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RESEARCH ARTICLE

Effect of Addition of Different Concentrations of Alpha Lipoic Acid to Tris Egg Yolk Citrate Glycerol Extender on Cryopreservation of Sahiwal Bull Spermatozoa

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ABSTRACT

Aim of the present study was to monitor pre-freeze and post-thaw semen quality of Sahiwal bulls after addition of different concentrations of alpha lipoic acid (ALA) to the extender. Semen of four Sahiwal bulls was collected two times per week for four weeks with an artificial vagina. Ejaculates collected on each collection day from four bulls were pooled, split into four equal parts and diluted with tris-egg yolk-citrate-glycerol extender containing 0.500, 0.250, 0.125 and 0.000 mM (control) ALA. Diluted samples were filled in 0.5 ml straws, frozen and stored for at least 24 hours in liquid nitrogen. Pre-freeze and post-thaw quality was assessed in terms of sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity. Results indicated higher ($P<0.05$) mean values for pre-freeze and post-thaw sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity for samples diluted in extender having 0.250 and 0.125 mM ALA compared to samples diluted in extender containing 0.500 mM ALA and control extender. Moreover, higher mean values ($P<0.05$) of these characteristics were observed in samples diluted in extender containing 0.250 mM ALA compared to those diluted in extender containing 0.125 mM ALA. However, samples diluted with 0.500 mM ALA showed lower ($P<0.05$) pre-freeze and post-thaw semen quality than control samples. It was concluded that ALA at a concentration of 0.250 mM significantly improved pre-freeze and post-thaw characteristics of Sahiwal bull semen. However, field trials regarding effects of adding different concentrations of ALA to semen extender on fertility of frozen-thawed semen are necessary before making any recommendations in this regard.

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INTRODUCTION

Artificial insemination is a useful technique for rapid genetic improvement of livestock through exploiting genetic potential of elite sires. This technique not only improves the reproductive performance but also plays a major role in controlling venereal diseases in the herd (Holt, 2000). However, for harvesting maximum benefits from this technique, it is necessary that spermatozoa are stored for long time without damaging their fertilizing ability to ensure their availability as and when required.

Deep freezing of semen in liquid nitrogen is the method of choice for its long term storage. However, it favors the release of reactive oxygen species (ROS), which induces oxidative stress and adversely affects functional integrity of spermatozoa (Bilodeau *et al.*, 2000). Natural defense mechanism of spermatozoa against oxidative stress induced by ROS formation may be altered during the process of deep freezing (Bilodeau *et al.*, 2000; Gadea *et al.*, 2004). These harmful effects of oxidative stress on spermatozoa during freezing can be minimized by supplementation of semen extender with some suitable antioxidants like vitamin E (Soltanpour *et al.*, 2014), cysteine, superoxide dismutase (Partyka *et al.*, 2013), glutathione peroxidase (Asadpour *et al.*, 2012) and alpha lipoic acid (Pindaru and Groza, 2015).

Alpha lipoic acid (ALA), which is a short chain fatty acid, is universally considered as a potent antioxidant due to its antioxidative effect in oxidized and reduced form (Goraca *et al.*, 2011; Rochette *et al.*, 2013). Moreover, ALA is considered as a weak acid and its addition to semen extender does not affect the seminal pH.

Addition of ALA to the extender has been shown to improve low quality semen collected from sub-fertile bulls (Ibrahim *et al.*, 2011). According to Gohar *et al.* (2014), semen ejaculates collected from Nili-Ravi buffalo bulls were diluted using extender supplemented with 0.5, 1.0, 2.0, 3.0 and 4.0 mM concentrations of ALA. Post-thaw evaluation of spermatozoa revealed that lower concentrations of ALA (0.5 and 1.0 mM) improved semen quality in terms of sperm motility and viability. Similarly, improvement in semen quality following addition of ALA to semen extender has been reported in goats (Huiming *et al.*, 2011), Holstein Friesian bulls (Buyukleblebici *et al.*, 2015), Limousin bulls (Osman *et al.*, 2012) and boars (Pindaru and Groza, 2015). However, there is relatively little information regarding the potential role of ALA in improving freezability of Sahiwal bull spermatozoa. Therefore, the current study was designed to monitor pre-freeze and post-thaw characteristics of Sahiwal bull semen after addition of different concentrations of ALA to tris-egg yolk-citrate-glycerol extender. Attempts were also made to determine optimum concentration of ALA which can be used to improve cryopreservation of Sahiwal bull semen.

MATERIALS AND METHODS

Collection of semen: A total of four adult and clinically healthy Sahiwal bulls, routinely used for semen collection at the Semen Production Unit, Harichand, district Charsadda, Pakistan, were used for the present study. From each bull, collection of semen was carried out through artificial vagina twice a week for four weeks, with two ejaculates per collection. Following collection, ejaculates were placed at 37ºC and examined for motility of spermatozoa. Ejaculates showing at least 60% progressively motile spermatozoa were used in the study. Selected ejaculates taken from four bulls on each collection day were pooled and split into four equal parts. Semen samples of first three parts were diluted in tris-citrate-egg yolk-glycerol extender supplemented with 0.500, 0.250 and 0.125mM alpha lipoic acid (ALA), respectively, while samples of the fourth part were diluted using the same extender without ALA and served as untreated control.

Preparation of extender: In this study, tris-citrate-egg yolk-glycerol extender was supplemented with three concentrations of ALA. For this purpose, a stock solution (307mM) of alpha lipoic acid (Sigma-Aldrich, 99%) was prepared by dissolving 1.03g of ALA in 5ml of distilled water. Further solubility was achieved with titration through sodium hydroxide (NaOH; Merck). Solution of NaOH was prepared by dissolving 0.51g of NaOH in 10ml of distilled water. Then 40, 20 and 10 µl of this solution were added to 30 ml of extender to achieve 0.500, 0.250 and 0.125 mM concentration of ALA, respectively.

Dilution and freezing of semen: Semen samples were diluted at 37°C with extender containing the respective concentration of ALA in such a way that each 0.5 ml of diluted semen contained 30 million spermatozoa. Diluted samples were left at 25°C for 20 minutes to allow uptake of ALA by spermatozoa and then evaluated for pre-freeze characteristics.

These semen samples were then filled in 0.5 ml straws and kept for an equilibration period of four hours at 4°C (Shahverdi *et al.*, 2014). Freezing of straws filled with semen was carried out by holding them in vapors of liquid nitrogen (LN), about 5 cm above surface of LN, for 10 minutes. Then they were dipped into LN and stored for at least 24 hours.

Pre-freeze and post-thaw semen evaluation: Before evaluation, thawing of frozen semen straws was carried out by holding them at 37°C for 30 seconds. Before and after freezing, semen samples were evaluated for progressive sperm motility, acrosome integrity, live sperm percentage and plasma membrane integrity of spermatozoa. Progressive sperm motility was estimated by placing a small drop of semen on a warm glass slide, covering it with a cover slip and examining under a bright field microscope (40X). At least three fields for each slide were examined for percentage of progressively motile sperm and their mean value was taken as a single data point.

Hypo-osmotic Swelling Test (HOST), described by Ramu and Jeyendran (2013), was applied for the assessment of integrity of sperm plasma membrane. Briefly, hypo-osmotic solution (500 µL) having osmotic pressure of 150 mOsm/L and semen sample (50 µL) were incubated at 37°C for 45 minutes. After incubation, a small drop was placed on a clean glass slide and examined using bright field microscopy (400x). At least 200 sperm were examined for each sample and percentage of sperm with intact plasma membrane, indicated by swollen/coiled tail, was computed. Mean of three values was taken as a single data point.

Live sperm percentage in each sample was estimated by mixing 20 µL of semen with 20 µL of eosin nigrosine stain, as described by Mughal *et al.* (2013). Later, a small drop of this mixture was used to make a thin smear on a glass slide; the smear was air-dried and examined under a bright field microscope. Dead spermatozoa were characterized by having partially or completely stained heads, while those with unstained heads were considered as live. At least 200 spermatozoa were examined for each slide to compute percentage of live sperm. Three slides were examined for each sample and their mean value was taken as a single value for statistical analysis.

Determination of sperm acrosome integrity was carried out by fixing semen sample (500 µL) in 50 µL of 1% formal solution in 2.9% (w/v) tri-sodium citrate dihydrate. At least 200 sperm cells were counted using bright field microscopy and percentage of spermatozoa with normal acrosome ridge (NAR) was computed.

Statistical analysis: Mean±SEM values of various parameters of pre-freeze and post-thaw semen quality for different groups were computed. In order to study the magnitude of variation among different extenders, the data were subjected to analysis of variance in General Linear Model Procedure, using Minitab Statistical Software Computer Package. Comparison among different means was done through Duncan's Multiple Range Test. Pearson's correlation coefficients were also computed among various post-thaw semen quality parameters.

RESULTS

Pre-freeze semen quality: Pre-freezing sperm motility was the highest for samples diluted with extender containing 0.250 mM ALA, while the lowest sperm motility before freezing was recorded in samples diluted with extender containing 0.500 mM ALA, the difference was significant (P<0.05). Further analysis revealed that pre-freeze sperm motility differed significantly among all four extenders (P<0.05), the highest value was for extender having 0.250 mM ALA, followed by extender supplemented with 0.125 mM ALA and the control group, while lowest value was noted in samples diluted with extender containing 0.500 mM ALA (Table 1).

Pre-freeze sperm plasma membrane integrity showed a trend similar to that of sperm motility. Samples diluted with extender supplemented with 0.250 and 0.125 mM ALA showed higher pre-freeze plasma membrane integrity compared to controls. Moreover, control samples also showed higher pre-freeze plasma membrane integrity than those diluted with extender having 0.500 mM ALA. The same was true for pre-freeze live sperm percentage and acrosome integrity of spermatozoa.

Post-thaw semen quality: Mean values for post-thaw sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity in semen samples diluted with extenders having 0.250 and 0.125 mM ALA were higher (P<0.05) compared to those diluted in control extender. Moreover, samples diluted with extender containing 0.250 mM ALA also showed significantly higher mean values of these characteristics than those diluted with extender supplemented with 0.125 mM ALA (Table 2). However, samples diluted with 0.500 mM ALA showed lower post-thaw sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity than control samples (P<0.05). Thus, the highest values for all these parameters of post-thaw semen quality were recorded for samples diluted with extender supplemented with 0.250 mM ALA, while the lowest values were observed for samples diluted using extender containing 0.500 mM ALA.

Correlation matrix: Values for correlation coefficients among sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity post-thaw are shown in Table 3. It shows that correlation coefficients among all four post-thaw sperm quality characteristics were highly significant (P<0.01).

DISCUSSION

The main objective of this experiment was to monitor antioxidant efficacy of three levels of alpha lipoic acid (ALA) during dilution and freezing of Sahiwal bull semen. It is well known that during processing of semen, osmotic and thermal shock results in the release of ROS. The latter are described as reactive molecules released from molecular oxygen during aerobic respiration when mitochondrial electron transport takes place. These molecules are also released during metal-catalyzed oxidation and by oxido-reductase enzymes. These free molecules can cause deleterious effects on cell signaling. Presence of a small number of ROS is necessary for physiological functions of sperm cells and any excess of ROS within sperm is normally neutralized by the antioxidants, like catalase, present in the semen. However, in case of any disruption between production and neutralization of these ROS, there is excess of ROS which can lead to oxidative stress and adversely affect fluidity of plasma membrane and DNA integrity of sperm (Aitkin, 1999; Hussain *et al.*, 2011). These changes can affect the quality and subsequent fertility of frozen-thawed semen.

In the present study, it was assumed that addition of some antioxidant, like ALA, to semen extender would minimize such adverse effects of oxidative stress on post-thaw semen quality parameters. In order to verify this assumption, three concentrations of ALA (0.500, 0.250 and 0.125 mM) were used to supplement tris-egg yolk-citrate-glycerol extender routinely used for dilution of semen at a semen production unit. The results showed that supplementation of 0.250 and 0.125 mM ALA to semen extender significantly improved pre-freeze, as well as post-thaw, sperm motility, plasma membrane integrity, live sperm percentage and acrosome integrity compared to control samples diluted without ALA. Gohar *et al.* (2014) also reported an improvement in the post-thaw sperm motility and viability when Nili-Ravi buffalo bull semen was diluted with extender containing 0.5 and 1.0 mM ALA and stored in frozen form. However, Hussain *et al.* (2011) did not observe any improvement in post-thaw sperm quality parameters when they used different concentrations of ALA in extender for deep freezing of stallion semen. This could have been due to species differences.

Table 1: Effect of addition of different concentrations of alpha lipoic acid (ALA) to extender on pre-freezing values of various parameters of semen quality

Concentration of ALA (mM)	Sperm motility (%)	Sperm plasma membrane integrity (%)	Live sperm (%)	Sperm acrosome integrity (%)
0.500	31.75d	40.83d	39.07d	34.64d
0.250	71.25a	85.90a	87.14a	85.07a
0.125	60.63b	72.70b	79.05b	76.95b
Control	54.06c	66.85c	67.67c	67.39c
SEM	0.78	0.90	1.08	1.11

Mean values (±SEM) bearing different letters within a column are significantly different from one another (P<0.05).

Table 2: Effect of addition of different concentrations of alpha lipoic acid (ALA) to extender on post-thaw values of various parameters of semen quality

Concentration of ALA (mM)	Sperm motility (%)	Sperm plasma membrane integrity (%)	Live sperm (%)	Sperm acrosome integrity (%)
0.500	22.81d	31.56d	22.84d	19.66d
0.250	61.25a	71.50a	84.20a	84.44a
0.125	51.44b	63.72b	72.02b	66.91b
Control	40.94c	49.31c	65.81c	58.94c
SEM	0.78	0.90	1.08	1.11

Mean values (±SEM) bearing different letters within a column are significantly different from one another (P<0.05).

Table 3: Correlation matrix among post-thaw characteristics of semen of Sahiwal bulls

	Sperm motility (%)	Sperm plasma membrane integrity (%)	Live sperm (%)	Sperm acrosome integrity (%)
Sperm motility (%)	1.00			
Sperm plasma membrane integrity (%)	0.988**	1.00		
Live sperm (%)	0.948**	0.933**	1.00	
Sperm acrosome integrity (%)	0.951**	0.939**	0.968**	1.00

**Correlations are significant (P<0.01).

In the present study, addition of 0.250 and 0.125 mM ALA to semen extender improved pre-freeze semen quality parameters compared to control samples. This indicates that ALA exerted its beneficial effects even before freezing process. Similarly, Pindaru and Groza (2015) recorded a significant (<0.05) increase in percentage of sperm with normal plasma membrane, as well as in percentage of live sperm, following dilution of boar semen in diluent supplemented with different concentrations of ALA and stored in liquid form at 17ºC for up to five days. This shows that addition of ALA to semen extender has beneficial effects in terms of semen quality parameters, no matter whether the semen is to be stored for short term in liquid form or for long term in frozen form.

The improvement in the pre-freeze and post-thaw semen quality parameters in samples diluted with 0.250 and 0.125 mM ALA recorded in the present study can be attributed to the antioxidative effects of ALA. Previous studies have shown that ALA enters the cell mitochondria and acts as a cofactor of mitochondrial respiration enzymes (Arivazhagan *et al.*, 2001). Alpha lipoic acid also enters the Krebs cycle, accelerating the respiration rate of spermatozoa, thus helps in the production of ATP, the latter is required for the energy production, which is necessary for sperm motility (Perchec *et al.*, 1995) and viability. Moreover, ALA also facilitates interaction with other antioxidants and enhances their function; addition of ALA helps directly in the increased production of vitamin C, glutathione and coenzyme q10 upto 70% (Kleinkauf *et al.*, 2013). Alpha lipoic acid has been shown to minimize oxidative stress induced by ROS, prevent sperm plasma membrane damage, inhibit lipid peroxidation (Huiming *et al.*, 2011) and protect testosterone synthesis pathway across hypothalamo-pituitary-testicular axis (Othman *et al.*, 2012).

Another aim of this study was to find out optimum concentration of ALA which can be used for improving freezability of Sahiwal bull spermatozoa. For this purpose, three concentrations of ALA (0.500, 0.250 and 0.125 mM) were tested. The results revealed that lower two concentrations of ALA (0.250 and 0.125 mM) significantly improved pre-freeze and post-thaw semen quality parameters compared to controls. Moreover, ALA concentration of 0.250 mM showed better results than those of 0.125 mM. However, higher concentration of ALA (0.500 mM) had adverse effects, as it resulted in significantly lower values for all quality parameters of pre-freeze and post-thaw semen compared to other two concentrations of ALA, as well as controls. This shows that ALA at a concentration of 0.250 mM can be used in the extender for improving post-thaw semen quality of Sahiwal bulls. Gohar *et al.* (2014) and Buyukleblebici *et al.* (2015) reported significant improvement (P<0.05) in post-thaw motility of sperm for samples treated with lower concentration of ALA (0.5 mM) compared to 1.00, 2.00, 3.00 and 4.00 mM added to semen diluter before deep freezing of Nili-Ravi buffalo and Holstein bull semen, respectively. Similarly, Fayyaz *et al.* (2017) observed an increase in the sperm membrane integrity and DNA integrity caused by ALA at concentrations of 0.50 and 1.00 mM. However, these workers did not test ALA concentrations lower than 0.50 mM, as has been used in the present study. According to Huiming *et al.* (2011) and Pindaru and Groza (2015), concentration of ALA as low as 0.10 mM can minimize oxidative stress induced by ROS during preservation of semen in frozen or liquid form, respectively.

Conclusions: ALA at the concentration of 0.250 mM significantly improved pre-freeze and post-thaw characteristics of Sahiwal bull semen. However, field trials regarding effects of adding different concentrations of ALA to semen extender on fertility of frozen-thawed semen are necessary before making any recommendations in this regard.

Authors contribution: IA and HJ conceived the idea; IA, NA and ZIQ finalized experimental plan; SBA conducted experimental work and QA made arrangements for bulls and helped in evaluation of semen. KA helped with data analysis; IA and NA prepared the manuscript, while it was reviewed by all authors.

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