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IN VITRO SUPPLEMENTATION OF COMBINED FATTY ACIDS IN EXTENDER FOR CRYOPRESERVATION OF BUFFALO SEMEN

Rabea Ejaz*[1], Samina Qamer[1], Fareeha Ambreen[1], Anas Bilal[1], Asima Azam[2], Rizwana Kousar[3], Zahid Abbas Malik[4], Muhammad Sajid[5], Shamim Akhter[6]

[1]Department of Zoology, Rawalpindi Women University, Rawalpindi- 46300, Pakistan

[2]Department of Zoology, Shaheed Benazir Bhutto Women University Peshawar-25000, Pakistan

[3]Department of Biology, Allama Iqbal Open University, Islamabad, Pakistan

[4]Department of Biotechnology and Food Science, University of Layyah, Layyah

[5]Department of Zoology, Government College University Faisalabad

[6]Department of Zoology, Wildlife and Fisheries, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan

Corresponding Author: Rabea Ejaz, Department of Zoology, Rawalpindi Women University, Rawalpindi- 46300, Pakistan, **Email address**: *rabeaejaz29@gmail.com

ABSTRACT

The current study aimed to assess the impact of extender supplementation of a combination of three fatty acids (arachidic acid 20 ng/mL, α-linolenic acid 5.0 ng/mL and cholesterol 5.0 ng/mL) for cryopreservation of buffalo sperm. Three adult buffalo bulls of the same age were used to collect semen using an artificial vagina (42°C). Tris citric acid extender (37°C) was used to dilute qualified semen ejaculates (>0.5 billion per mL conc., >1 mL volume, >60% motility). Six different experimental extenders were prepared containing fatty acids (arachidic acid 20 ng/mL, α-linolenic acid 5.0 ng/mL and cholesterol 5.0 ng/mL) alone and in combinations. The control extender was without any fatty acid supplementation. Extended semen chilled to 4°C (in 2 hours) was placed for equilibration (at 4°C) for 4 hours. Plastic straws 0.5 mL (4°C) were used to fill the equilibrated semen. Liquid nitrogen gas vapours were used to further freeze the semen for 10 min. and then dipped in liquid-nitrogen for storing. After 24 hours thawing was done (37°C) for 30 seconds and semen quality was assessed.

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Supplementation of combination of fatty acids in extender didn't improved percentage of progressive sperm motility, plasma membrane intactness, viable sperm and intact chromatin of cryopreserved buffalo semen compared to supplementation of fatty acids alone in extender and control. In conclusiothe n, addition of fatty acids in combination to a semen extender was not found beneficial to improve quality of cryopreserved buffalo semen.

Keywords: Fatty acid, Cryopreservation, Extender, Buffalo, Semen

Introduction

The process of freeze-thaw significantly reduces the quality of buffalo sperm [1] reducing 50% viable sperm and upto 70% sperm fertilizing ability [2]. Degree of sperm progressive motility and viability largely depend on intactness of sperm plasma membrane [3], the organelle that first face the temperature variation during cryopreservation. Its intactness is further affected by alteration in lipid composition during freeze thaw cycle. Lipids are essential components of semen and are present both in spermatozoa and seminal plasma. Sperm lipids contain neutral fatty acids, cholesterol, phospholipids and glycolipids [4]. Polyunsaturated fatty acids (PUFAs), predominant in sperm plasmalemma, are prone to lipid peroxidation [5] and result in membrane damage, intracellular enzyme leakage and inhibition of respiration.

During cryopreservation, cold shock stress and freezing step causes significant loss of total lipids (15.8%-34.55%) and phospholipids (6.49%-19.1%) in buffalo sperm [6]. Moreover, Kadirvel *et al.* [7] reported significant loss of sperm cholesterol after the process of cryopreservation that caused destabilization of plasma membrane in buffalo sperm. In the course of cryopreservation this fatty acids loss from membrane is might be because of lipid peroxidation and production of acetyl CoA through β-oxidation [8].

Literature showed that when fatty acids were introduced through diet in different species semen quality was improved as diet supplies the PUFAs to sperm membrane [9]. Diet supplementation of docosahexaenoic acid enhanced the quality of stallion sperm after freezing [10] and fresh Holstein bull semen [11]. Flaxseed oil in diet enhanced quality of fresh semen and its livability [12]. Likewise, adding unsaturated fatty acids in diet upgraded the semen quality and freezibility [13]. Improvement in semen quality parameters by adding fatty acids in extender was reported in different studies [14,15]. In previous studies alpha linolenic acid [16], cholesterol [17] and arachidic acid [18,19] improved quality of cryopreserved Nili-Ravi sperm. Supplementation of combined fatty acids in extender may enhance semen quality by

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compensating the loss of fatty acids incurred during cryopreservation. The present study was aimed at evaluating the combined impact of α-linolenic acid, cholesterol and arachidic acid on post thaw quality of buffalo sperm.

Materials and methods

Extender preparation

Tris-citric acid (pH 7.0) was comprised of 3.0% tris aminomethane (Research Organics, USA), 1.56% citric acid (Fisher Scientific, UK), 7.0% glycerol (Merck, Germany), 0.2% w/v fructose (Scharlau, Spain), 20% v/v egg yolk, antibiotics; streptomycin sulphate (1000 µg mL-1), benzyl penicillin (1000 IU mL-1) and 74 mL dist. water. Seven extenders were made containing fatty acids (Sigma Chemical Co. USA) 20.0 ng mL-1 of arachidic acid (AA) (extender I), 5.0 ng mL-1 of alpha linolenic acid (ALA) (extender II), 5.0 ng mL-1 of cholesterol (extender III), AA and ALA (extender IV), AA and cholesterol (extender V), ALA and cholesterol (extender VI). However, control was extender lacking fatty acid (extender VII). Ethanol (0.05%) was used to solubilize fatty acids [16].

Semen collection and initial evaluation

Three bulls of same age were used at Semen Production Unit Qadirabad, Pakistan. Artificial vagina (42°C) was used for semen collection for five weeks. Semen was quickly transported to laboratory for initial evaluation (n=30; r= 5 and two ejaculates per replication). Qualified ejaculates (>0.5 × 10^9sperm/ mL concentration, >60% motility, >0.5 mL volume) were split into seven aliquots and held in water bath at 37°C for 15 min in seven different extenders.

Semen processing

Extended semen was placed to cool in 2 hours and equilibrated for 4h (at 4°C). Semen straws (0.5 mL French) were used to fill by suction pump at 4°C, placed on liquid nitrogen vapours (5 cm above) for freezing for 10 minutes. Straws were then placed into liquid nitrogen (-196°C) container [20]. After 24h, semen straws (3 for each replicate) were thawed in a water bath (at 37°C) for 30 seconds for quality evaluation.

Evaluation of post-thaw sperm functional assays

Sperm progressive motility

Progressive motility was assessed from a covered drop of thawed semen on slide and viewed under phase contrast microscope (400X; 37°C).

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Sperm plasma membrane integrity

Supravital hypoosmotic swelling test (HOST) used to evaluate the intactness of membrane as described by Ejaz *et al.* [18]. Semen sample (50µL) was mixed with pre-warmed HOS solution (500µL) and incubated for 30-40 min (37°C). Incubated mixture drop was placed on slide and viewed with coverslip microscopically (400X). Tails with swollen morphology showed intactness of sperm membrane while opposite showed disrupted sperm membranes.

Acrosome intact live sperm and live/dead ratio

Viable sperms with intact acrosome were evaluated by trypan blue Giemsa stain as described by Akhter *et al.* [21]. Briefly, an equal minute amount of trypan blue and semen was mixed on a slide and air dried. Dried smear was fixed in formaldehyde-neutral red (for 5 min), dipped in dist. water and held in 7.5% Giemsa stain (for 4h). Air dried samples were mounted with Canada Balsam. 1000X Phase contrast microscope was used to study slides. Trypan blue stain the non-viable sperm blue whose membrane is disrupted while unstained are viable sperm. Giemsa stain the intact acrosome of sperm as purple.

Sperm chromatin integrity

Intactness of chromatin was evaluated as practiced earlier Ejaz *et al.* [19]. A drop of semen was air dried and fixed in 96% ethanol-acetone (1:1) for 30min (at 4°C) then hydrolyzed with 4N HCl (25°C) for 10-30 min. After washing with distilled water toluidine blue was used to stain the slides in sodium citrate-phosphate (Mcllvaine buffer) for 10min. Sperm with damaged chromatin was stained dark blue to purple with toluidine blue while light blue sperm were taken as with intact chromatin.

Statistical analysis

Post thaw buffalo sperm quality data was evaluated using ANOVA in Randomized Complete Block Design (RCBD) by fixed-effect model. The data were presented as mean (±SE). LSD test was applied to compare treatment means at $\alpha < 0.05$.

Results

Results on the effect of α-linolenic acid and cholesterol at 5.0 ng/mL and arachidic acid at 20.0 ng/mL individually and along with their combinations on cryopreserved buffalo semen quality are shown in Table 1. Sperm characteristics were considerably higher ($p<0.05$) in extender having arachidic acid and alpha linolenic acid alone compared to control and other extenders.

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Addition of combined fatty acids in extender did not proved beneficial to improve quality of cryopreserved buffalo semen.

Table 1: Effect of combined fatty acids supplementation in extender on post-thaw quality of cryopreserved buffalo bull Spermatozoa (N=30).

Fatty acids ng/mL	Progressive motility (%)	Plasma membrane integrity (%)	Live dead ratio (%)	Viability (%)	Chromatin Integrity (%)
Control	42.3± 4.9^{d}	53.5±5.6^{e}	53.9±8.9^{b}	36.2±5.9^{e}	93.9±0.06^{b}
AA	52.0±1.15^{a}	62.3±3.23^{ab}	59.7±6.3^{a}	45.7±3.9^{a}	96.5±0.12^{a}
ALA	52.3±2.18^{a}	63.7±2.13^{a}	59.7±8.4^{a}	45.9±5.3^{a}	96.13±0.70^{a}
Chol	48.3±0.66^{b}	58.5±2.35^{d}	59.9±3.2^{a}	39.9±6.4^{c}	94.1±0.46^{b}
AA, ALA	47.3±2.02^{bc}	61.3±1.34^{bc}	53.1±3.2^{b}	42.3±4.3^{b}	94.1±0.56^{b}
AA, Chol	47.3±1.45^{bc}	60.3±1.05^{cd}	53.3±1.9^{b}	38.0±2.4^{d}	94.3±0.14^{b}
ALA, Chol	45.0±2.08^{c}	58.9±2.7^{d}	52.4±6.2^{b}	37.7±6.7^{d}	94.1±0.49^{b}

The values with different superscripts within the same column differ significantly ($P < 0.05$). Total numbers of ejaculates were 30 (five replicates per each of the three bulls and two ejaculates per replication).

Discussion

Mammalian sperm depending on composition and ratio of membrane lipids showed varying pattern of susceptibility toward cold shock [1]. Sperm motility, its sensitivity for cold shock and its ability to fuse with egg all depend upon membrane fluidity and permeability that in turn depend upon its lipid composition [22]. Buffalo sperm is a bit different from other species have high amount of omega 3 and omega 6 PUFAs crucial for energy generation during lipid metabolic pathways and also very important for membrane stability during freeze-thaw cycle [23]. Cholesterol to phospholipid ratio of mammalian sperm affects cold shock susceptibility during cryopreservation [1]. Sperm fatty acids significantly reduce during cryopreservation. In previous reports with alpha linolenic acid [16], cholesterol [17] and arachidic acid [18,19]. extender supplementation alone enhanced the frozen semen characteristics. Therefore, adding fatty acids in combination was needed to be investigated for buffalo sperm cryopreservation.

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In this study, supplementation fatty acids (ALA at 5 ng/mL; AA at 20 ng/mL; Chol at 5 ng/mL) alone improved quality of cryopreserved buffalo semen. However, supplementation of combination of fatty acids in extender did not improve semen quality. The results are in line with the previous studies presented by Kaka *et al.* [24] who found that supplementation of combination of ALA and DHA did not improve quality of cryopreserved bull sperm. Previously, supplementation of n-3, 6, 9 fatty acids in the form of soft gel containing 357 mg n-3, 292 mg n-6 and 152 mg n-9 with concentrations 1, 2.5 and 5% failed to improve quality of chilled and frozen bovine semen [25]. These findings support the results of the present study. Arachidic acid and α-linolenic acid significantly improved sperm progressive motility compared to other experimental extenders and control suggesting the effective incorporation of fatty acids. Combinations of fatty acids did not prove beneficial as these fatty acids might stiffen plasma membrane and disturb its fluidity. These results also suggest that when sufficient lipids are available the effect of further lipid incorporation may be masked.

Semen quality and fertility depend on the type and length of carbon chain in PUFAs [26]. It is reported that long chain PUFAs improve the plasma membrane fluidity and its resistance to bear the cold shock [27]. This loss of membrane lipids during freeze-thaw cycle impairs membrane that ultimately affects the morphological characteristics of sperm [10]. The ice crystal that formed during the freezing and thawing are the important factor that cause changes in membrane lipid composition like phase transition of lipids, change in membrane domains that leads to lipids re-aggregation [28]. All these changes during freeze thaw steps alter the permeability of membrane that leads to increase intracellular calcium concentration ultimately affecting the quality of sperm [29] According to Lahnsteiner *et al.* [30], the supplementation of fatty acids might have stabilized the energy metabolism of sperm and served as energy substrate in extender. In present study, exogenous fatty acids might have compensated for the loss of fatty acids during cryopreservation through their incorporation into sperm membrane resulted in improved semen quality. However, supplementation of combination of fatty acids in extender did not prove beneficial to improve quality of cryopreserved buffalo semen. These results suggest that when adequate lipids are available to stabilize sperm plasma membrane, further incorporation of fatty acids may have disturbed membrane fluidity by increasing fatty acid saturation that ultimately resulted in decreased semen quality.

Conclusion

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In conclusion, the supplementation of combination of fatty acids (arachidic acid, α-linolenic acid and cholesterol) in extender did not prove beneficial to improve quality of cryopreserved buffalo semen.

Author's contribution

Rabea Ejaz and Shamim Akhter conceived and designed the experiments. Asima Azam and Rabea Ejaz performed the experiments. Samina Qamer, Fareeha Ambreen, Anas Bilal, Rizwana Kousar, Zahid Abbas Malik and Muhammad Sajid helped wrote the manuscript.

Conflict of interest

The authors have no conflict of interest

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