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Fertility of Cryopreserved Buffalo Semen Can be Improved by Supplementation of Arachidic Acid in Extender

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Key words
Arachidic acid, Buffalo, Cryopreservation, Semen, Saturated fatty acid

ABSTRACT

The study aimed to evaluate *in vivo* fertility of cryopreserved buffalo spermatozoa by addition of arachidic acid in semen extender. For this purpose, semen was collected from 3 adult buffalo bulls (*Bubalus bubalis*) of same age by artificial vagina keeping temperature at 42°C for a period of 5 weeks (replicates; n=30). Ejaculates were mixed in *tris* citric acid extender having 0.0 (control), 20.0, 25.0 and 30.0 ng/mL of arachidic acid at a temperature of 37°C (>1 mL volume, >0.5 billion per mL conc., >60% motility) and cryopreserved using standard procedures. Percent sperm motility, liveability, plasmalemma integrity and viability were increased in extender (P<0.05) having 20.0 ng/mL of arachidic acid compared to 25.0 ng/mL, 30.0 ng/mL and control. However, sperm chromatin integrity was equally improved in experimental extenders having arachidic acid compared to control. Sperm abnormalities were reduced in experimental extender with 20 ng/mL of arachidic acid compared to other experimental extenders containing arachidic acid and control. In experiment 2, a total of 533 inseminations were carried out by the extender containing best level of arachidic acid (20 ng/mL of extender). *In vivo* fertility was significantly improved in buffaloes inseminated with semen containing 20.0 ng/mL of arachidic acid (58.64%) compared to control (46.06%). In conclusion, addition of arachidic acid (20.0 ng/mL) in extender significantly enhanced quality and *in vivo* fertility of post thaw buffalo semen.

INTRODUCTION

During cryopreservation, the freeze thaw damages are unavoidable that result in reduced semen quality (Yoshida, 2000). Cryopreservation without protective agents causes loss of lipids (Pickett and Komarek, 1964) and reorientation of phospholipids from sperm membrane that disrupts plasma membrane functions (Lessard *et al*., 2000). In buffalo spermatozoa, the cold shock and freezing causes 6.49% and 19.1% loss of phospholipids respectively (Sarmah *et al*., 1984). Lipids are lost from sperm membranes due to lipid peroxidation reactions (Aitken, 1995) and generation of acetyl CoA (Cook *et al*., 1982) by β-oxidation. The loss of lipids from sperm membrane can be mitigated by enriching the semen extender with fatty acids (Ejaz *et al*., 2014, 2017).

High ratio of polyunsaturated fatty acids (PUFAs) in sperm plasmalemma causes high fluidity that in turn resulted in low resistance to thermal shock (Giraud *et al*., 2000). However, saturated fatty acids (SFAs) are not as much susceptible to peroxidation (Rael *et al*., 2004) compared to unsaturated fatty acids. Arachidic acid (AA) is naturally found in buffalo semen and is involved in membrane functions (Jain and Anand, 1976). Previously, when AA was evaluated at 0, 5.0, 10.0 and 20.0 ng/mL in extender, improvement in buffalo sperm quality was recorded at the highest level i.e., 20 ng/mL of tris citric acid extender (Ejaz *et al*., 2014). We speculated that

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higher concentration of AA in the extender may further increase post thaw quality and *in vivo* fertility of buffalo spermatozoa., This study, therefore, was planned to identify cut off value of AA in extender for improvement in post thaw quality and to evaluate *in vivo* fertility of buffalo semen cryopreserved in extender containing best evolved level of AA.

MATERIALS AND METHODS

Extenders preparation

Tris citric acid (320 mOsmol Kg^{-1} of osmotic pressure; pH 7.0) was added as buffer, having 1.56% citric acid (Fisher Scientific, Leicestershire, Loughborough, UK), 3.0% tris-amino methane (Research Organics, OH, Cleveland, USA), 7.0% glycerol (Merck, Germany), 0.2% w/v of fructose (Scharlau, Spain), antibiotics; streptomycin sulphate (1000 µg mL^{-1}), benzyl penicillin (1000 IU mL^{-1}) and 20% of egg yolk (v/v) in distilled water (74 mL). Total four extenders were prepared containing 0.0, 20.0, 25.0 and 30.0 ng/mL of AA (Sigma Chemical Co., MO, USA) respectively. Ethanol 0.05% was added due to insolubility of fatty acids in extender (Kaka *et al*., 2015a; Kaka *et al*., 2015b; Ejaz *et al*., 2014).

Semen collection

Three adult Nili-Ravi (*Bubalus bubalis*) buffalo bulls having same age (7 to 8 years) and of well known fertility having normal reproductive tracts were selected for this study. The animals were maintained under the similar handling and feeding situation at Semen Production Unit (SPU), Qadirabad, Pakistan. Semen was collected in the peak (September to November) breeding season at weekly intervals for 5 (replicates) weeks by an artificial vagina (IMV, France) at 42°C, using a bull as a teaser. After semen collection, the ejaculates were quickly transported to laboratory for assessment of concentration, motility and volume. The motility of sperm was evaluated by phase contrast microscope at X 400 (Tokyo, Japan; Olympus BX20) at 37°C (Ahmed *et al*., 2003). Concentration of sperm was evaluated by bovine photometer (IMV, France) ACCUCELL. The qualified ejaculates (>60% motility, >1 mL volume, >0.5 billion/mL concentration; Qadeer *et al*., 2015) were divided in 4 parts and placed in water bath for fifteen minutes (37°C).

Sample processing

Semen samples were mixed in *Tris* citric acid extender (at 50 × 10^6 motile spermatozoa / mL; 37°C) having different concentrations of AA (20.0, 25.0 and 30.0 ng/mL). The extender without AA was considered as control. The extended semen was placed (at 4°C) in two hours at the rate of 0.275 °C per minute and placed at 4°C for 4hours. Semen samples were filled in 0.5 mL of French straws (IMV, France) in cold cabinet (4°C) and placed on vapours of liquid nitrogen (for ten minutes). Semen straws were placed in liquid nitrogen container at -196°C. After 24h, semen samples were thawed in water bath (37°C) for thirty sec. to evaluate quality of post thaw semen.

Post-thaw sperm functional assays

Sperm progressive motility

Semen sample of 5µL was put on glass slide and evaluated for sperm motility (37°C) using phase contrast microscope at X 400 (Olympus BX20; Akhter *et al*., 2013).

Sperm plasmalemma integrity

Sperm plasmalemma integrity was evaluated by hypo-osmotic swelling test as practiced by (Qadeer *et al*., 2015). Solution for this test contained 0.73 g of sodium citrate (Merck) and 1.35 g of fructose (Barcelona, Scharlau, Spain) in distilled water (100 mL; osmotic pressure 190 mOsmol per kg). Sperm plasmalemma integrity was evaluated by mixing semen sample (50µL) with HOS solution (500µL) and placed at 37°C for 30 to 40 minutes. A sample drop (5µL) was put on glass slide and observed under microscope (400X magnification). Sperms with swollen tails have functional and intact membranes while sperms with unswollen tails have non functional and inactive sperm membranes (Ejaz *et al*., 2016).

Sperm liveability and viability

Liveability and viability of sperm were determined by dual staining process (Akhter *et al*., 2008). Equal volume of semen and trypan blue (Eschwege, Germany) was put on slide. The solution was mixed and dried. Samples were placed in formaldehyde neutral red for five minutes and rinsed in distilled water. Samples were placed for 4 h in 7.5% Giemsa solution (Sigma). Samples were placed in distilled water and dried at 37 ˚C. The samples were mounted by Canada Balsam. Sperms with light blue or transparent colour were live while sperms with dark blue colour were dead. Light blue or transparent sperms with apparent acrosome end were viable (live sperms with intact acrosome) while sperms with blue coloured discrimination and acrosome with blur ends were nonviable (dead sperms having disrupted acrosome). The spermatozoa were assessed in each sample under a phase contrast microscope (X1000) individually for sperm viability and liveability.

Sperm chromatin integrity

Chromatin integrity of sperm was evaluated as described by Ejaz *et al*. (2014). Semen smears were prepared and air dried. Semen sample were placed in

96% of ethanol acetone (1:1) for 30min (4°C) and in HCl solution (4N) for 10-30 min. (25°C). Samples were placed in distilled water for 3 times (for 2 min. in each) and placed in toluidine blue solution for ten min. Semen samples were get dried and mounted by Canada Balsam and observed under light microscope (1000X). Sperms with dark blue or purple colour were having damaged chromatin and sperm with light blue colour were having intact chromatin.

Sperm morphological abnormalities

For the evaluation of sperm morphological abnormalities, semen sample (100 µL) was mixed in 1% formal citrate (500 µL). Solution of formal citrate was prepared by adding 2.9 g Tris sodium citrate dihydrate in 1 mL solution of formaldehyde (37%), mixed in distilled water (100 mL). A sample of semen drop was put on slide and observed by phase contrast microscope at X1000. Head abnormalities of sperm were observed like double heads, macro and micro heads, detached heads and pyriform heads. Abnormalities of mid piece were recorded as abaxial attachment, distal droplet and proximal droplet. Abnormalities of tail were recorded as coiled tail under head, bent tail at mid piece, double tail and headless tail (Akhter *et al*., 2008).

Evaluation of in vivo fertility

Semen collection and cryopreservation

Tris citric acid extender was readied as discussed in previous section. Two extenders were readied containing 0.0 (control) and 20.0 ng/mL of AA (Sigma Chemical Co., MO, St. Louis, USA). Ethanol 0.05% was added due to insolubility of fatty acids in extender (Kaka *et al*., 2015a; Kaka *et al*., 2015b; Ejaz *et al*., 2014).

Semen was collected by artificial vagina at 42°C from 3 mature buffalo bulls of same age and proven fertility. Collected semen samples were quickly transported to laboratory. Sperm concentration and motility was evaluated as discussed in previous section. Qualified semen ejaculates (>60% motility, >1 mL volume, >0.5 billion/mL concentration) were divided in aliquots and placed in water bath (37°C) for 15 minutes. Each sample was mixed in extenders (37°C) at 50×10^6 motile spermatozoa/mL of concentration. Semen samples were cryopreserved as described in previous section. Samples were placed in water bath for thawing at 37°C (30 sec.) for further use in artificial insemination.

Artificial insemination

Total 533 inseminations were carried out for a period of 3 months in breeding season (October to December). Inseminations were carried out under field conditions in animals with 4 to 6 years of age. The inseminations were carried out 24 hours after observation of signs of heat in buffaloes that have a record of one successful parturition. The experimental animals were observed for pregnancy at least ninty days after insemination with rectal palpation by trained technicians under field conditions.

Data analysis

Results of current study were shown as means ± SEM. Effect of different levels of AA addition in extender on the quality of post thaw buffalo semen was analyzed by analysis of variance (ANOVA) using MSTAT-C. If F ratio was significant ($P < 0.05$), least significant difference was applied for comparison of treatment means (Ansari *et al*., 2011). Data on *in vivo* fertility rate were analyzed by Chi square.

RESULTS

Post thaw semen quality parameters

Data on effect of AA in extender on motility, plasmalemma integrity, live/dead ratio, chromatin integrity and viability of post thaw buffalo sperm are shown in Table I. Highest sperm motility, plasmalemma integrity, viability and live/dead ratio were recorded in extender having 20.0 ng/mL of AA compared to control. Further increase in AA at 25 ng/mL and 30.0 ng/mL deteriorated the above mentioned parameters compared to extender containing 20 ng/mL of AA ($P<0.05$). However, sperm chromatin integrity was equally improved ($P < 0.05$) in all extenders having AA compared to control. Data on effect of AA in extender on sperm morphological abnormalities (head, tail, mid piece and total) of cryopreserved buffalo sperm are presented in Figure 1. Lowest sperm head abnormalities were recorded in experimental extender having AA (20 ng/mL) compared to control, however, the difference was non significant ($P>0.05$). The mid piece abnormalities remained similar in all extenders having AA ($P>0.05$) compared to control. Sperm tail and overall total abnormalities were decreased in extender having AA (20 ng/mL) compared to other experimental extenders containing AA and control ($P<0.05$).

In vivo fertility rate

Data on the *in vivo* fertility rate of cryopreserved buffalo bull spermatozoa are presented in Table II. The fertility rates (%) did not differ ($P>0.05$) in extender having 20 ng/mL of AA in bull 1, bull 2 and bull 3 compared to control. However, overall *in vivo* fertility (%) in buffaloes was greater with semen cryopreserved in extender containing 20 ng/mL of AA ($P < 0.05$) compared to control (58.64% vs 46.06%).

Table I. Effect of AA supplementation in extender on post-thaw quality (Mean±SE) of cryopreserved buffalo semen.

AA (ng/mL)	Sperm progressive motility (%)	Sperm plasma membrane integrity (%)	No. of live sperm (%)	Sperm viability (%)	Sperm chromatin integrity (%)
0	45.3±1.20c	59.77±1.69c	73.1±1.41b	37.6±1.62c	95.8±0.47b
20	61.3±2.18a	73.0±0.97a	81.5±2.26a	51.9±1.08a	97.3±0.51a
25	49.0±1.0b	70.43±0.93b	72.5±1.60b	41.3±1.05b	97.7±0.08a
30	39.7±1.76d	59.17±1.8c	62.7±2.33c	36.0±1.90c	97.5±0.50a

Abbreviations: AA, arachidic acid
Values having dissimilar superscripts within the same column have significant difference ($p < 0.05$). The total no. of semen ejaculates were thirty (5 replicates per each of three bulls; 2 ejaculates per replication).

Table II. Effect of AA supplementation (20.0 ng/mL) in extender on the *in vivo* fertility rate of cryopreserved buffalo semen (N=533).

Bull No.	Dose detail (Extender supplementation)	No. of inseminations recorded	Pregnancies achieved (%)	Chi-square value	P-value
01	Control	98	46 (46.9)	1.864	0.172^{NS}
	AA	97	56 (57.7)		
02	Control	98	45 (45.9)	3.22	0.072^{NS}
	AA	97	58 (59.8)		
03	Control	71	32 (45.1)	2.015	0.155^{NS}
	AA	72	42 (58.3)		
Overall	Control	267	123 (46.06)	7.955	0.004^{S}
	AA	266	156 (58.64)		

Abbreviations: AA, arachidic acid; NS, non significant; S, significant.

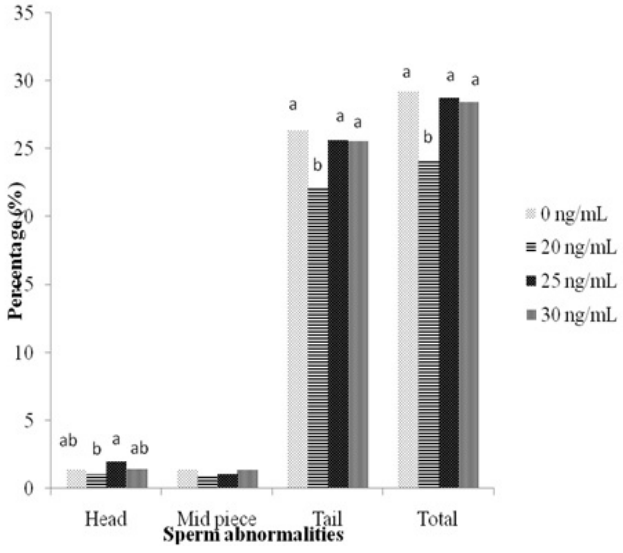

Fig. 1. Effect of arachidic acid addition in extender on abnormalities of cryopreserved buffalo sperm. Total number of ejaculates were thirty (5 replicates per each three bulls; 2 ejaculates per replication). Bars with dissimilar letters differ significantly.

DISCUSSION

Membrane phospholipids, depending on their fatty acid composition, are important for the permeability and functionality of the cell (Garcia *et al*., 2011). The saturated fatty acids (SFAs) are reported to increase the rigidity of the membrane while PUFAs make sperm membrane more flexible and fluid (Lenzi *et al*., 1996; Flesch and Gazella, 2000). Consequently, ratio of unsaturated to SFAs affects sperm sensitivity to thermal shock and species with high such level are more vulnerable for cold shock (White, 1993) than those with lower ratio. The greater PUFAs level in sperm plasma membrane shows its greater vulnerability to oxidative stress, particularly lipid peroxidation through ROS (Cocchia *et al*., 2011). Buffalo sperm having a high content of PUFAs in their membrane (Cheshmedjieva and Dimov, 1994; Parks *et al*., 1987) are more vulnerable to lipid peroxidation, one of the processes liable to negative biochemical and physiological changes during cryopreservation (Cerolini *et al*., 2001). Dietary supplementation of lipids have a positive effect on semen quality in cattle (Gholami *et al*., 2010) and buffalo (Adeel *et al*., 2009). AA is naturally present in fish oil

(Goransson, 2008) and supplementation of fish oil in diet improved semen quality in goat (Dolatpanah *et al*., 2008), sheep (Samadian *et al*., 2010), boar (Rooke *et al*., 2001) and bull (Gholami *et al*., 2010). Hossain *et al*. (2007) reported that supplementation of oleic and linoleic acid to extender significantly enhanced the motility and viability of boar spermatozoa. In the present study, supplementation of AA (20 ng/mL) in extender resulted in improved sperm motility, viability, plasmalemma integrity and liveability (P<0.05) compared to control. The AA might have effectively incorporated into spermatozoal lipids as has previously been reported with bull sperm (Neil and Master, 1972) or it may provided as energy substrate for sperm (Lahnsteiner *et al*., 2009) and stabilized the energy metabolism of spermatozoa. The decrease in semen quality with further increase in AA concentration (>20 ng/mL) might be owing to stiffness of plasma membrane with higher concentration of SFAs.

It has also been observed that sperm tail and overall total abnormalities were reduced in extender containing AA (20ng/mL) compared to control. The successful inclusion of AA in sperm plasma membrane might have prevented the damage occurring due to direct contact between cells during cryopreservation. Earlier study has reported the absorption of fatty acid by sperm membrane specifically at tail (Maldjian *et al*., 2005). Presently, sperm chromatin integrity was equally enhanced in extender having 20 ng/mL, 25 ng/mL and 30ng/mL of AA compared to control. The increased chromatin integrity might be attributed to the unavailability of unsaturated fatty acids for lipid peroxidation due to increased SFAs.

During cryopreservation, the distortion of lipid protein association is believed to enhance influx of calcium ions that can lead to capacitation like changes with shortened life span and reduced fertilization potential of sperm (Watson, 1995; Visconti *et al*., 1999). Fatty acids have a pivotal role in metabolism of lipid, sperm membrane fluidity and fusion with oocyte (Kelso *et al*., 1997). In this study, the *in vivo* fertility (overall) of post thaw buffalo semen was improved in extender (P <0.05) provided with 20.0 ng/mL of AA (58.64%) compared to control (46.06%). The addition of fatty acid in extender might have countered peroxidation, stabilized membranes and reduced calcium uptake that resulted in successful transport of frozen thawed sperm and their improved life span in female tract. Similar to our study, improvement in conception rate has also been reported in ram when semen was supplemented with fish oil (Abd El-Razek *et al*., 2009; Sallam, 1999; El-Sharawy, 2005).

CONCLUSION

In conclusion, the higher concentration of AA deteriorated quality of cryopreserved buffalo semen. However, addition of AA in extender at 20 ng/mL significantly improved *in vivo* fertility of cryopreserved buffalo *(Bubalus bubalis)* sperm (P<0.05).

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Statement of conflict of interest

The authors have no conflict of interest.

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