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

Rabea Ejaz1*, Saima Qadeer2, Muhammad Sajjad Ansari3, Bushra Allah Rakha4, Shazia Shamas5, Iram Maqsood1, Asma Ul Husna6, Asima Azam1 and Shamim Akhter6

1Department of Zoology, Shaheed Benazir Bhutto Women University, Peshawar - 25000, Pakistan,
2University of Education, Jauharabad Campus, Jauharabad-41200, Pakistan
3Division of Science and Technology, University of Education, Lahore-54000, Pakistan
4Department of Wildlife Management, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi-46300, Pakistan
5Department of Zoology, University of Gujrat, Gujrat-50700, Pakistan
6Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi-46300, Pakistan

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 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...
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 % 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 semen collection, the ejaculates were quickly transported to laboratory for assessment of concentration, motility and viability. The qualified ejaculates (>0.5 billion ⁄ mL concentration; Qadeer ACCUCELL. The qualified ejaculates (>60% motility, >1 s sperm was evaluated by bovine photometer (IMV, France) at 37°C (BX20) at X 400 (Tokyo, Japan; Olympus BX20) at 37°C (Akhter et al., 2013).

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 spermatzoa 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., 2014).

**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., 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 samples were divided in aliquots and placed in water bath (37°C) for 15 minutes. Each sample was placed in extender containing 20 ng/mL of AA compared to control. Sperm tail and overall abnormalities remained similar in all extenders having AA compared to control, however, the mid piece abnormalities were recorded in experimental extender containing 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%).

**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 containing 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 Table II. 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).

**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.

![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).](image-url)
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                      | 70.4±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            | 0.172NS |
|          | AA                                     | 97                            | 56 (57.7)                |                  |         |
| 02       | Control                                | 98                            | 45 (45.9)                | 3.22             | 0.072NS |
|          | AA                                     | 97                            | 58 (59.8)                |                  |         |
| 03       | Control                                | 71                            | 32 (45.1)                | 2.015            | 0.155NS |
|          | AA                                     | 72                            | 42 (58.3)                |                  |         |
| Overall  | Control                                | 267                           | 123 (46.06)              | 7.955            | 0.004a  |
|          | AA                                     | 266                           | 156 (58.64)              |                  |         |

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

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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