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# OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm

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ABSTRACT

The present study was conducted to compare the liposome-containing, animal protein–free, commercially available OPTIXcell extender with the *Tris*–citric–egg yolk extender for postthaw quality and fertility of buffalo semen. Semen was collected from five adult Nili-Ravi buffalo (*Bubalus bubalis*) bulls of similar age group with an artificial vagina (at 42 °C) for 3 weeks (replicates). Semen ejaculates from each buffalo bull were divided into two aliquots and diluted (at 37 °C having $50 \times 10^6$ spermatozoa/mL) in the OPTIXcell or *Tris*–citric–egg yolk (control) extender. Diluted semen was cooled to 4 °C in 2 hours, equilibrated for 4 hours, and filled in 0.5-mL straws. The semen straws were kept over liquid nitrogen vapors (5 cm) for 10 minutes. The straws were then plunged and stored in liquid nitrogen (−196 °C). After 24 hours of storage, the semen straws were thawed at 37 °C for 30 seconds to assess postthaw quality. Percentages of sperm motility, plasma membrane integrity, viability, and acrosomal integrity were improved ($P < 0.05$) in the OPTIXcell extender compared to the *Tris*–citric–egg yolk extender. Values for DNA integrity (%) did not differ in the OPTIXcell and *Tris*–citric–egg yolk extenders. The overall conception rate in buffaloes was improved ($P < 0.05$) with semen cryopreserved in the OPTIXcell extender (59.5%) compared to semen cryopreserved in the *Tris*–citric–egg yolk extender (41.5%). It is concluded that the liposome-containing commercially available OPTIXcell extender is more efficient to conserve postthaw quality and resulted in higher fertility rate of buffalo in the field.



## 1. Introduction

Egg yolk has been routinely used as a nonpermeable cryoprotectant for sperm cryopreservation in different mammalian species including buffalo. In addition to cryoprotectants, the whole egg yolk contains some anti-cryoprotective agents such as high-density lipoproteins [1], hormones [2] and being of animal origin bears risks of microbial contamination [3]. Furthermore, the composition of commercial hen egg yolk varies depending on the hybrid selected, management, and nutritional practices adopted [4]. Therefore, continuous efforts are being made to develop chemically defined extenders free from proteins of animal origin. In such an attempt, low-density lipoproteins (LDLs) extracted from hen egg yolk were found efficient in terms of postthaw quality and fertility of buffalo bull semen compared to whole egg yolk in the *Tris*-based extender [5].

The LDLs of egg yolk contain phospholipids that protect sperm by forming a protective film on the sperm surface or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process [6–8]. Therefore, attempts have now been reverted to substitute egg yolk by defined lipids, among which liposomes made from known molecules are of interest. The protective

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properties of liposomes are attributed to lipid and cholesterol transfer between liposomal and cellular membranes. The first studies showing some protective effects of artificial liposomes on sperm functions after freezing and thawing were reported in bull spermatozoa [9]. Since then, various studies reported the beneficial effect of liposomes in bull [10], boar [11], and stallion [12] semen. Liposomes made from soybean lipids were also found efficient to replace hen egg yolk in an extender for freezing bovine semen [13].

Despite the beneficial effects of liposomes for semen cryopreservation, the possible limitation for more extensive application of liposomes is the lack of standardization. Because the chemically defined, liposome-containing OPTIXcell extender is available and has not been evaluated for freezability and fertility of Nili-Ravi buffalo bull semen, it was hypothesized that OPTIXcell will be efficient to conserve postthaw quality and *in vivo* fertility of buffalo bull semen compared to the *Tris*–citric–egg yolk acid extender. The objective was to compare the postthaw sperm motility, plasma membrane integrity, viability, acrosome integrity, DNA integrity, and *in vivo* fertility of Nili-Ravi buffalo semen cryopreserved in the commercially available extender OPTIXcell and *Tris*–citric–egg yolk extender.

## 2. Materials and methods

### 2.1. Preparation of extenders

*Tris*–citric egg yolk extender was prepared by using 1.56-g citric acid (Fisher Scientific, UK) and 3.0-g *tris* (hydroxymethyl)aminomethane (Research Organics, USA), 0.2% wt/vol fructose (Scharlau, Spain), 7.0-mL glycerol (Merck, Germany), egg yolk 20% in 74-mL distilled water. Antibiotics namely gentamicin sulfate (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) were added to the *Tris*–citric–egg yolk extender. OPTIXcell was prepared according to the manufacturer's instructions (IMV, France).

### 2.2. Semen collection and initial evaluation

Two consecutive ejaculates were collected using an artificial vagina (at 42 °C) from five adult Nili-Ravi buffalo bulls (*Bubalus bubalis*) of known fertility and similar age (7–8 years) for a period of 3 weeks (replicates). Ejaculated semen (two ejaculates/bull/week) was immediately transferred to the laboratory. Sperm progressive motility was determined microscopically (magnification: × 400) with a closed-circuit television, and sperm concentration was determined by a Neubauer hemocytometer. Qualifying ejaculates from each bull having motility greater than 60% were split into two aliquots and held at 37 °C in a water bath for 15 minutes before dilution.

### 2.3. Semen processing

Each semen aliquot from each bull was diluted with one of the two experimental extenders at the concentration of $50 \times 10^6$ motile spermatozoa/mL at 37 °C. Diluted semen was cooled to 4 °C in 2 hours, equilibrated for 4 hours at 4 °C, and filled in 0.5-mL french straws (IMV) with a suction pump at 4 °C in a cold cabinet unit (Minitube, Germany). After 24 hours, the semen straws were thawed in a water bath at 37 °C for 30 seconds [14].

#### 2.3.1. Sperm progressive motility

A drop of semen was placed on a prewarmed microscope slide and subjectively assessed at 37 °C for percent sperm progressive motility using phase-contrast microscopy (magnification: × 400).

#### 2.3.2. Sperm plasma membrane integrity

Sperm plasma membrane integrity was determined using the hypoosmotic swelling assay [15]. Hypoosmotic swelling assay solution consisted of 0.73-g sodium citrate and 1.35-g fructose dissolved in 100-mL distilled water (osmotic pressure: ~190 mOsmol/Kg). To assess the sperm plasma membrane integrity, semen (50 μL) was mixed with hypoosmotic assay solution (500 μL) and incubated for 30 minutes at 37 °C before examining under a phase-contrast (magnification: × 400) microscope. One hundred spermatozoa were assessed for swelling characterized by a coiled tail indicating the functional integrity of tail plasma membrane [16].

#### 2.3.3. Sperm viability

Viability of spermatozoa was assessed with trypan blue stain as described by Brito et al. [17]. A smear of semen (5 μL) and an equal volume of 0.4% trypan blue solution was made on a microscope slide and allowed to air-dry before examining under a phase-contrast microscope (magnification: × 1000). One hundred spermatozoa were counted from each sample; unstained white spermatozoa were categorized as viable and blue-stained spermatozoa as nonviable.

#### 2.3.4. Sperm acrosome integrity

To assess sperm acrosomal integrity, 100 μL of semen sample was fixed in 500 μL of 1% formal citrate (2.9-g trisodium citrate dihydrate, 1 mL of 37% solution of formaldehyde dissolved in 100 mL of distilled water). A total of 1800 sperm were examined per treatment (200 per each of the three replicates for each of the three bulls) with a phase-contrast microscope (magnification: × 1000; Olympus BX20, Tokyo, Japan) under oil immersion. The normal acrosome was characterized by a normal apical ridge [18].

#### 2.3.5. Sperm DNA integrity

Sperm DNA integrity was assessed using the acridine orange assay [19,20]. Smears of semen were prepared on glass slides, air-dried, and fixed for overnight in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion). The slides were air-dried and incubated in tampon solution (80 mmol/L of citric acid and 15 mmol/L of $Na_2HPO_4$, pH 2.5) at 75 °C for 5 minutes. Then, the slides were stained with acridine orange stain (0.2 mg/mL). Stained slides were washed with water to remove background staining; while still wet, the slides were covered