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Effect of L-cysteine in extender on post-thaw quality of Sahiwal bull semen

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The study was conducted to evaluate the effect of addition of L-cysteine to *tris*-citric acid (TCA) extender on the post-thaw quality of Sahiwal bull semen. For this purpose, two consecutive ejaculates were collected from three Sahiwal bulls using artificial vagina at weekly intervals for a period of three weeks (three replicates). Qualifying semen ejaculates were diluted (50×10^6 motile spermatozoa ml^{-1}) in TCA extender having L-cysteine either 0.0 (control) or 0.5, 1.0 or 2.0 mM. Diluted semen was cooled to 4°C for 2 h, equilibrated for 4 h at 4°C, filled in straws at 4°C, kept in liquid nitrogen vapours for 10 min and then stored in the liquid nitrogen. Thawing was performed after 24 h of storage, at 37°C for 30 s. and the sperm motility, viability, plasma membrane and acrosomal integrity were assessed. Higher ($P<0.05$) sperm motility, viability, plasma membrane and acrosomal integrity were observed using extenders containing 1.0 or 2.0 mM compared to those containing 0.5 or 0.0 mM L-cysteine. It is concluded that addition of L-cysteine (to reach 1.0-2.0 mM) in TCA extender improves the post-thaw quality of Sahiwal bull semen.

KEY WORDS: bull semen / cryopreservation / cysteine / Sahiwal / semen quality

Cryopreservation reduces the functional and structural integrity of bovine spermatozoa, and is associated with reactive oxygen species (ROS) production [Bilodeau *et al*. 2000]. Alterations in the antioxidant defense system and damage to sperm membranes have been observed due to excessive generations of ROS in

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semen during freeze-thawing process [Bilodeau *et al*. 2000, Gadea *et al*. 2004]. These changes in cryopreserved semen are responsible for poor sperm viability in female reproductive tract and lower fertility rates [Bailey *et al*. 2000].

Oxidative stress during freezing of mammalian semen can cause functional and structural damage to spermatozoa [Lenzi *et al.* 2002] involving ROS-mediated pathways [Baumber *et al.* 2005]. It is suggested that higher susceptibility of mammalian spermatozoa towards oxidative stress may have been due to higher lipid peroxidation levels [Kankofer *et al.* 2005]. Although bovine semen has natural defense system against the ROS, it is considered insufficient under cryopreservation-mediated stress [Nichi *et al.* 2006]. Therefore, reinforcement of semen extender with suitable antioxidant is suggested to reduce oxidative damage during freeze-thawing of bull spermatozoa. Cysteine, an amino acid precursor of glutathione is considered to be an intracellular antioxidant which protects the cells from ROS-mediated damages under oxidative stress [Meister and Anderson 1983].

Sahiwal is a breed of Zebu cattle native to South Asia characterized by high adaptability to the local harsh conditions [Ansari *et al*. 2010]. This is the dominant cattle of Pakistan, highly resistant to heat stress, ticks infestation and tropical diseases [Garcia *et al.* 2003]. The information on the use of L-cysteine in extender for cryopreservation of bull spermatozoa is lacking. We hypothesized that L-cysteine addition to extender may improve the post-thaw quality (motility, plasma membrane integrity, viability and acrosomal integrity) of bull spermatozoa. This study was designed to evaluate the effect of L-cysteine in tris-citric acid extender on post-thaw quality of semen in Sahiwal bulls.

Material and methods

The *tris*-citric acid extender (TCA) was used containing *tris*–(hydroxymethyl)-aminomethane (3.03% w/v), citric acid (1.56% w/v), fructose (0.2% w/v), glycerol (7% v/v) and egg yolk (20% v/v). Antibiotics streptomycin sulphate @ 1 mg/ml, procaine penicillin @ 300 IU/ml and benzyl penicillin @ 100 IU/ml were added to extender. Four extenders were prepared by adding 0.0 (control), 0.5, 1.0 and 2.0 mM L-cysteine to the TCA extender [Ansari *et al*. 2011].

Two consecutive ejaculates were collected from three Sahiwal bulls of similar age in graduated plastic tubes using artificial vagina (42°C) at weekly intervals for a period of three weeks (three replicates). Semen samples were initially evaluated for volume, motility and sperm concentration. Qualifying semen ejaculates were split into four aliquots and diluted in four experimental extenders at the rate 50×10^6 motile spermatozoa ml^{-1} approximately. At least one ejaculate per bull in a week was used having motility >60%, volume >1ml, and concentration >0.5 billion/ml. Extended semen was cooled to 4°C in 2 h and equilibrated over 4 h at 4°C. Semen was filled in 0.5 ml plastic French straws using suction pump at 4°C in cold cabinet unit and kept in liquid nitrogen vapours for 10 min. Straws were then plunged and stored into

liquid nitrogen (-196°C) for 24 h before thawing (37°C for 30 s) The thawed semen was assessed for progressive sperm motility, plasma membrane integrity, viability and acrosomal integrity.

Sperm progressive motility was determined using phase contrast microscope (×200) by placing semen sample on pre-warmed (37°C) glass slide and covered with a cover slip. **Sperm plasma membrane integrity** was assessed using supravital hypo-osmotic swelling test. Hypo-osmotic swelling assay was performed as described by Jeyendran *et al.* [1984]. After hypo-osmotic swelling incubation period, an aliquot (5 μl) of the solution was placed on a warm slide and a droplet (5 μl) of Eosin [0.5% (w/v) sodium citrate 2.92%] was mixed for 10 s. A cover slip was placed on the mixture and evaluated using phase contrast microscope (×400). A total of 200 spermatozoa were observed in at least five different fields. Clear heads and tails and swollen tails were considered intact, biochemically active sperm membranes, while pink heads and tails and unswollen tails were indicative of disrupted, inactive sperm membranes. **Sperm viability and acrosomal integrity** was determined by dual staining procedure [Kovacs and Foote 1992] using Trypan-blue and giemsa stains. The supravital stain Trypan-blue distinguished live and dead spermatozoa while giemsa stain was used to evaluate the integrity of the acrosomal membrane. In short, equal drops of Trypan-blue and semen were placed on a slide, mixed, air-dried and slides were fixed with formaldehyde-neutral red for 5 min. After rinsing with running distilled water, Giemsa stain (7.5%) was applied for 4 h. The slides were rinsed, air-dried and mounted with Balsam of Canada. Trypan-blue penetrates non-viable, dead spermatozoa with disrupted membrane which appeared stained in blue, while live, intact spermatozoa appeared unstained. Giemsa accumulates in spermatozoa with an intact acrosome, staining the acrosome region in purple. Two hundred spermatozoa were evaluated in at least five different fields in each smear by phase contrast microscope at ×1000 [Tartaglione and Titta 2004].

The data are presented as means with standard deviations of post-thaw sperm motility, plasma membrane integrity, viability and acrosomal integrity of Sahiwal bull spermatozoa cryopreserved in different experimental extenders. Analysis of variance (ANOVA) in completely randomized design was used to analyse the data and when appropriate, post-hoc comparisons for different treatments were performed with least significant difference (LSD). A level of 5% ($P<0.05$) was used to determine statistical level of significance (MSTAT-C Ver.1.42).

Results and discussion

Sperm progressive motility

The data on the effect of L-cysteine in extender on post-thaw progressive motility of Sahiwal bull spermatozoa are given in Figure 1. Higher ($P<0.05$) percentage of sperm motility was observed in extender containing 1.0 mM (56.7±2.9) and 2.0 mM (58.3±2.9) compared to that containing 0.5 mM (41.7±3.0) and 0.0 (41.7±2.9) L-

cysteine. It is believed that cysteine additive in semen extender improves motility of bovine semen through reducing oxidative stress by scavenging the reactive oxygen species (ROS) molecules [Alvarez and Storey 1983]. ROS molecules in cryopreserved semen are considered deleterious to sperm plasma membrane system because it causes lipid peroxidation of the membrane associated with poor sperm motility [Bucak *et al.* 2008].

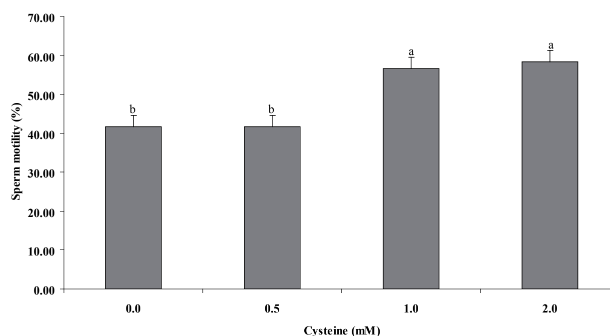

Fig. 1. Effect of L-cysteine in extender on percentage of Sahiwal bull sperms showing post-thaw progressive motility. Different letters on the tops of bars show significant (P<0.05) differences among extenders.

Sperm plasma membrane integrity

Sperm plasma membrane integrity (structural and functional) is essential for the fertilization and play critical role in the process of capacitation, acrosome reaction and the oocyte penetration [Jeyendran *et al.* 1984]. In the present study sperm plasma membrane integrity was assessed by supravital eosin stain in combination with hypo-osmotic swelling test known as supravital hypo-osmotic test. This assay is considered superior in determining the fertility (R^2=0.78) of bovine semen [Tartaglione and Titta 2004]. The data on the effect of L-cysteine in extender on post-thaw sperm plasma membrane integrity of Sahiwal bull spermatozoa are given in Figure 2. Percentage of sperm

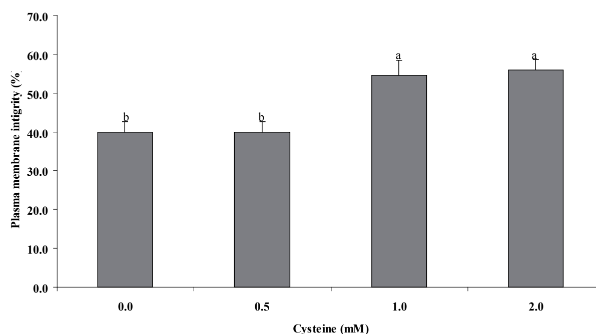

Fig. 2. Effect of L-cysteine in extender on percentage of Sahiwal bull sperms showing post-thaw plasma membrane integrity. Different letters on the tops of bars show significant (P<0.05) differences among extenders.

with intact plasm membrane was recorded higher (P<0.05) in extender containing 1.0 (54.7±3.8) and 2.0 mM (56.0±2.6) than containing 0.5 (40.0±2.6) and 0.0 mM (40.0±2.6) L-cysteine. Higher percentage of sperm with intact plasma membrane was reported in ovine [Uysal and Bucak 2007, Bucak *et al.* 2008, Ozkan *et al.* 2009] and caprine semen [Atessahin *et al.* 2008] cryopreserved in extender containing cysteine along with higher catalase activity. It is suggested that cysteine in extender protects the sperm plasma membrane integrity by scavenging the ROS molecules [Alvarez and Storey 1983] directly and/or indirectly [Cotran *et al.* 1989].

Sperm viability (live/dead ratio)

The number of viable sperm per dose after cryopreservation significantly affects the fertility of bovine semen under field conditions [Andrabi *et al.* 2006]. The data on the effect of L-cysteine in extender on viability of Sahiwal bull spermatozoa are given in Figure 3. Sperm viability was observed higher in extender containing 1.0 (73.3±1.5) and 2.0 mM (79.3±4.2) compared to that containing 0.5 mM (62.7±1.5) and 0.0 mM (61.7±2.5) L-cysteine. In similar studies on ovine semen higher percentage of viable spermatozoa was reported in association with increased catalase activity after the addition of cysteine to semen extender [Uysal and Bucak 2007, Bucak *et al.* 2008]. It is pertinent to mention that sperm motility, plasma membrane integrity and viability showed the same trends in this study.

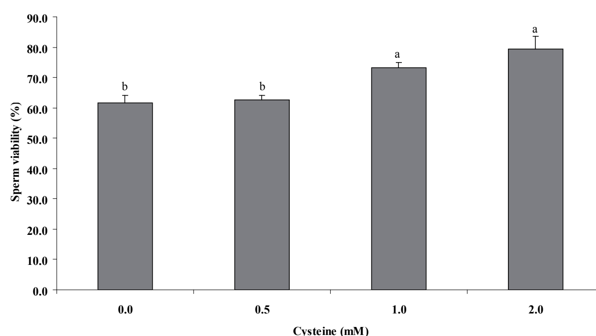

Fig. 3: Effect of L-cysteine in extender on percentage of Sahiwal bull sperms showing post-thaw viability Different letters on the tops of bars show significant (P<0.05) differences among extenders.

Sperm acrosomal integrity

The presence of functional acrosome is required for capacitation and acrosomal reaction that are critical steps in the process of fertilization [Thomas *et al.* 1997]. A significant positive relationship exists between the percentage of intact acrosome and fertility of frozen bovine spermatozoa [Saacke and White 1972]. The data on the effect of L-cysteine in extender on live sperm with intact acrosomes of Sahiwal bull spermatozoa are given in Figure 4. Post-thawed percentage of viable spermatozoa with intact acrosomes occurred higher (P<0.05) in extender containing 1.0 (70.7±1.2)

and 2.0 mM (77.0±3.5) compared to extender containing 0.5 mM (60.0±1.7) and 0.0 mM (60.3±3.1) L-cysteine. It is suggested that cysteine maintains the acrosomal integrity of spermatozoa by scavenging the ROS molecules through glutathione-mediated pathway by increasing antioxidant activity in the semen-extender complex [Bucak *et al.* 2008].

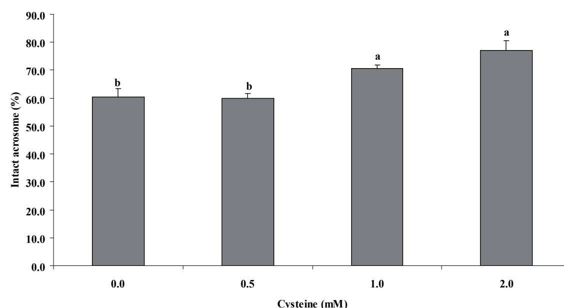

Fig. 4. Effect of L-cysteine in extender on percentage of Sahiwal bull sperms showing post-thaw acrosomal integrity. Different letters on the tops of bars show significant ($P<0.05$) differences among extenders.

In conclusion, L-cysteine addition (1.0-2.0mM) in TCA semen extender improved post-thaw quality of the Sahiwal bull spermatozoa. This can only be recommended for artificial insemination programmes if supported by an improvement in the fertility results and required further study.

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