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Short Communication

Effect of Alanine on Post-Thaw Quality of Sahiwal Bull Spermatozoa

Nuzhat Kamal[1], Muhammed Sajjad Ansari[2]*, Bushra Allah Rakha[1], Shamim Akhter[1], Saima Qadeer[1] and Ali Akhter[1]

[1]*Department of Zoology, Wildlife and Fisheries, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan*
[2]*Department of Zoology, Division of Science and Technology, University of Education, College Road, Township, Lahore-54000, Pakistan*

ABSTRACT

This study was designed to evaluate the effect of alanine on post-thaw quality of Sahiwal bull spermatozoa. Qualifying semen ejaculates [motility (>60%) volume (>4ml) and concentration (>0.5 billion/ml)] collected from three Sahiwal bulls were diluted with tris-citric acid extender supplemented with different concentration of alanine (5, 10, 15 and 20mM), extender without alanine was kept as control. Extended semen was cooled to 4°C in 2 h and equilibrated for 4 h at 4°C. After equilibration, cooled semen was filled in 0.5 ml French straw at 4°C and kept over liquid nitrogen vapors for 10 min. Then straws were plunged into liquid nitrogen (-196°C) for storage. After 24 h of storage, straws were thawed at 37°C for 30 seconds and assessed for sperm motility (%), plasma membrane integrity (%), viability (%), normal apical ridge (%) and chromatin integrity (%). Sperm motility was recorded higher in extender containing 15mM alanine compared to control. Sperm viability was recorded higher in extender containing alanine 10, 15, 20mM compared to control. Sperm plasma membrane integrity, normal apical ridge and chromatin integrity remain similar in all experimental extenders. It is concluded that alanine addition in tris-citric acid extender improved the motility and viability of Sahiwal bull spermatozoa.

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NK and SQ performed experimental work and wrote first draft. MSA, BAR and SA designed and executed the experiment. AA, BAR and SA proofread the manuscript.

Key words
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Freeze-thawing of the bovine spermatozoa cause irreversible damage to the sperm bio-membrane system (Parks and Graham, 1992; Anzar *et al*., 2002). These damages reduce the fertilizing ability of the frozen-thawed semen and resulted in poor fertility rates (Bailey *et al*., 2000; Watson, 2000; Ansari *et al*., 2012). Sperm cells have unique physiology having little protection mechanisms mediated by different protectants (Holt, 2000; Batool *et al*., 2012).

A variety of amino acids are naturally present in bovone seminal plasm and have distinct role in sperm membrane protection during freeze-thawing stress (Bhargava *et al*., 1959). Alanine is one of the naturally occurring amino acid which is reported to have cryoprotectant ability for spermatozoon during freezing of ovine (Scanchez *et al*., 1992) caprine (Kundu *et al*., 2001) and human semen (Philippe *et al*., 1996). However, the information on the precise mechanism through which alanine protects the sperm in freezing stress in still not known. It was observed that the addition of amino acids with glycerol improve post-thaw sperm motility in goat (Kundu *et al*., 2001).

Alanine addition in extender improved the preservability of Indian (Murrah), Egyptian and Italian buffalo bull sperm (Singh *et al*., 1990; Dhami and Sahni, 1993; Dhami *et al*., 1994; El-Sheshtawy *et al*., 2008; Del Sorbo *et al*., 1995) in liquid and/or frozen state. However, information on the use of alanine to improve post-thaw quality of Sahiwal bull spermatozoa is lacking. Therefore, present study was designed to identify the effect of alanine in extender on post-thaw semen quality of Sahiwal bull spermatozoa.

Materials and methods

Five experimental extenders were prepared by using *tris*-citric acid as a buffer (pH 7.0; osmotic pressure 320 mOsmol Kg^{-1}) which consisted of 1.56g citric acid and 3.0g *tris*–(hydroxymethyl) amino methane dissolved in 73 mL

* Corresponding author: m.sajjad.ansari@gmail.com
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distilled water. Fructose 0.2% w/v; glycerol 7%; egg yolk 20% v/v, antibiotics, benzyl penicillin (1000 IU mL^{-1}) and streptomycin sulphate (1000 ug mL^{-1}) were added to each of the experimental extenders. Alanine was added at the rate of 5, 10, 15, 20 mM to make experimental extenders. The extender without alanine was kept as control.

Two consecutive ejaculates were collected from three Sahiwal bulls per week with artificial vagina (42°C) for a period of three weeks (replicate). Semen was immediately transferred to laboratory for motility assessment microscopically (X400) at 37ºC. Sperm concentration was assessed by Neubauer haemocytometer. Semen samples with >60% motile spermatozoa were used for further dilution.

Semen aliquots were diluted at 37°C with one of the five experimental extenders at the rate of 50 × $10^6 mL^{-1}$ motile spermatozoa. Diluted semen was cooled to 4°C in 2h and equilibrated for 4 h at 4°C. Semen was filled in 0.5mL French straws with suction pump at 4°C in the cold cabinet unit and straws were exposed to liquid nitrogen vapors for 10min. Straws were then plunged and stored into liquid nitrogen (-196°C). After 24 h, for each experimental extender three semen straws were thawed in a water bath at 37°C for 30 seconds to assess post-thaw semen quality.

A drop of semen was placed on a pre-warmed (37ºC) glass slide and cover slipped. Post-thaw sperm motility (%) was assessed as described in the earlier section.

Sperm plasma membrane integrity (PMI) was assessed by hypo-osmotic swelling assay (HOS) (Qadeer *et al*., 2013). The HOS solution (osmotic pressure approximately 190mOsmol/kg) was prepared by dissolving 0.735g sodium citrate and 1.351g fructose in 100mL distilled water. For the assessment of sperm plasma membrane integrity, 50µL of the semen sample was mixed with 500µL of the pre-warmed HOS solution and was incubated at 37°C for 30-40 min. After incubation a drop of mixture was placed on a glass slide, cover slipped and visualized microscopically (400X magnification). A total of 200 spermatozoa per preparation were observed in at least five different fields. Swollen tails indicated intact, biochemically active sperm membranes, while unswollen tails indicated disrupted, inactive sperm membranes.

Sperm viability was studied by dual staining procedure (Ejaz *et al*., 2014). Equal drops of Trypan-blue and semen were placed on a slide at room temperature and mixed. Smear was air-dried and fixed with formaldehyde-neutral red for 5 min. The slides were rinsed with running distilled water and Giemsa stain (7.5%) was applied for 4 h. The slides were rinsed, air dried and a total of 200 spermatozoa were evaluated in each smear by light microscopy at 1000X magnification. Transparent or light blue sperms with clear acrosome were considered viable being live and having intact acrosome, while sperms having a clear dark blue demarcation and blunt ended acrosome were considered dead and non-viable.

Acrosomal Integrity of sperm was assessed by the presence of normal apical ridge by mixing of 100µL of semen sample with 500µL of formal citrate, which was consisted of 37% commercial formaldehyde (1mL) and 2.9% (w/v) sodium citrate (99mL) (Akhter *et al*. 2010). under phase contrast microscope oil immersion (1000X).

Sperm chromatin integrity during was assessed by toluidine blue assay (Batool *et al*., 2012). Air dried smears of semen samples were fixed in 96% ethanol-acetone (1:1) at 4°C for 30 min, hydrolyzed with 4N HCl at 25°C for 10-30 min. The smears were then suspended in distilled water, three times for two min each. The slides were stained with toluidine blue in Mcllvaine buffer (sodium citrate-phosphate) for 10 min. Chromatin integrity was assessed with light microscopy at 1000X magnification under oil immersion. Spermatozoa stained light blue were considered to have intact chromatin and the spermatozoa stained from dark blue to violet were considered to have damaged chromatin. A total of 200 spermatozoa were studied for each sample.

Effect of adding various concentrations of alanine in extender is analyzed by the analysis of variance (ANOVA) in randomized complete block design using MSTAT-C (Ansari *et al*., 2012).

Results and discussion

The present study was designed to evaluate the effect of alanine supplementation in extender on post-thaw quality viz; motility, plasma membrane integrity, viability, normal apical ridge and chromatin integrity of Sahiwal bull spermatozoa. The supplementation of *tri-citric acid* extender with alanine improved the post-thaw semen characteristics viz; progressive motility, viability, acrosome and membrane integrity in ram (Khalili *et al*., 2010).

Higher ($P<0.05$) sperm motility was recorded in extender containing 15mM alanine compared to control (Table I). In similar studies, alanine improved the post-thaw motility of ram spermatozoa (Khalili *et al*., 2010). Higher concentration of alanine resulted in the decreased sperm motility which is in-line with the findings of Lahnsteiner *et al*. (1992) and Li *et al*. (2003), who reported that the high concentrations of amino acids caused a significant reduction in sperm motility.

Sperm plasma membrane integrity, normal apical ridge and chromatin integrity did not differ ($P>0.05$) in all experimental extenders (Table I). In contrary to our findings addition of alanine in semen extender for other

Table I. Effect of alanine in extender on post thaw motility, plasma membrane integrity, viability, acrosome integrity and chromatin integrity (%) of Sahiwal bull spermatozoa.

Alanine (mM)	Semen quality assays				
	Motility	Plasma membrane integrity	Viability	Acrosome integrity	Chromatin integrity
0	42.2±1.2c	56.8±3.6	39.1±1.4b	65.4±1.7	95.9±1.3
5	45.6±1.0bc	57.6±2.6	40.8±0.9b	69.3±1.7	96.4±0.8
10	47.8±1.7ab	55.7±3.3	43.2±0.5a	68.9±1.8	96.7±0.8
15	52.8±3.0a	59.5±3.3	44.1±0.9a	71.9±2.9	96.8±0.5
20	50.5±2.1ab	56.3±2.9	43.1±1.3a	71.1±2.7	95.8±0.6

The values with different superscript within the column differ significantly ($P < 0.05$).

related species improved the motility, plasma membrane integrity and percentage of live sperm. Addition of alanine resulted in higher percentage of sperm with functional plasma membrane in ovine sperm (Uysal and Bucak, 2007; Bucak *et al*. 2008; Özkan *et al*., 2009) and caprine semen (Atessahin *et al*., 2008). El-Nenaey *et al*. (2006) suggested that the addition of alanine to the extender during freezing phase improved the normal acrosome of frozen-thawed spermatozoa in buck. It is reported that alanine supplementation improved percent live sperms in ram (Bucak *et al*., 2008); stallion (Trimeche *et al*., 1999), goat (Kundu *et al*., 2001) and buffalo bull sperms (El-Sheshtawy *et al*., 2008). It is suggested that differences in protective effects of alanine may be due to the species differences and species specific unique physiology of the spermatozoa.

Sperm viability is an important indicator for the assessment of fertility of bovine semen. The viability assessed through this dual staining procedure is highly related with fertility of bull semen (Tartaglione and Ritta, 2004). The data on the effect of alanine on viability of Sahiwal bull spermatozoa is given in (Table I). Percentage of sperm with live acrosome was higher in extender contain alanine 10, 15 and 20mM compared to control. Viability of spermatozoa percentage affects the fertility of bovine semen (Aalseth and Saacke, 1986). Moreover, an improvement in sperm viability has been reported to be highly correlated with alanine content of the bovine spermatozoa. It is reported that alanine supplementation improved sperm viability in ram (Bucak *et al*., 2008), stallion (Trimeche *et al*., 1999), goat (Kundu *et al*., 2001) and bovine sperm (El-Sheshtawy *et al*., 2008). In conclusion, alanine addition in tris-citric acid semen extender improved motility and viability of the Sahiwal bull spermatozoa.

Statement of conflict of interest

The authors have declared no conflict of interest.

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