Assessment Of Alpha Lipoic Acid Inclusion In Semen Extender On Cryopreservation Of Nili-Ravi Buffalo Bull Spermatozoa

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Abstract: The current investigation was carried out to clarify whether the addition of antioxidant Alpha Lipoic Acid (ALA) increases the quality of cryopreserved Nili Ravi buffalo semen. Semen from five healthy Nili-Ravi buffalo bulls was collected by artificial vagina and subjected to the different inclusion levels of ALA @ 0.5mM, 1.mM, 2.mM, 3.mM, and 4.mM respectively. Experiments were executed for Post thawed semen analysis including spermatozoa motility, viability, plasma membrane integrity and acrosomal integrity. Our result indicated that Spermatozoa motility and viability was significantly higher ($P < 0.05$) at lower amount of alpha lipoic acid at 0.5 and 1.mM whereas the spermatozoa acrosomal integrity was significantly higher ($P < 0.05$) at 0.5mM. On the other hand, the lowest spermatozoa acrosomal integrity was observed with increasing concentration of alpha lipoic acid at 2.0, 3.0 and 4.mM. Our result further demonstrated that plasma membrane integrity was higher at 0.5 mM ($P < 0.05$). Based on the finding of current study, it is evident that the Antioxidant Alpha Lipoic Acid could be used for enhancing quality of the post thawed buffalo spermatozoa at lower concentration (0.5mM).
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1. Introduction

Enhanced buffalo production through assisted reproductive technique such as artificial insemination could boost considerably the economy and living standard of many rural communities throughout the world. Enormous amount of resources and researches have been directed to explore the potential of buffalo productivity for meeting the emerging demand for meat, milk and work in the developing countries. Artificial insemination (AI), the assisted reproductive technique has not yet extensively utilized in buffalo on large scale due to poor freezability of buffalo bull spermatozoa (Kumaresan et al., 2005), in spite of the fact that A.I with frozen-thawed spermatozoa was introduced and applied in most of the developing countries more than three decades ago (Anzar et al., 2003; Andrabi et al., 2008). Furthermore, another hindrance for enhancement of reproductive efficiency in buffalo is due to much shorter life span of cryopreserved spermatozoa and lower fertility than the fresh spermatozoa when compared with cattle. It has been investigated that rate of buffalo breeding through artificial insemination (14.1%) is lowered in comparison with natural breeding (Younas et al., 2009). The cryopreserved semen has been resulted in lower fertility rate (33.0%) in buffalo (Bhosrekar et al.,2001).

Since semen freezing and thawing process of cryopreservation caused 50 % damages in buffalo spermatozoa (Watson, 2000), adversely affecting the functional charteristics of spermatozoa such as motility, acrosomal and chromatin integrity (Rasul et al., 2001; Mahmood and Ijaz A 2006; Khan and Ijaz 2007, 2008). Earlier investigation have demonstrated that buffalo spermatozoa has been be more prone to oxidative stress-induced damages (Raizada et al., 1990) because of holding more poly unsaturated fatty acids like arachidonic and decosahexaenoic acids in plasma membrane (Nair et al.,2006; Alvarez and Storey 1992) and low concentrations of scavenging enzymes in their cytoplasm (Aitken and Fisher 1994). Oxidative stress induced by high level of Reactive oxygen species and free radicals is a potential factor associated with decline of sperm motility and fertility during semen storage and adversely impaired the functional sperm charteristics (Bilodeau et al.,2000; Khalifa and El-Saidy 2006). Hence addition of potent antioxidant is essential for sperm analysis and incubation in assisted reproductive technique (Bansal and Bilaspuri 2010) in reducing the damaging effects of oxidative stress during cryopreservation, thus improving the quality of preserved semen (Bilodeau et al.,2001) and enhancing the reproductive efficiency of buffalo bulls used in AI.

It is well known that alpha lipoic acid (α-LA) is a short-chain fatty acid that acts as a cofactor of enzymes involved in mitochondrial respiration (Lovell et al., 2003). Exploration of the beneficial antioxidative properties of α-lipoic acid is an important and interested research area for many researchers and recently its positive antioxidative role has been established in various mammalian body parts such as the brain (Piotrowski et al.,2001), kidney(Mervaala et al.,2003) and heart (Midaoui 2003).

Recent studies carried out in rat have established the crucial role of alpha lipoic acid regarding the inhibition of lipid oxidation in the polyunsaturated fatty acids in adult rat sertoli cells (Hamdy et al., 2009) and enhancing functional charteristics of rat sperm (Selvakumar et al., 2006). Alpha lipoic acid is able to enter the Krebs cycle and associated with the production of ATP. The essentiality of ALA in biological system has been confirmed for energy production (Long et al., 2009) which is vital for sperm functional charteristics (Ibrahim et al., 2008). Recently enrichment of bull spermatozoa motility with alpha lipoic acid has been reported (Ibrahim et al., 2011) whereas protective function of alpha lipoic acid on sperm motility and mitochondrial function during goat sperm-mediated gene transfer has been confirmed (Huiming et al., 2011). Also the effect of Alpha lipoic on cattle sperm kinetics has been evaluated using computer assisted semen analysis (Osman et al., 2012). Additionally, its role in Boer buck ((Ibrahim et al., 2008) and stallion (Hussain et al., 2011) semen has been tested. On the other hand, the potential effect of ALA on frozen-thawed buffalo spermatozoa has not been demonstrated.

Accordingly, here we attempted to elucidate the significance of Alpha lipoic acid inclusion in semen extender, on Nili-Ravi buffalo bull spermatozoa quality parameters including post-thawed spermatozoa motility, viability, plasma membrane integrity and acrosomal integrity.

2. Material and Methods

The semen samples were collected from Five (Nili-Ravi) buffalo bulls, maintained at the Semen Production Unit, Qadirabad, Sahiwal, Pakistan. The experimental bulls were nurtured in clean and hygienic environment. The breeding bulls were fed seasonal fodder at 10 % of the body weight along with 2-3 kg concentrate on the daily basis and had free access to drinking water during the study period. All the bulls were clinically sound and were donating semen of acceptable quality for artificial insemination.

Semen from all the experimental bulls were collected with the help of artificial vagina maintained at 42°C (Andrabi et al., 2008). Two consecutive ejaculates were collected from each bull at weekly interval for 5 weeks. Immediately after collection, the ejaculates were shifted to a water bath at 37ºC for 15 minutes and subjected to gross examination such as volume, color, pH and microscopic assessment including estimation of progressive motility, percentage motility and morphologically normal sperm. Semen samples having more than 75% motility were selected for further processing. After evaluation, the semen which best fitted the criteria (75-90% motility) was pooled and extended in Egg Yolk Citrate extender which was prepared as described earlier (Khan and Ijaz 2007). Briefly Tris-Hcl; 24.20g, Citric acid; 13.40g, Fructose; 10g, Glycerol; 70ml, Egg Yolk; 200ml, Streptomycin; 1g, Benzyl Penicillin; 500,000IU, Distilled Water up to 1000ml) at 37°Cwithin 10 minutes after collection.

Different inclusion levels (0.00, 0.50, 1.00, 2.00, 3.00, 4.00mM) of ALA were used. For this purpose, a stock solution (206mM) of ALA was prepared by dissolving 0.856g of ALA and 0.161g NaOH in 20ml distilled water. For the above mentioned inclusion levels, 0.00µl (0.00mM/control), 73µl (0.50mM), 146µl (1.00mM), 292µl (2.00mM), 438µl (3.00mM), 584µl (4.00mM) of this solution was added to 30mL of extended semen. These semen samples were then kept in water bath at 37°C for 5 minutes to allow the uptake of ALA by the spermatozoa.

In order to cool the semen from 37°C to 4°C gradually, the test tubes were shifted from water bath to cold cabinet (4°C) and were kept there for 4hrs. During adjusting the temperature to 4°C, the semen acquired equilibration, after which straws (0.50ml) were filled with semen, sealed and stored in liquid nitrogen at -196°C for evaluation. The spermatozoa concentration was adjusted to 40×10^6spermatozoa per 0.5ml.The following characteristics were evaluated to assess the effect of ALA for the cryopreservation.

Post-thawed motility of frozen semen straws was evaluated just after thawing as earlier described (Hasan et al.,2001).Thawing of frozen semen straws was carried out at 37°C for 30 seconds in a hot-water bath. Percentage spermatozoa motility rate was assessed following the standard procedure (Ijaz et al.,2009) at X400 under a phase-contrast microscope (Labomed Lx 400,U.S.A).

Assessment of spermatozoa viability was carried out by standard protocol as recently described

(Mughal et al.,2013).Briefly, 3% solution of sodium citrate was prepared by dissolving 3g of sodium citrate in 100ml distilled water. This solution was divided into two equal halves. To one half, 1g eosin (Merck, Germany) and to the other half, 5g nigrosin (Merck, Germany) was added. These solutions were incubated at 60ºC for 25 minutes. A thin smear of thawed semen and Eosin and Nigrosin was prepared on a microscopic slide and the viability was assessed by counting 100 spermatozoa per slide under a phase contrast microscope (X1000).Sperm demonstrating fractional or thorough purple staining were measured non-viable whereas only sperm showing strict absence of stain was considered to be viable (Balestri *et al.*, 2007). Heads of live spermatozoa remained unstained, while stained or partially stained heads of the spermatozoa were considered as dead spermatozoa. The viability was assessed by counting 100 cells (per slide) under a microscope. The percentage of live spermatozoa was estimated by counting a minimum of 200 spermatozoa on the slide.

For acrosomal integrity, the method of (Jankovicova et al., 2006) was used with slight modification. A 0.2% trypan blue solution was prepared by dissolving 0.2g of trypan blue to 100ml distilled water. An equal quantity of thawed semen and trypan blue was placed and smeared on a pre-warmed slide and fixed for 2 min with the fixative solution containing 86ml of 1N HCl and 14ml of 37% formaldehyde. The spermatozoa (200 in number) were evaluated for their normal apical ridge using a phase-contrast microscope at X1000 (Khan and Ijaz 2007). The intact acrosomes were light colored while the damaged acrosomes were violet stained.

Plasma membrane integrity was assessed using the hypoosmotic swelling (HOS) test as described in the recent past (Ijaz et al.,2009; Adeel et al., 2009). Observation was made under a phase contrast microscope (X400) and two hundred spermatozoa were counted for swelling/coiling of tail.

Statistical analysis was conducted with the Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA). The data is presented as mean ± SE. The Kolmogorov Smirnov test was employed to test the normal distribution of the data. The data was analyzed using one-way analysis of variance. The group differences were compared by the Duncan's Multiple Range Test. The differences were considered significant at $P < 0.05$.

3. Results

Table-1 shows the percentage of sperm motility, viability, acrosome integrity and spermatozoa plasma membrane integrity of buffalo bull semen with various alpha Lipoic acid inclusions. Our result indicated that Spermatozoa motility and viability was significantly higher ($P < 0.05$) at lower amount of alpha Lipoic acid at 0.5 and 1.0mM whereas significant decreases were demonstrated in the spermatozoa motility at higher concentration of alpha Lipoic acid in the treated semen. The spermatozoa acrosomal integrity was significantly higher ($P < 0.05$) at 0.5mM. On the other hand the lowest spermatozoa acrosomal integrity was also observed with increasing concentration at 2.0,3.0 and 4.00mM treated semen samples. Our result further demonstrated that plasma membrane integrity was higher at 0.50 mM ($P < 0.05$). Conversely, the Plasma membrane integrity remains significantly lower with increasing concentration of Alpha Lipoic Acid in the treated semen.

Table-1 Post-thaw spermatozoa characteristics under various concentrations of Alpha Lipoic Acid in Egg yolk citrate extender

con	Post-thaw spermatozoa characteristic (%)			
	M	V	AI	PMI
0.0	25±1.63	67.88±2.45	59±3.24	73.63±1.91
0.5	35.63±1.99a	69.5±3.45a	64±2.58a	92.38±2.29a
1.0	33.75±1.25a	65.25±3.15ab	43±2.41b	88.25±2.87bc
2.0	10.625±0.63c	61.12±1.8ba	31±2.58c	83.25±1.86b
3.0	10.25±0.25c	58.40±3.69c	29±4.01c	81.75±2.96ab
4.0	5.0±0.2673d	49.38±2.06d	28±1.23c	77.25±1.27bc

(con, concentration of Alpha lipoic acid; M, Motilit; V, Viability; AI', Acrosomal Integrity; PMI,Plasma Membrane Integrity) Values are represented as Mean ± S.E. Different letters (a-d) within the same column indicate significant differences ($P<0.05$) among the groups

4. Discussions

To the best of our knowledge, it is the first report to elucidate the significance of ALA inclusion in Nili-Ravi buffalo bull semen. It has been well established that assessment of functional charteristics of sperm is indispensable to the effectiveness of the cryopreservation methods in maintaining sperm motility or viability and the potential fertilizing capacity of the processed semen for A.I. Also, the higher post-thaw motility along with entire efficient integrity of diverse apparatus of spermatozoa is considered an enviable feature to assess the fertilizing ability of bovine semen. Our results indicated that spermatozoa motility was higher ($P < 0.05$) in semen treated with 0.50 and 1.00mM ALA. The current findings demonstrated that ALA has the ability to increase the motility of post-thawed semen that is in accordance with the recent studies carried out in goat. In later study, increased motility of the frozen–thawed goat sperm has been established with Alpha Lipoic Acid supplementation (Huiming et al., 2011). Similarly, a study carried out in rats, also demonstrated the protective efficacy of Alpha Lipoic Acid on the sperm characteristics, thus improving semen quality (Selvakumar et al., 2006). The protective influence of alpha Lipoic acid against reproductive dysfunction in male rats has been recently confirmed (Azza et al., 2012). Our results that the response of Alpha Lipoic Acid was high at low concentration were in accordance with finding of Ibrahim et al., 2008 who demonstrated the enhancement of sperm motility of cryopreserved Boer buck sperm at lower concentration of Alpha Lipoic Acid.

Since it well established that structurally the spermatozoa have a cap over the anterior end, surrounded internally and externally by an acrosomal membrane. Healthy spermatozoa must retain this membrane throughout freeze-thaw process and start acrosomal reaction in female tract (Therien and Manjunath 2003). The presence of normal acrosomal cap is highly correlated with the fertility of the frozen bull semen and is important in the fertilization process. The spermatozoa with damaged acrosome may be motile and viable, but may not be able to fertilize an ovum (Graham 2001). Damage to the acrosomes is due to the greater release of acrosomal enzymes, hyaluronidase (Akhtar and Chaudhry 1989) and acrosome (Chinnaiya GP, Ganguli 1980), which occurs during and after freezing and thawing in bovine bull semen. In our current investigation, significant difference for acrosomal integrity was recorded in semen samples treated with 0.50mM ALA as compared with semen samples treated with 1.00, 2.00, 3.00 and 4.00mM ALA. It indicated that at 0.50mM, ALA has positive effect on NAR and confirmed the previous investigation carried on other antioxidants like glutathione and ascorbic acid that were only beneficial at lower concentration on the acrosomal integrity (Abdel-Khalek et al.,2009).

Review of literature indicated that an intact and functional plasma membrane is key component of the cell and must be maintained in the freezing condition if the cell is to be kept alive (Marti et al., 2003). Correspondingly, plasma membrane and acrosomal integrity have been positively correlated with fertility in Bovine (Saake RG and White 1972) thus the availability of morphologically normal motile and healthy spermatozoa at the site of fertilization would enhance the efficiency of Artificial insemination in buffalo. The results of our study indicated that the number of HOST+ve spermatozoa at 0.50mM was greater in comparison with higher concentration of 1.00, 2.00 and 4.00mM ALA. These findings suggested that the presence of ALA in the semen extender, at low concentration can maintain the membrane integrity of spermatozoa in Nili- Ravi buffalo bull semen.

In conclusion, assessment of various sperm parameters of the current study demonstrated that Alpha Lipoic Acid inclusion in semen extender improved the post-thaw quality parameters of cryopreserved Nili-Ravi buffalo bull spermatozoa. Among the Alpha Lipoic Acid concentrations evaluated, maximum improvement in post-thaw semen quality parameters was observed at 0.50mM. Hence Alpha Lipoic Acid could be used as stabilizer in the semen extender or Cryoprotectant agent to improve sperm functional charteristics at low concentration and could definitely contribute to increase reproductive efficiency of buffalo bull used in artificial insemination.

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