid absorption by spermatozoa. The findings of this study reaffirm that the interactions of OEP components, mainly SDS, with ostrich egg yolk lipoproteins, rendered the spermatozoa less susceptible to cryoinduced damage.

The beneficial effects of OEP components on boar sperm cryosurvival have not been fully ascertained and more research is needed to unravel the molecular processes by which ostrich egg yolk lipoproteins interact with SDS during the cooling and freezing processes. Furthermore, the results of this study demonstrated the varying effects of OEP-treated ostrich egg yolk lipoproteins on post-thaw sperm survival and suggest that more studies are needed on a large number of boars to identify individuals with good and poor semen freezability.

Acknowledgements

References

Aboagla EM, Terada T (2004) Effect of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 62: 1160-1172.

Arriola J, Foote RH (1987) Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J Dairy Sci 70: 1664-1670.

Axnér E, Hermansson U, Linde-Forsberg C (2004) The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci 84: 179-191.

Cerolini S, Maldjian A, Pizzi F, Gliozzi TM (2001) Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121: 395-401.

Fraser L, Strzeżek J (2007) Effect of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing-thawing. Anim Reprod Sci 99: 317-329.

Fraser L, Zasiadczyk Ł, Strzeżek J (2010) Interactions of egg yolk lipoprotein fraction with boar spermatozoa assessed with a fluorescent membrane probe. Folia Histochem Cytobiol 48: 292-298.

Garner DL, Johnson LA (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 53: 276-284.

Kato S, Miyano T, Nanjo I, Yasui T, Kanada S (1990) Effect of concentration of sodium laurylsulfate on motility and acrosome morphology of frozen boar spermatozoa. Japan Journal of Animal Reproduction 36: 26-30.

Johnson LA, Weitze KF, Fiser P, Maxwell WM (2000) Storage of boar semen. Anim Reprod Sci 62: 143-172.

Moussa M, Martinet V, Trimeche A, Tainturier D, Anton M (2002) Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology 57: 1695-1706.

Niżański W, Klimowicz M, Partyka A, Savić M, Dubiel A (2009) Effects of the inclusion of Equex STM into Tris-based extender on the motility of dog spermatozoa incubated at 5°C. Reprod Domest Anim 44 (Suppl 2): 363-365.

Peña A, Linde-Forsberg C (2000) Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54: 859-875.

Pettit MJ, Buhr MM (1998) Extender components and surfactants affect boar sperm function and membrane behavior during cryopreservation. J Androl 19: 736-746.

Pillet E, Duchamp G, Batellier F, Beaumal V, Anton M, Desherces S, Schmitt E, Magistrini M (2011) Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology 75: 105-114.

Pursel VG, Johnson LA, Schulman LL (1972) Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci 35: 580-584.

Pursel VG, Schulman LL, Johnson LA (1978) Effect of Orvus Es Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci 47: 198-202.

Strzeżek J, Lecewicz L, Dziekońska A, Fraser L (2005) A simple method of extraction of lipoprotein fractions from avian egg yolk – protective effect on cooled boar semen. Theriogenology 63: 496-497.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE (1998) Effect of cryopreservation on bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod 58: 786-793.

Waberski D, Weitze KF, Gleumes T, Schwarz M, Willmen T, Petzoldt R (1994) Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. Theriogenology 42: 831-840.

Watson PF (1975) Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. Vet Rec 97: 12-15.

Wu TW, Cheng FP, Chen IH, Yang CH, Tsai MY, Chang MH, Wang JH, Wu JT (2013) The combinatorial effect of different Equex STM Paste concentrations, cryoprotectants and the straw-freezing methods on the post-thaw boar semen quality. Reprod Domest Anim 48: 53-58.