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Cryopreservation of bull semen in OptiXcell® and conventional extenders: Comparison of semen quality and fertility

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Commercially available OptiXcell® extender was compared with conventional extenders for freezability and *in vivo* fertility of bull semen. Semen was collected from three Friesian bulls for five weeks (replicate) and qualifying ejaculates (motility >60%, concentration >0.5 billion/mL, volume >1mL) were diluted (37°C; 50 × 10^6 spermatozoa/ml) with OptiXcell®, tris-citric egg yolk and egg yolk-citrate extenders. Diluted semen was cooled to 4°C in 2 hours, equilibrated for 4 hours and filled in 0.5 ml straws. The straws were kept over liquid nitrogen vapours for 10 minutes and plunged into liquid nitrogen. Percentages of post thaw sperm motility and plasma membrane integrity were recorded higher (P<0.05) in OptiXcell® compared to tris-citric egg yolk followed by egg yolk-citrate extender. Sperm viability (%) were recorded higher (P<0.05) in OptiXcell® compared to tris-citric egg yolk and egg yolk citrate extender. Percentages of normal apical ridge and DNA integrity were higher (P<0.05) in OptiXcell® and tris-citric egg yolk extender compared to egg yolk-citrate extender. Higher (P<0.05) fertility rate was recorded with semen frozen in OptiXcell® compared to tris-citric egg yolk and egg yolk-citrate extender. In conclusion, OptiXcell® is superior to conventional extenders for spermatozoal quality of frozen-thawed bull semen and produced higher fertility rates under field conditions.

KEYWORDS: bull semen / cryopreservation / egg yolk-citrate / OptiXcell® / tris-citric egg yolk

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Artificial insemination (AI) is a technique that is used to improve genetic and production potential of the dairy animals. The technique AI is based on exploitation of germplasm from genetically superior males. In developing countries, the bull semen is mostly processed in tris-citric egg yolk and/or egg yolk-citrate conventional extenders based on their availability [Vishwanath and Shannon 2000]. These extenders are prepared in the laboratory following a very cumbersome process that involves weighing of chemicals used for buffer preparation, measuring of glycerol and egg yolk, all are associated with chances of human-error [Ansari *et al*. 2010]. Before these all steps, harvesting of egg yolk is a gruelling procedure consisted of multiple steps, involving proper disease screening, disinfection skilful breaking of outer shell and inner membrane to separate the yolk from albumin and chalazae [Ansari *et al*. 2010]. Egg yolk also has unwanted/deleterious substances that either have no role in cryoprotection or toxic to the sperm plasma membrane [Watson and Martin 1976, Smith *et al.* 1979, Muller-Schlosser *et al.* 2001]. Therefore, removal of deleterious components and/or extracting the most active component of egg yolk which offer protection to the spermatozoa becomes desirable [Akhter *et al.* 2011]. In this context, the clarified egg yolk [Wall and Foote 1999], egg yolk plasma [Belala *et al.* 2016ab] and low density lipoproteins [Foulkes 1977, Amirat *et al*. 2004, 2005, Amirat-Briand *et al*. 2010, Moussa *et al*. 2002, Hu *et al.* 2010, 2011, Akhter *et al.* 2013], conferred better results in terms of sperm quality compared to whole egg yolk.

The phospholipids of egg yolk are considered active cryoprotectant component that protect the spermatozoa by forming a protective film on the sperm surface or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process [Pace and Graham 1974, Manjunath *et al*. 2002, Bergeron and Manjunath 2006, Manjunath 2012]. More precisely, the required lipid vesicles may be formed that can fuse with and modify membrane lipid composition during cryopreservation. Since their first application for freezing bull spermatozoa [Robinson 2001], the beneficial effect of liposomes has been reported for cryopreservation of bull [Urrotia *et al.* 1992], boar [Worrall *et al.* 1998], and stallion [Griffith and Yaish 2004] sperm. Liposomes made from soybean lipids were also found efficient to replace hen egg yolk in an extender for freezing bovine semen [Duman 1977].

OptiXcell® is a liposome based commercial extender that have shown better cryoprotective ability compared to soya-lecithin [Kumar *et al*., 2015] and tris based extenders for buffalo semen [Akhter *et al.* 2016]. OptiXcell® was found efficient compared to other egg yolk free commercial extenders viz., AndroMed®, Bioxcell® and an egg yolk based diluent Triladyl® in terms of post-thaw quality of bull spermatozoa [Miguel-Jiménez *et al.* 2016]. It is relevant to mention that male-to-male variation exist in terms of ability of sperm to fuse to liposomes. These differences might be attributed to the genetic makeup, ecological conditions and feeding practices of the bulls which could affect sperm plasma membrane composition and integrity [Anzar *et al.* 2002, Nichi *et al*. 2006]. It is therefore, imperative to evaluate liposome based extender in comparison to routinely used extenders for different breeds adapted to

different ecological conditions. Further, OptiXcell® has not yet been evaluated for *in vivo* fertility rates in bovine under subtropical conditions. Therefore, present study was designed to evaluate commercial OptiXcell® extender with tris-citric egg yolk and egg yolk-citrate extenders for cryopreservation of bull semen and *in vivo* fertility under field conditions.

Material and methods

The tris-citric egg yolk extender was consisted of 1.56 g citric acid (Fisher Scientific, UK), 3.0 g *tris*-(hydroxymethyl)-aminomethane (Research Organics, USA), fructose (Scharlau, Spain) 0.2 g; egg yolk 20 mL; 7 mM glycerol and 73 ml distilled water. Egg yolk-citrate was prepared by adding 2.9 g sodium citrate, 7 mL glycerol, 0.2 g fructose, 20 mL egg yolk in 73 mL distilled water. Antibiotics; gentamycin sulphate (500 µg/ml; Reckitt Benckiser, Pakistan), tylosin tartrate (100 µg/ml; VMD, Belgium), lincomycin hydrochloride (300 µg/ml; Pharmacia & Upjohn, Belgium) and spectinomycin hydrochloride (600 µg/ml; Pharmacia & Upjohn, Belgium) were added to tris-egg yolk and egg yolk-citrate extender. OptiXcell® was prepared according to the manufacturer instructions (IMV, France).

Two consecutive semen ejaculates were collected with artificial vagina from three adult Friesian bulls for a period of five weeks (replicates). Semen ejaculates were initially evaluated for motility, volume and concentration. Semen volume was recorded from graduated tube, motility was determined using light microscope at X400 and sperm concentration was determined by Neubauer haemocytometer. Semen ejaculates having motility >60%, volume >1.0mL and concentration >0.5 billion/ml were used for further processing.

Qualifying semen ejaculate from each bull was equally divided into three parts and diluted with either OptiXcell or tris-citric egg yolk or egg yolk-citrate extender having final concentration of 50×10^6 motile spermatozoa ml^{-1} at 37°C. Semen diluted in experimental extenders was cooled from 37°C to 4°C in 2 hours and equilibrated for 4 hours at 4°C. After equilibration, semen was filled in 0.5 ml French straws (IMV, France) with suction pump at 4°C in cold cabinet unit (IMV, France), kept over liquid nitrogen vapours for 10 min. Then, straws were plunged into liquid nitrogen and stored for three days. After three days of storage, straws in triplicate were thawed in a water bath at 37°C for 30 seconds for assessment of motility, plasma membrane integrity, viability, normal apical ridge and DNA integrity.

Sperm motility was assessed by placing a drop of semen sample on pre-warmed glass slide and cover-slipped at 37°C. The slide was examined for motility under light microscope (X 400).

Sperm plasma membrane intactness was determined using hypo-osmotic swelling (HOS) assay [Jeyendran *et al.*, 1984] in combination with supravital stain eosin. To assess the sperm tail plasma membrane integrity, semen (50µl) was mixed with 500µl HOS solution [HOS solution was consisted of 0.73g sodium citrate (Merck,

Darmstadt, Germany) and 1.35 g fructose (Merck, Darmstadt, Germany) dissolved in 100ml distilled water (osmotic pressure ~190 mOsmol Kg^{-1})] and incubated for 30 minutes at 37°C. Equal drop of semen sample (5 µl) was mixed with 0.5% eosin (in 2.9% sodium citrate; Merck, Darmstadt, Germany) on a glass slide and cover-slipped to examine under phase contrast microscope (X 400). A total of 100 spermatozoa were studied per preparation. Sperm with clear head, tail and swollen tail considered to have intact biochemically active sperm membranes, while sperm with pink head, tail and unswollen tail considered to have disrupted, inactive sperm membranes [Tartaglione and Ritta 2004].

The normal apical ridge was assessed by fixing the semen sample (100 µl) in 500 µl formal citrate solution (2.9 g tri-sodium citrate dihydrate, 1 ml of 37% solution of formaldehyde, in 100 ml of distilled water; Merck, Darmstadt, Germany). A drop of semen sample fixed in formal citrate was placed on glass slide and one hundred spermatozoa were examined for normal apical ridge with a phase contrast microscope (X 1000) under oil immersion.

Sperm viability was assessed through dual staining procedure with Trypan blue-Giemsa stain as described by Kovacs and Foote [1992]. Briefly, the double stain procedure includes the use of the supravital stain Trypan-blue to distinguish live and dead spermatozoa and Giemsa to evaluate the integrity of the acrosome membrane. Equal drops of Trypan-blue staining solution (0.2% MP Biomedicals, Eschwege, Germany) and semen were placed on a glass slide at ambient temperature and quickly mixed. Smears were air-dried, and slides were fixed with formaldehyde-neutral (86 ml 1 M HCl + 14 ml 37% formaldehyde [Merck, Darmstadt, Germany] + 0.2 g Neutral Red [MP Biomedicals, Eschwege, Germany]) red for 5 minutes. After rinsing with distilled water, 7.5% Giemsa (MP Biomedicals, Eschwege, Germany) stain was applied for 4 hours. After rinsing with distilled water, air-dried slides were cover-slipped and mounted with Balsam of Canada (Merck, Darmstadt, Germany). One hundred spermatozoa were evaluated in each smear under phase contrast microscope at 1000x. Trypan-blue penetrates non-viable, dead spermatozoa with disrupted membrane, which appeared stained in blue, while live, intact spermatozoa appeared unstained. Giemsa accumulates in spermatozoa with an intact acrosome, staining the acrosome region in purple.

Sperm DNA integrity was assessed by acridine orange assay as performed by Ansari *et al*. [2012]. The air-dried smears of semen sample on glass slide were fixed overnight in Carnoy's solution (freshly prepared, methanol [Merck, Darmstadt, Germany] and glacial acetic acid [Merck, Darmstadt, Germany]; 3:1). The slides were air-dried yet again and incubated in tampon solution (80 M citric acid [Merck, Darmstadt, Germany]) and 15 mM disodium hydrogen phosphate (Sigma, MO, USA, pH 2.5) at 75 8°C for 5 minutes. Subsequently, the slides were stained with acridine orange (0.2 mg/ml; Sigma, MO, USA) stain. The slides stained with acridine orange were rinsed with water to remove background staining and, while still wet, were cover-slipped and examined with an epifluorescence microscope (Labomed, USA LX

400; 480/550 nm excitation/barrier filter). One hundred spermatozoa were analysed in each slide; sperm with normal DNA exhibited green fluorescence, whereas those with abnormal DNA content exhibited fluorescence from yellow-green to red in spectrum.

The semen from Friesian bull of known fertility was cryopreserved in OptiXcell, tris-citric egg yolk and egg yolk-citrate extender; used for artificial inseminations in cows under field conditions. To determine fertility rate, a total of 300 inseminations (100/extender) were examined for conception through rectal palpation at least 70-days post-insemination.

The data on motility, plasma membrane integrity, viability, normal apical ridge and DNA integrity for OptiXcell®, tris-citric egg yolk and egg yolk-citrate were analysed by one-way ANOVA (effect of bull had proven to be insignificant) in completely randomized design using statistical package MegaStat® (version 10.1, McGraw-Hill, USA). When F-ratio was found significant ($P<0.05$), least significant difference test was used to compare the treatment means. The data on fertility rate were analysed with chi-square test with a level of significance $P<0.05$ in MegaStat®.

Results and Discussion

In the present study, post-thaw motility and plasma membrane integrity (%) were recorded higher ($P<0.05$) in commercial OptiXcell® extender than tris-citric egg yolk and egg yolk-citrate extender (Fig. 1). Sperm motility and plasma membrane

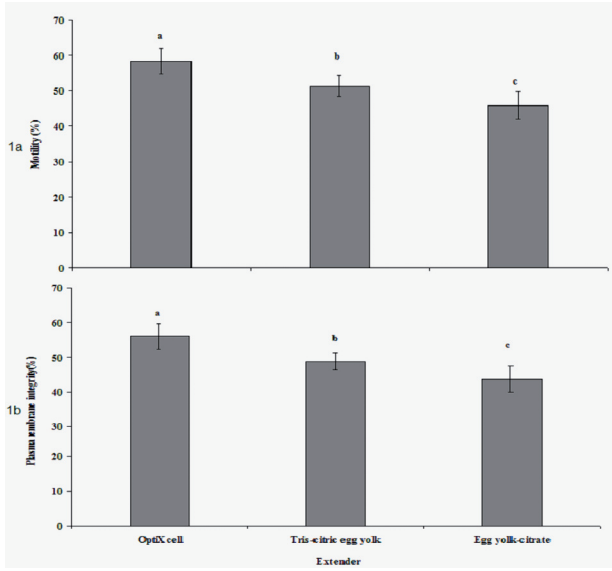

Fig. 1. Effect of OptiXcell®, tris-citric egg yolk and egg yolk citrate extender on post-thaw motility (1a) and plasma membrane integrity (1b) of bull spermatozoa (n=5). The bars with different letters differ significantly ($P<0.05$).

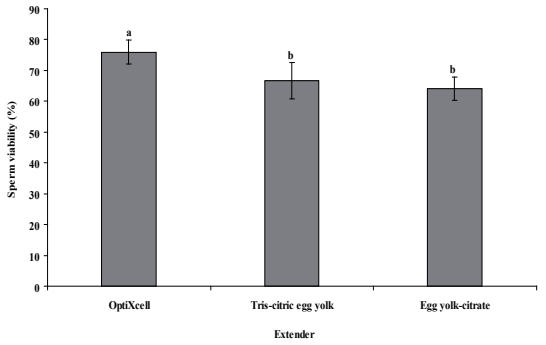

Fig. 2. Effect of OptiXcell®, tris-citric egg yolk and egg yolk citrate extender on viability of bull spermatozoa (n=5). The bars with different letters differ significantly (P<0.05).

integrity both are considered important parameters to predict the fertility of bovine semen. Motility facilitates the spermatozoa to reach at the site of fertilization in female reproductive tract and functional plasma membrane is necessary in signalling pathways during interaction with ovum to accomplish successful fertilization [Brito *et al.* 2003]. It has been demonstrated that liposome based extender is equal to extender having egg yolk in maintaining the post-thaw motility of bull spermatozoa [Graham and Foote 1987]. Our results are inline with the previous studies, that showed better cryoprotective ability of OptiXcell® compared to commercial Triladyl® and Biladyl® egg yolk based extender for plasma membrane integrity of the white-tailed deer [Stewart *et al.* 2016]. Nevertheless, liposomes composed of phospholipids E80 [commercial lecithins from egg yolk composed of phosphatidylcholine and Phosphatidylethanolamine] showed lesser capacity to protect stallion sperm motility parameters compared to egg yolk [Pillet *et al.* 2012] but plasma membrane integrity did not differ in neither of extenders. Interestingly, total, progressive and rapid motility along with Kinematic parameters during post-thaw incubation were recorded higher in OptiXcell® compared to conventional tris-citric egg yolk and commercial soya-lecithin based extenders [Bioxcell® and AndroMed®] in frozen-thawed Murrah buffalo semen [Kumar *et al.* 2015]. Ansari *et al.* [2016] reported higher motility as well as plasma membrane integrity of buffalo sperm in OptiXcell® compared to tris-citric egg yolk extender. It is believed that presence of unwanted/deleterious substances like granules and high density lipoproteins may cause hindrance in cellular respiration that resulted in reduced motility of the spermatozoa [Pace and Graham, 1974]. The variations in findings using liposomes based extender is might be due to the difference in liposomes constituent, concentration, buffering media, species difference and management and feeding practices of animals.

Sperm viability (%) was recorded highest (P<0.05) in OptiXcell® compared to tris-citric egg yolk and egg yolk-citrate extender (Fig. 2). Sperm viability assessed through dual staining procedure describes simultaneously acrosome and live/dead

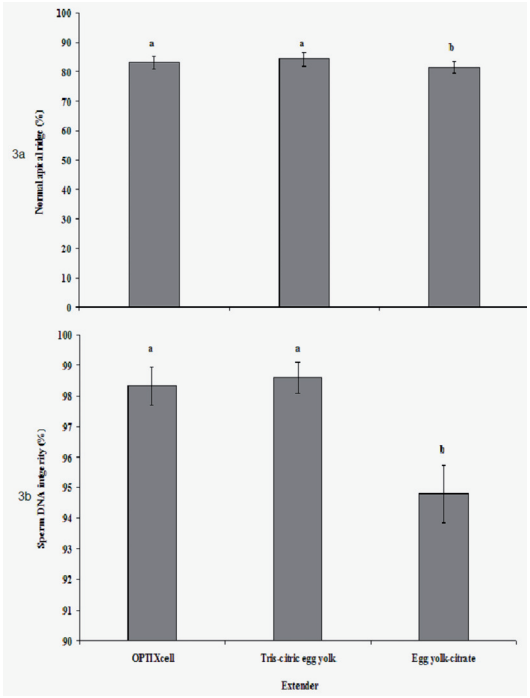

Fig. 3. Effect of OptiXcell®, tris-citric egg yolk and egg yolk citrate extender on normal apical ridge (3a) and Sperm DNA integrity (3b) of bull spermatozoa (n=5). The bars with different letters differ significantly ($P<0.05$).

status. Percentage of live sperm and live sperm with intact acrosome both were recorded higher in OptiXcell® compared to tris-citric egg yolk in extender frozen-thawed Nili-Ravi buffalo semen [Ansari *et al.* 2016]. Stewart *et al.* [2016] reported higher viability of the white-tailed deer sperm in OptiXcell® compared to commercial Triladyl® and Biladyl® egg yolk based extender. It is suggested that presence of steroid hormones and high concentration of calcium ions in egg yolk induces acrosomal reaction that resulted in loss of acrosome during cryopreservation [Amirat *et al.* 2005]. Likewise, granules components of the egg yolk are directly absorbed by the acrosome and cellular membrane that leads to the self-destruction of acrosome without visible rupture on the plasma membrane [Amirat *et al.* 2005].

Sperm percentages with normal acrosome morphology and DNA integrity were recorded higher ($P<0.05$) in OptiXcell® and tris-citric egg yolk extender compared to egg yolk citrate extender (Fig. 3). Sperm normal apical ridge and DNA integrity are important semen quality assessment parameters and are highly related with fertility [Ansari *et al.*, 2012]. Our results are inline with a recent study on white-tailed deer that showed higher sperm acrosome integrity in OptiXcell® compared to commercial Biladyl® and Triladyl® egg yolk based extender [Stewart *et al.* 2016]. Ansari *et al.* [2016]

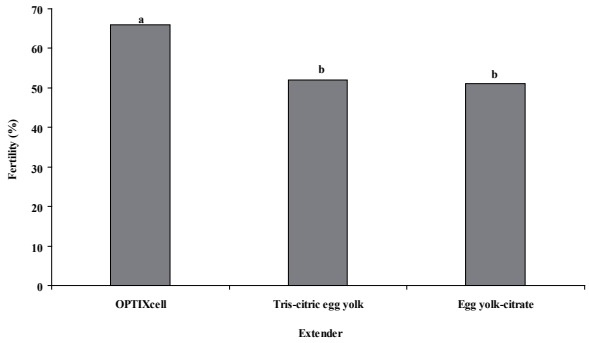

Fig. 4. Effect of OPTIXcell®, tris-citric egg yolk and egg yolk citrate extender on *in vivo* fertility of bull spermatozoa (n=5). The bars with different letters differ significantly (P<0.05).

reported higher normal apical ridge and DNA integrity of buffalo sperm in OptiXcell® compared to conventional tris-citric egg yolk extender. Nevertheless, Kumar *et al.* [2015] reported no difference in acrosome integrity of Murrah buffalo spermatozoa in OptiXcell® compared to conventional tris-citric egg yolk and commercial soya-lecithin based extenders [Bioxcell® and AndroMed®].

Fertility results showed higher (P<0.05) percentages of fertility rates with semen cryopreserved in OptiXcell® (66%) compared to tris-citric egg yolk (52%) and egg yolk-citrate extender (51%) (Fig. 4). It was noted that tris-egg yolk based extender is superior to skim milk and egg yolk-citrate extender in maintaining post-thaw motility of the bull sperm [Davis *et al.* 1963; Steinbach and Foote 1964]. Moreover, zwitterions buffer has capacity to maintain higher percentage of motile spermatozoa. Yet, the extracellular GOT enzyme leakage due to plasma membrane damage is higher than egg yolk-citrate extender [Graham *et al.* 1972]. This might be the reason that percentage of live sperm [with intact plasma membrane] with intact acrosome and fertility rate did not differ in tris-citric egg yolk and egg yolk-citrate extender. The OptiXcell® has showed higher fertility rate compared to tris-citric egg yolk with frozen buffalo semen [Akhter *et al.* 2016]. Nevertheless, fertility rates in mare were recorded similar in E80-lipsome based and egg yolk based extenders [Pillet *et al.* 2012]. Interestingly, numerically higher fertility rate in mare was recorded with extender having egg yolk plasma compared to whole egg yolk [Pillet *et al.* 2011]. It has been demonstrated that in OptiXcell® sperm retained higher ability to travel long-distance in cervical mucus test compared to commercial soya-lecithin based [AndroMed® and Bioxcell®] conventional tris-citric egg yolk based extender [Kumar *et al.* 2015]. It revealed indirectly higher ability of the spermatozoa in OptiXcell® to reach at the site of fertilization that increases the chances of conception. Moreover, the higher percentage of functional and structurally intact spermatozoa in OptiXcell® compared to conventional extenders contributes in higher fertility rates.

In conclusion, our results suggest that commercially available liposome based OptiXcell® extender is superior to conventional tris-citric egg yolk and egg yolk-citrate extender in protecting the quality of bull semen during cryopreservation and provide higher fertility rates under field conditions.

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