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Sodium alginate potentiates antioxidants, cryoprotection and antibacterial activities of egg yolk extender during semen cryopreservation in buffalo

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ABSTRACT

The study was conducted to determine effects of sodium alginate on sperm during cryopreservation. Each ejaculate (n = 20) of five buffalo bulls (3–5 years) were divided into six equal fractions and diluted using egg yolk based extender supplemented with different concentrations of sodium alginate and cryopreserved. Frozen-thawed semen samples were evaluated using the CASA, hypo-osmotic swelling test, cervical mucus penetration capacity test, and chlortetracycline fluorescence assay (CTC). Phosphorylation of tyrosine containing proteins and malondialdehyde concentration of sperm membrane were evaluated using immunoblotting and thiobarbituric acid reactive substance assay respectively. The semen extender's anioxidative capacities were estimated by conducting 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, metal chelating capacity by assessing ferrozine and antibacterial capacity using agar plate methods. Supplementation of sodium alginate in extender improved sperm longevity, plasma membrane integrity as well as capacity to transit through the cervical mucus. Supplementation of extender with sodium alginate minimises the phase transition of sperm membranes and phosphorylation of tyrosine containing proteins during cryopreservation. Malondialdehyde concentration of sperm was less in sodium alginate-treated sperm as compared with control samples. The results indicated that sodium alginate increased antioxidant capacity of semen extender. Supplementation with sodium alginate also improved the metal chelating capacity and antibacterial properties of the extender. In conclusion, supplementation of extender with sodium alginate enhances free radical scavenging, metal reduction and chelating capacities to protect sperm during cryopreservation.

1. Introduction

The limited amounts of endogenous antioxidants present in sperm do not provide sufficient protection against the excessive amount of reactive oxygen species (ROS) generated during the cryopreservation process (dilution, cooling, freezing and thawing)

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resulting in irreversible damage to spermatozoa (Mazzilli et al., 1995). Further, mammalian sperm plasma membranes are rich in polyunsaturated fatty acids that during the freezing-thawing process become susceptible to ROS attack resulting in lipid peroxidation of the sperm membrane (Aitken and Baker, 2004). Lipid peroxidation of the plasma membrane alters its fluidity resulting in an influx of calcium and bicarbonate that affects sperm-oocyte fusion (Ball, 2008; Storey, 1997). The use of antioxidants as additives in sperm cryopreservation has been attempted with varying success (Dalal et al., 2019). Most of the antioxidants that were previously assessed directly or indirectly affected the scavenging free radicals produced during sperm cryopreservation (Yeste, 2016). In addition to need for free radical scavenging capacity during sperm cryopreservation, there is a need for an additive that has a metal reducing property because metals function as catalysts during the oxidative reaction (Ercal et al., 2001). Redox-active metals (iron, copper and chromium) undergo redox cycling whereas redox-inactive metals (lead, cadmium, and mercury) deplete cells of the primary antioxidants and enzymes (Ercal et al., 2001). Hen egg yolk is the main constituent of extender for cattle and buffalo semen and there are both redox-active and redox-inactive metals present in egg yolk (Hashish et al., 2012; Islam et al., 2014; Hossain et al., 2017; Thongcharoen et al., 2017) which may result an increase in production of ROS. It, therefore, is imperative to chelate the undesired metals present in the semen extender.

Also an ideal additive should possess antimicrobial activity because the presence of bacteria in semen and egg yolk is a serious concern for semen processing laboratories (Diemer et al., 1996). In semen, many bacteria can survive in liquid nitrogen (−196 °C) and are resistant to antibiotics (Ronald and Prabhakar, 2001). Similarly, a number of viruses transit into ejaculated semen (Jain et al., 2009), posing a potential threat to the livestock bio-security.

Sodium alginates are a group of naturally occurring anionic polysaccharides derived from brown seaweed and are generally considered as biocompatible, non-immunogenic and non-toxic polymers (Sachan et al., 2009). Results of several studies indicate sodium alginates are a rich source of antioxidant compounds (Sellimi et al., 2015; Krol et al., 2017), transition metal chelators (Falkeborg et al., 2014), and possess antibacterial (Yan et al., 2011; Benavides et al., 2012; Khan et al., 2012; Pritchard et al., 2017; Karbassi et al., 2014), antiviral (Ahmadi et al., 2015) and antifungal (Tøndervik et al., 2014) properties.

This natural polymer has assessed for encapsulation of sperm (Nebel et al., 1985; Herrler et al., 2006; Weber et al., 2006; Perteghella et al., 2017) with results indicating sodium alginate is non-toxic to sperm. It was also reported that sodium alginate improved motility, functional integrity and antioxidant capacity of boar spermatozoa (Hu et al., 2014). In the present study, there was evaluation of the free radical scavenging, ferric reducing, iron chelating and antibacterial capacities of sodium alginate when there was supplementation in semen extender. There was also an attempt to establish how sodium alginate minimises the membrane lipid structural disruptions and improves the overall semen quality after cryopreservation.

2. Material and methods

2.1. Sodium alginate

Sodium alginate was purchased from Sigma-Aldrich Chemicals Pvt Limited (Cat No.: 180,947). Its molecular weight is 120,000 to 190,000 g/moL with a mannuronic acid to glucuronic acid ratio and viscosity of 1.56 and 15 to 25 centipoises in 1% water, respectively.

2.2. Semen collection

Five Murrah buffalo bulls (age 3–5years) maintained at the Semen Freezing Laboratory, ICAR-Central Institute for Research on Buffaloes, Hisar, India were selected for the study. Four ejaculates from each bull were collected using an artificial vagina technique during the winter season and semen was cryopreserved using standard procedures (Kumar et al., 2016a).

2.3. Determination of dose toleration

Before the time of initiation of the experiment, there was determination of the maximum dose of sodium alginate tolerated by buffalo sperm based on assessments of sperm motility. The ejaculates with > 70% sperm motility were aliquoted and diluted in a 2.9% sodium citrate dihydrate to a concentration of 80 million sperm/mL. Sodium alginate was added to yield six different final concentrations: 0, 1, 2, 3, 4, 5 and 6 mg/mL. The extended sperm were incubated at 37 °C for 60 min and evaluated for sperm motility.

2.4. Semen processing and cryopreservation

The ejaculates of each bull were divided into six equal fractions and diluted to final concentration of 80 million sperm/mL using egg yolk based extender (Tris, 3.02% w/v; citric acid, 1.67% w/v; fructose 1% w/v; egg yolk, 20% v/v; penicillin 1000 IU/mL; streptomycin, 1000 g/mL and glycerol, 6.4% v/v) supplemented with different concentrations of sodium alginate (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). The extended semen was subsequently loaded into 0.25 mL plastic straws (IMV, L'Aigle, France), cooled slowly to 4 °C and equilibrated for a period of 4 h in a cold cabinet (IMV, L'Aigle, France), and cryopreserved using a programmable biological freezer (Mini Digi-cool, IMV Technologies, L'Aigle, France) as described previously (Kumar et al., 2015a).

2.5. Sperm motility and kinematic variables

Each semen sample was thawed in water bath at 37 °C for 30 s. The contents of straws (˜0.21 mL) were transferred to 2 mL tubes maintained in a water bath at 37 °C. Sperm kinematics and motility variables were assessed using a computer-assisted sperm analyser (CASA) system (IVOS12.1, Hamilton-Thorne Biosciences, Beverly, MA, USA) as previously described (Kumar et al., 2015b) at 0, 30, 60, 90, 120, 150 and 180 min.

The following motion characteristics were recorded: total motility (TM, %), progressive motility (PM, %), rapid motility (RM, %), straight linear velocity (VSL, μm/s), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %) of the sperm. The CASA software settings were as follows: temperature = 38 °C, frame rate = 60 Hz, frames acquired = 30, minimum contrast = 35, minimum cell size = five pixels, cell size = nine pixels, cell intensity = 110 pixels, progressive cells (VAP cut-off = 50 μm/s, STR cut-off = 70%), slow cells (VAP cut-off = 30 μ/s and VSL cut-off = 15 μ/s).

2.6. Hypo-osmotic swelling test

Plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) as described by Kumar et al. (2016b).

2.7. Bovine cervical mucus penetration test

The bovine cervical mucus penetration test was conducted as per the protocol described by Swami et al. (2017a).

2.8. Determination of tyrosine phosphorylation of sperm protein

2.8.1. Purification of sperm

Sperm cells were separated from semen extender and other somatic cells that were present due to contamination using Bovi Pure™ (Nidacon #BP-100). Briefly, 2 ml of Bovi Pure™ solution was prepared in a 15 mL centrifuge tube by mixing 1 mL of Bovi Pure™ and 1 mL phosphate-buffered saline (PBS). Thawed semen from three straws (60 million sperm) layered over the top of the 2 mL solution of Bovi Pure ™ solution was centrifuged at 600 *g* for 15 min at room temperature. The sperm pellet was re-suspended in PBS, re-centrifuged and supernatant was discarded. The sperm pellet was used for protein extraction.

2.8.2. Sperm protein extraction

Protein from sperm was extracted as described by Dalal et al. (2019). Briefly, the sperm pellet was solubilised in 100 μL of lysis buffer containing 4% CHAPS (Sigma-Aldrich®, USA, C3023), 40 mM Tris-base (Sigma-Aldrich®, USA, # T6066), 75 mM DDT (AMRESCO®, USA, # 0281), 1 mM PMSF (Sigma, USA, #: 329-98-6), 1 mM EDTA (Qualigens ®, India), 7 mM urea (Sigma-Aldrich®, USA, U5378), 2 mM thiourea (Sigma-Aldrich®, USA, # T8656), 1 mM sodium orthovanadate (Sigma-Aldrich®, USA, #450,243) and a protease inhibitor cocktail at 10 μL/mL (Sigma-Aldrich®, USA, #P8340). The mixture was incubated at room temperature for 60 min followed by centrifugation at 21,130 *g* for 30 min at 4 °C. After centrifugation, the supernatant was separated and stored at − 20 °C for further use. Protein concentration was determined using the Quick Start™ Bradford Protein Assay Kit (BioRad™, #5,000,201).

2.8.3. SDS–PAGE and immunoblotting

For one dimensional gel electrophoresis, 10 μg of solubilised proteins were loaded per lane and resolved using SDS–PAGE (Mini-PROTEAN®tetra cell (Bio-Rad™) using stacking and resolving gels with 4% and 10% of acrylamide, respectively. A BenchMark™ Protein Ladder (Invitrogen™, USA, # 10747-012) was used as a molecular weight standard for SDS-PAGE. The MagicMark™ XP Western Protein Standard (Invitrogen™, USA, # LC5603) standard was used as the marker for western blotting. All samples were processed in duplicate one for staining and another for further immunoblotting. Western blotting was conducted using the iBlot® Dry Blotting System (Invitrogen™, USA, # IB1001) by utilising iBlot® Gel Transfer Stacks, Mini (Invitrogen™, USA, # IB4010-02). Immunoblotting was conducted using the WesternBreez® Chromogenic immunodetection system kit (secondary antibody- alkaline phosphatase conjugated anti mouse IgG, Chromogenic Substrate- BCIP/NBT substrate), using an anti-phosphotyrosine antibody, clone 4G10® (Merck Life Science # 630,684) as primary antibody. The experiment was replicated four times. To quantify changes in protein tyrosine phosphorrylation, myImageAnalysis software from Thermo Fisher Scientific was used to estimate quantity based on the intensity of the band.

2.9. Determination of physiological status of sperm

To assess physiological changes of the sperm after cryopreservation, the chlortetracycline (CTC) assay was performed (Dalal et al., 2019). The distribution of membrane calcium in the sperm head is thought to change during capacitation and the acrosome reaction; and is thought to be associated with an influx of calcium (Gillan et al., 1997). Sperm were evaluated according to 1 of 3 CTC staining patterns (Fraser et al. 1995): fluorescence over the entire head (normal cells, pattern F), fluorescence-free band in the post-acrosomal region (cryo-damaged cells, pattern B) and a small amount of fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome- damaged cells, pattern AR). A total of 200 sperm per slide were observed.

2.10. Determination of lipid-peroxidation of sperm membrane

The extent of lipid peroxidation (Malondialdehyde, MDA) in samples was determined using the TBARS assay kit (Cayman Chemical Company) as described by Kumar et al. (2016a,c). The standard curve was prepared using the MDA standards, and the value for the MDA concentration for each sample was calculated from a standard curve and expressed as μM/million sperm.

2.11. Determination of free radical scavenging capacity of fortified semen extender

Initially there was preparation of a methanol extract of extenders as described by Nimalaratne et al. (2011) with some modification. Briefly, 1 mL of extender was extracted with 10 mL of 80% methanol (80:20, vol/vol) adjusted to a pH of 1.5 with 1 M HCl. The sample was vortexed for 2 min and centrifuged at 6000 *g* for 10 min at 4 °C. The supernatant was evaporated until 1 mL of the sample remained and the sample was subjected to further analyses. The effect of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging was evaluated using the method of Kumar et al. (2019). There was an assessment of the free radical scavenging capacity of the methanol extract of egg yolk extender supplemented with different concentrations sodium alginate. Results were calculated as the percent increase/decrease with respect to the control samples. The percentage of DPPH inhibition was calculated as $[(A_0\text{-}A_s)/A_S] \times 100$, where, A_0 was the absorbance of the control, and A_S was the absorbance of alginate fortified extenders.

2.12. Determination of ferric reducing capacity of fortified semen extender

Ferric reducing antioxidant power (FRAP) of sodium alginate in semen extender was determined using the methods of Kumar et al. (2019). The values obtained were expressed as μM of ferrous equivalent Fe (II) per mL of sample.

2.13. Determination of metal chelating capacity of fortified semen extender

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, however, the complex formation is disrupted with the formation of the red colour of the complex being less. Measurement of the colour reduction, therefore, allows the estimation of the chelating capacity of the coexisting chelator. The chelating of Fe^{2+} by sodium alginate fortified extender was estimated using the method of Wong et al. (2014). Briefly, 100 μL of 0.1 mM $FeCl_2$ was added to 100 μL of a different concentration of sodium alginate (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). The reaction was initiated with the addition of 200 μL of 0.25 mM ferrozine. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was recorded at 562 nm. Sodium ethylene diamine tetraacetic acid (EDTA, Sigma Aldrich) was used as the reference molecule (solution from 0 to 400 μM). A decrease in absorbance corresponded to an increase in iron chelating capacity.

2.14. Determination antibacterial capacity of fortified semen extender

The antibacterial capacity of the semen samples was estimated using the standard plate count (SPC) method. The semen is extended in the extender prepared without antibiotics and sodium alginate. Furthermore, the same semen sample was also placed in the extender fortified with sodium alginate at different concentrations with and without antibiotics (penicillin and streptomycin). To prepare agar media, a 23.5 g plate count agar (tryptone 5 g, yeast extract 2.5 g, dextrose 1.0 g and agar 15.0 g, Himedia Lab, Cat.No. M091) was suspended in 1000 mL distilled water and heated to boiling to completely dissolve the medium. The media was sterilised by autoclaving at 6.8 kg pressure (121 °C) for 15 min and cooled to 45 to 50 °C. There were serial dilutions of the semen sample (1:10, 1:100 and 1:1000) made in dilution media (bacteriological peptone 1.0 g, sodium chloride 8.5 g dissolved in 1000 mL distilled water, pH - 7.0). Inoculums with a size of 100 μL from each dilution was properly mixed with molten SPC agar at 50 °C and poured into petri dish plates. The separate plates were used with each dilution and three SPC agar plates were included for each replicate. Agar was allowed to set and then incubated at 37 °C for 48 h. Colonies per plate were counted with the use of a colony counter.

2.15. Statistical analysis

The data were analysed using the SPSS (Version 18) software package. Data were analysed to determine if there was a normal distribution using the Shapiro-Wilk test and the homogenity of variance was evaluated using the Levene's test. If the data were not normally distributed, there was an arcsine transformation of data performed before analysis and the differences among groups were compared by analysis of variance (ANOVA) and the Turkey's test was used for a *post hoc* analysis. Results are presented as the mean ± standard error of the mean (SEM).

3. Results

3.1. Dose tolerability test

To determine the concentration of sodium alginate for supplementation of extender, the dose tolerability test of sodium alginate (0, 1, 2, 3, 4, 5 and 6 mg/mL) was performed. Sperm motility at the end of 60 min incubation was greatest in 1 mg/mL sodium alginate as compared to other doses. Sodium alginate concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ mL of extender were evaluated for further study.

Table 1
Effect of alginate fortified egg yolk based extender on values for sperm motility variables of frozen- thawed sperm during 2 h of incubation at 38 °C.

Parameter	Time (Min)	Sodium Alginate (mg/ml) extended in egg yolk based extender					
		0	0.2	0.4	0.6	0.8	1.0
TM (%)	0	39.06 ± 1.66^{D}	40.50 ± 1.91	39.11 ± 1.41A	41.17 ± 1.14^{AB}	37.50 ± 1.19B	39.89 ± 1.85C
	30	36.56 ± 1.65^{Ca}	46.06 ± 1.07^{b}	46.56 ± 1.50B^{b}	44.56 ± 1.60$^{B, b}$	38.28 ± 1.59B^{a}	35.83 ± 1.40B^{a}
	60	36.22 ± 1.64^{Ca}	44.83 ± 1.08^{b}	38.83 ± 1.68A^{a}	48.39 ± 1.42$^{C, c}$	36.50 ± 1.62B^{a}	33.27 ± 2.50B^{a}
	90	30.00 ± 1.36^{Ba}	46.06 ± 1.02^{c}	40.17 ± 1.57Ab^{c}	37.83 ± 1.46$^{A, b}$	36.94 ± 1.35B^{b}	30.19 ± 1.58B^{a}
	120	25.22 ± 1.50^{Aa}	41.22 ± 1.88d	41.61 ± 1.69Ad	35.28 ± 1.67$^{A c}$	30.17 ± 1.29Ab	25.22 ± 1.07Aa
PM (%)	0	26.78 ± 1.72B	27.89 ± 1.29AB	25.72 ± 2.05	27.11 ± 1.96A^{B}	28.83 ± 1.58^{AB}	28.94 ± 1.24^{B}
	30	25.61 ± 1.25Ba	31.56 ± 1.26Bb	28.61 ± 1.75ab	31.33 ± 1.02^{Bb}	25.78 ± 1.68^{Ba}	25.06 ± 1.36^{ABa}
	60	20.44 ± 1.91Aa	29.00 ± 1.77ABbc	26.78 ± 1.09b	32.06 ± 1.96^{Bc}	25.00 ± 1.85^{ABb}	25.60 ± 1.05^{ABb}
	90	18.11 ± 1.26A^{a}	26.89 ± 1.68Ab	26.67 ± 1.15b	24.94 ± 1.30^{Ab}	23.61 ± 1.37^{ABb}	22.62 ± 1.36$^{A, b}$
	120	18.50 ± 1.65Aa	25.39 ± 1.26Ab	25.28 ± 1.60b	23.00 ± 1.24^{Aab}	20.11 ± 1.34^{Aa}	21.83 ± 1.50^{Aa}
RM (%)	0	32.00 ± 1.45B	33.72 ± 1.52A	30.33 ± 1.42	34.83 ± 1.43^{AB}	31.67 ± 1.77^{AB}	33.56 ± 1.69^{B}
	30	30.67 ± 1.96BCa	36.06 ± 1.77Bb	33.33 ± 1.36ab	36.44 ± 1.89^{ABb}	33.39 ± 1.90^{Bab}	29.33 ± 1.98^{ABa}
	60	30.44 ± 1.59BCa	33.50 ± 1.01Aa	30.44 ± 1.42^{a}	38.39 ± 1.91^{Bb}	29.61 ± 1.06^{ABa}	29.00 ± 1.50^{ABa}
	90	26.33 ± 1.69Ba	37.28 ± 1.32Bb	31.44 ± 1.36ab	29.17 ± 1.66^{ABab}	29.28 ± 1.69^{ABab}	25.95 ± 1.57^{Aa}
	120	20.61 ± 1.22Aa	31.78 ± 1.98Ac	31.89 ± 1.92c	27.22 ± 1.68^{Ab}	26.83 ± 1.60^{Ab}	25.72 ± 1.87^{Ab}

TM: total motility; PM: progressive motility; RM: rapid motility; ^{ABC} Different capital letters in the same column indicates a difference between additives ($P < 0.05$); ^{abcdef} Different lower case letters in the same row indicates a difference between holding times ($P < 0.05$); $n = 20$.

3.2. Sperm kinematics and motility

After cryopreservation, sperm kinematic variables were estimated using the CASA to make assessments more objective. The viscous property of sodium alginate did not decrease and maintained the sperm VCL, VAP and VSL as compared to the values for the control sample during the entire time period assessments were conducted (Supplementary Fig. 1). Similarly, there were no differences in ALH and BCF of sperm among treated and control samples during the incubation period. To estimate the sperm longevity in the sodium alginate fortified extenders, the values for sperm motility variables (progressive, rapid and total motile sperm) were determined (Table 1) and the samples with 0.2 and 0.4 mg/mL ALG had a greater ($P < 0.05$) number of motile sperm as compared with the control sample.

3.3. Plasma membrane integrity and cervical mucus penetration capacity of sperm

The percent of intact sperm membranes (HOS reactive sperm) of cells in samples containing 0.4 mg/mL ALG was greater ($P < 0.05$) than in the other samples (Fig. 1A). The capacity of sperm to transit through cervical mucus *in vitro* that mimicked *in vivo* conditions towards the site of fertilisation in the ampulla of the oviducts was determined and the sperm in samples containing 0.2 mg/mL ALG transited the longest distance compared to those in the control sample (Fig. 1B).

3.4. CTC assay, immuno-detection of tyrosine phosphorylated proteins and TBARS assay

The results from the CTC assay indicated there were intracellular calcium related changes during the freeze-thaw process (Fig. 2A). The percentage of normal sperm in samples containing 0.2, 0.4, 0.6 and 0.8 mg/mL ALG were greater ($P < 0.05$) than in

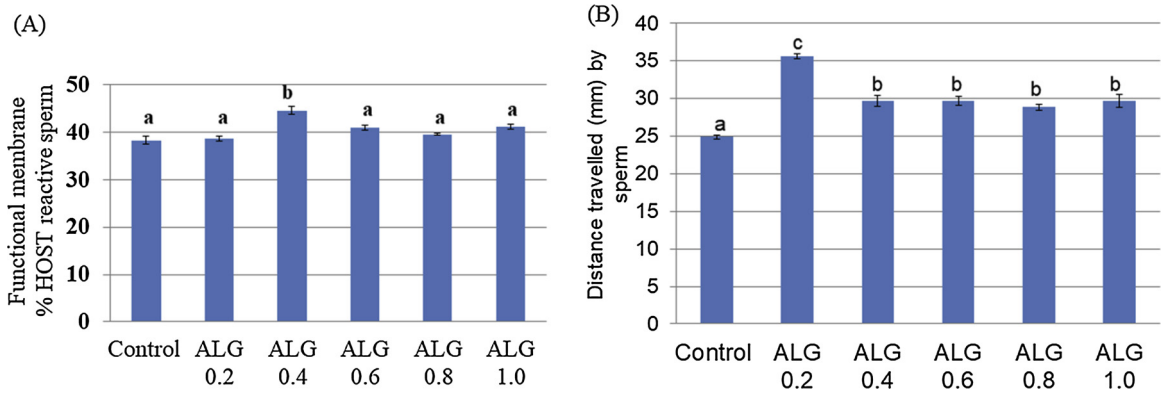

Fig. 1. (A). Percent (Mean ± SEM) of HOS reactive sperm (intact sperm membrane) in fortified egg yolk extender; (B). Distance (Mean ± SEM) of sperm transited in cervical mucus; Values with different letters (a–c) differ ($P < 0.05$), $n = 20$.

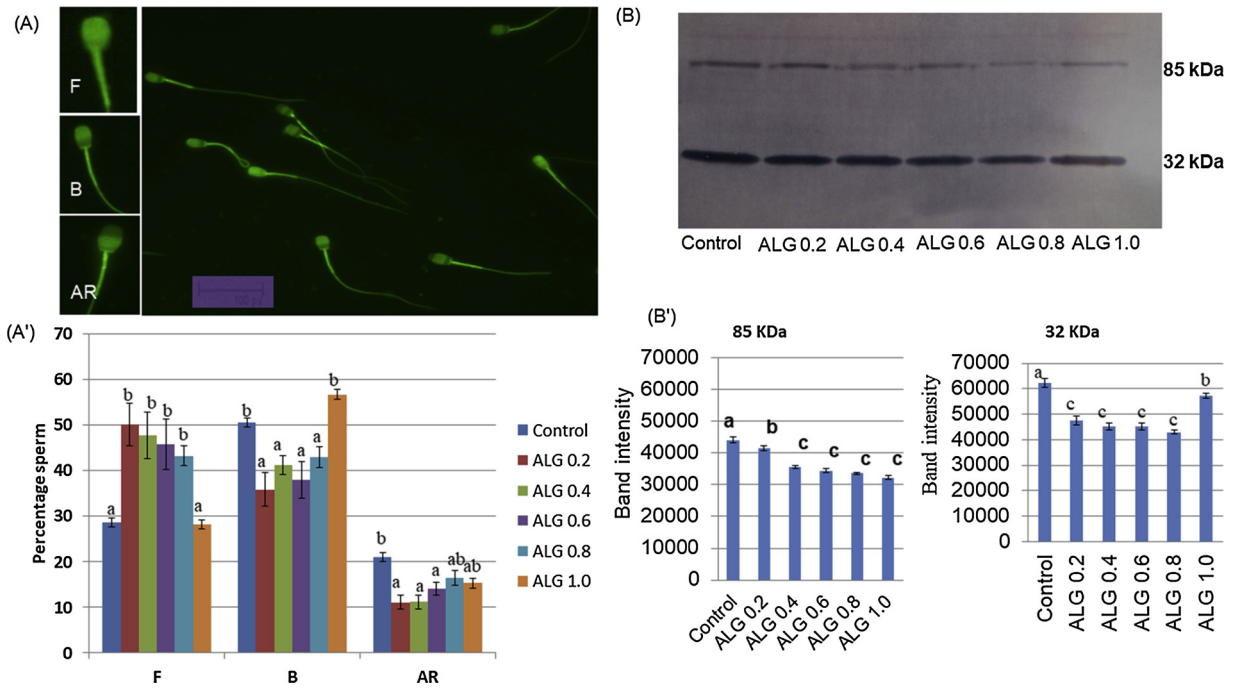

Fig. 2. (A). CTC (Chlortetracycline) assay. F – Normal sperm (bright green fluorescence distributed uniformly over entire sperm head with or without a more intense fluorescent line at the equatorial segment), B-Cryo-damaged sperm (green fluorescence over acrosomal region and dark post acrosome) and, AR – acrosome reacted sperm (sperm with green fluorescence only in post-acrosomal region, or no fluorescence over the head); (A′) Bar diagram showing percentage of sperm that are normal, cryo-damaged and acrosome reacted; Values (mean ± SEM) with different letters (a–b) differ ($P < 0.05$), $n = 20$ (B); Western blot band of tyrosine phosphorylated sperm proteins using anti-phosphotyrosine antibody, clone 4G10 as primary antibody; The 32 and 85 KDa bands differentially expressed alginate treated EY extenders; (B′) band density of 32 and 85 KDa bands was quantified using myImage Analysis software; Values (mean ± SEM) with different letters (a–c) differ ($P < 0.05$), $n = 4$.

the control sample. The percentage of cryo-damaged and acrosome reacted sperm were less ($P < 0.05$) in samples containing 0.2, 0.4 and 0.6 mg/mL ALG than the control sample. Furthermore, in the cryopreserved sperm, the abundances of tyrosine phosphorylated proteins were estimated (Fig. 2B). The results from the quantitative digital image analysis of the 85 kDa band in the western blot indicated there was a greater abundance of protein in the control sample while there were lesser abundances in samples containing 0.4, 0.6, 0.8 and 1.0 mg/mL ALG. Similarly, another band of a protein of about 32 kDa was in a greater abundance in the control sample. The extent of lipid peroxidation due to relatively greater concentrations of ROS generated during sperm cryopreservation in different groups as indicated by the amount of MDA is depicted in Fig. 3A. There was a greater amount of MDA in sperm in the control sample as compared to samples containing sodium alginate.

3.5. Antioxidant activity and metal chelating capacity of extender

To gain insights about how sodium alginate protects sperm during cryopreservation, sodium alginate fortified extenders were evaluated for antioxidant activity (DPPH and FRAP assays) and metal chelating capacity. The free radical scavenging capacity of sodium alginate fortified extenders was assessed by conducting an antioxidant assay based on electron-transfer (DPPH method). The sample containing 0.8 mg/mL ALG had a reduction capacity of about 50% of the DPPH, followed by samples containing 1.0 then 0.2, 0.4, and 0.6 mg/mL ALG (Fig. 3B). Similarly, the ferric reducing capacity of the extender was assessed by performing the antioxidant capacity assay, FRAP, and the sodium alginate fortified extenders had a greater reducing capacity as compared to that in the control sample (Fig. 3C). The metal chelating capacity of sodium alginate was also greater in a dose-dependent manner (Fig. 3D).

3.6. Antibacterial capacity of fortified semen extender

In the search of an alternative to antibiotics in the semen extender, the supplementation of the extender with sodium alginate resulted in an enhanced antibacterial property in a dose dependent manner in terms of number of bacterial colonies observed as compared to that of the control sample (Fig. 4A and B). The extender in which both antibiotics and sodium alginate were present had only a few bacterial colonies even after 7 days of incubation but there was a slightly larger number of bacterial colonies along with fungal growth observed when samples were incubated for a longer period without alginate supplementation (Fig. 4C).

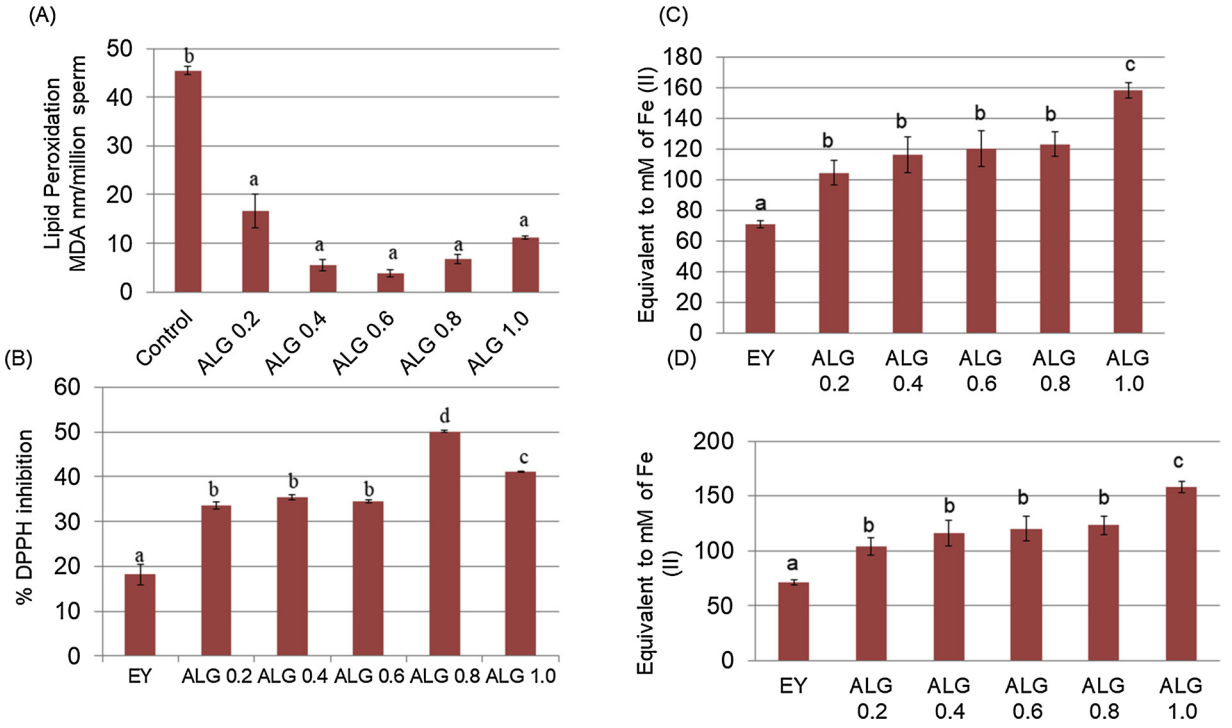

Fig. 3. (A) Greater MDA concentration (*i.e.*, more lipid peroxidation in sperm membrane) in sperm cryopreserved in EY extender without sodium alginate fortified in compared to alginate fortified EY extenders; Values (mean ± SEM) with different letters (a–c) differ ($P < 0.05$), $n = 20$; (B) Free radical scavenging capacity (DPPH reduction) of sodium alginate fortified egg yolk extender; Values (mean ± SEM) with different letters (a–d) differ ($P < 0.05$), $n = 4$; (C) Ferric reducing antioxidant power (FRAP) of sodium alginate fortified egg yolk extenders; Values (mean ± SEM) with different letters (a–c) differ ($P < 0.05$), $n = 4$; (D) Metal chelating capacity of sodium alginate fortified egg yolk extenders; Values (mean ± SEM) with different letters (a–c) differ ($P < 0.05$), $n = 4$.

4. Discussion

Since sodium alginate is widely used as a gelling agent, and it was anticipated that it would increase viscosity of extenders leading to lesser values for sperm velocities in samples containing the alginate fortified extender. There was evaluation of sperm velocities (VCL, VAP and VSL), therefore, of sodium alginate treated frozen-thawed sperm. There was no adverse effect of sodium alginate fortified extenders on sperm VCL, VAP and VSL in comparison to untreated extender. Furthermore, the normal ALH and BCF in alginate fortified extenders confirm that these concentrations are optimum for the use in semen extenders. In addition, results of many studies indicate that small increases in viscosity lead to an improvement in the semen quality in sheep (Yániz et al., 2005), rabbits (Rosato and Iaffaldano, 2011) and boars (Anaya et al., 2014). Sustaining the duration of sperm motility in female the reproductive tract is a pre-requisite for fertilisation. Results of the present study indicate that supplementations of semen extenders with sodium alginate can lead to a sustained sperm motility for longer periods as confirmed by results of the *in vitro* incubation test. In addition, for progressive motility to be sustained, a spermatozoon must have an intact and functional plasma membrane if the cell is to have fertilisation capacity. Destabilisation of the plasma membrane constituents occurs when there are decreases of temperatures during cryopreservation (Bailey et al., 2000; Swami et al., 2017b). The results of the present study indicate that supplementation of sodium alginate in EY extender minimised the membrane damage during cryopreservation. This finding corroborates the results of previous study in which the addition of sodium alginate to boar semen improved post-thaw plasma membrane integrity (Hu et al., 2014). Similarly, sodium alginate was effective in maintaining the plasma membrane integrity of salmonid fish sperm after short-term storage (10 days) at 4 °C (Merino et al., 2017). Spermatozoa have to traverse through the mucus of the female reproductive tract to reach to the site of fertilisation. Measuring the capacity of the spermatozoa to traverse through the cervical mucus of oestrous cows is indicative of the capacity to traverse through the female reproductive tract *in vivo*. In the present study, the sperm in the sample with 0.4 mg/mL sodium alginate fortified extender traversed the longest distance in cervical mucus *in vitro*, confirming that sodium alginate supplementation of extender can not only improve longevity of sperm, and maintain membrane integrity, but also the capacity to traverse through the cervical mucus.

Furthermore, in the present study there was an attempt to investigate how sodium alginate protects sperm during cryopreservation resulting in the improvement of the values for semen quality variables. The most commonly used index to quantify lipid peroxidation induced by free radicals is the estimation of concentrations of MDA. In the present study, the concentration of MDA was quantified in froze-thawed sperm. The concentration of MDA in sperm not supplemented with sodium alginate was markedly greater, indicating that alginate effectively prevents lipid peroxidation of sperm membranes. The reduction in lipid peroxidation may be due

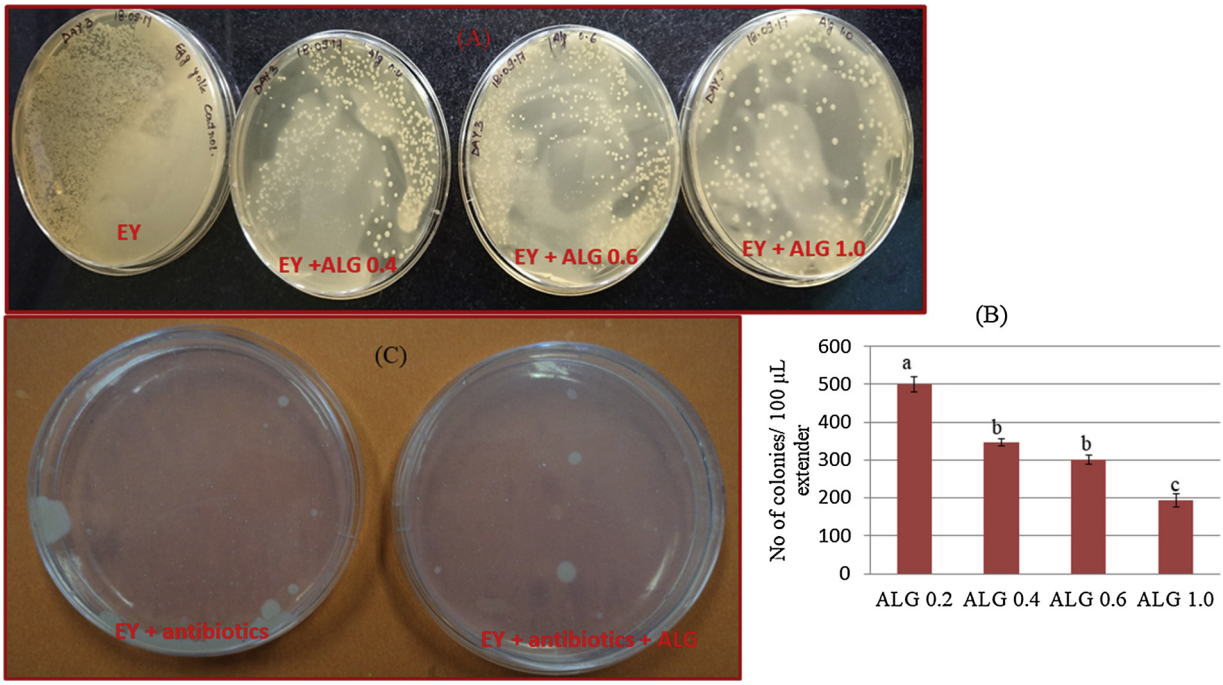

Fig. 4. Bacterial colonies on standard plate count agar plates; (A) Semen is extended in the EY extender without antibiotics but fortified with different concentrations of sodium alginate and colonies observed after 3 days; (B) Number of colonies uncountable in EY while in sodium alginate treated sample are depicted in the bar diagram; (C) No colonies observed in antibiotic treated extender and sodium alginate treated extender; Values (mean ± SEM) with different letters (a–c) differ ($P < 0.05$), $n = 4$.

to multiple protective actions of sodium alginate in terms of free radical scavenging, ferric reduction and iron-chelating properties as subsequently described in this manuscript. The membranes that undergo lipid peroxidation have a greater calcium influx during freezing-thawing processes resulting in greater intracellular calcium concentrations, inducing a signalling cascade associated with a capacitation = like reaction (Robertson and Watson, 1986; Bailey and Buhr, 1994; Bailey et al., 2000; Green and Watson, 2001). Sodium alginate fortification of an extender is likely to prevent the calcium influx and capacitation-like changes in sperm during the freezing-thawing processes. This is evident from the reduced abundance of immuno-detected phosphotyrosine-containing proteins (PTP) of about 32 and 85 kDa with sodium alginate supplementation of semen extender. The immuno-detection of a 32 kDa protein band in cryopreserved sperm is consistent with the finding of Cormier and Bailey (2003). The PTP is the most important intracellular signalling function reflected in changes in the acrosomal area of sperm that can be detected with use of the cholortetracycline (CTC) assay. The results of the CTC assay confirmed that sodium alginate is very effective in preventing the phase transition of sperm membrane during sperm cryopreservation. Bailey and Buhr (1994) also reported there was a decrease in efficiency of intracellular calcium control mechanisms in bull spermatozoa following cryopreservation.

To enhance the understanding of the possible mechanism of sperm cryo-protective action of sodium alginate, there was investigation of the redox status of supplemented semen samples using the DPPH, FRAP and metal chelating capacity assays. The DPPH assay is a rapid and widely used method to quantify the capacity of a compound to function as free radical scavengers or hydrogen donors (Prakash, 2001; Sendra et al., 2006). In the present study, the sodium alginate fortified extender can scavenge about 20% more free radicals in compared to extender without alginate. Furthermore, the results with the FRAP assay indicated sodium alginate in the extender functions as an electron donor and reacts with free radicals produced during cryopreservation to convert these to more stable products and thereby terminate the radical chain reactions as reported by Zhang et al. (2013).

Iron, copper, chromium, cobalt, vanadium, cadmium, arsenic and nickel promote oxidation by functioning as catalysts of free radical reactions (Ercal et al., 2001). It is also noteworthy that egg yolk contains several transition and heavy metals (Hashish et al., 2012; Islam et al., 2014; Hossain et al., 2017; Thongcharoen et al., 2017) which are detrimental to sperm properties. Some of those metals promote oxidation by functioning as catalysts during free radical generation (Ercal et al., 2001). The process of cryopreservation results in production of a large amount of free radicals and the presence of iron and other metals in the extender functions like 'fuel in the burning fire' to potentiate lipid peroxidation of sperm membranes. In the present study, sodium alginate had metal chelating functions in the semen extender. Results of previous studies have also confirmed that sodium alginate is a potential sequester of metals (Norajit et al., 2010; Sears, 2013). In a clinical trial involving five patients, sodium alginate administration reduced, by as much as 74%, the dangerous heavy metals, including lead, mercury, cadmium, *etc.* from the bodies of the patients (Eliaz et al., 2007). Similarly, sodium alginate in minced pork meat potentiated antioxidant activity due to its iron reductant, metal chelating and free radical scavenging capacities (Endo et al., 2015). The antibacterial effects of sodium alginate in semen extender in present study are significant. The use of sodium alginate as an antibacterial material has been previously reported in many studies (Shalumon et al.,

2011; Shao et al., 2015; Krol et al., 2017).

5. Conclusions

It is concluded that sodium alginate has the capacity to affect multiple processes to protect sperm from detrimental effects of cryopreservation as well as undesired substances present in the semen extender. Sodium alginate in a semen extender protects sperm by a) preventing generation of free radicals, b) scavenging the excess free radicals, c) chelating heavy metals, d) maintaining sperm plasma membrane fluidity, and g) potentiating the antibacterial capacity of the semen extender.

Author contributions

PK and JA involved in semen evaluation after semen collection. SP performed CASA, DPPH, FRAP and metal chelating ability. Jasmer performed CTC assay and Western blot. Suman and SB performed incubation and HOST test. SP and Suman performed agar plate incubation test. MHJ and PK performed statistical analysis. PK, DK, MHJ and PSY prepared manuscript. PK designed experiments and supervised the work.

Declaration of Competing Interest

The authors report that there are no conflicts of interest relevant to this publication.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.106166.

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