RESEARCH ARTICLE

Curcumin Supplementation in Semen Extender Improves Post-Thaw Quality and Antioxidant Capacity of Gir Bull Spermatozoa

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Abstract

This investigation was carried out on semen of three Gir bulls at Cattle Breeding Farm, Junagadh (India) for a period of 6 months. The aim of the study was to assess the effect of different concentrations of Curcumin, *viz.,* 0 µM (Control), 5 µM, 10 µM, and 15 µM in Andromed extender on sperm quality along with oxidative stress parameters of cryopreserved semen. Semen ejaculates (n=30, 10/bull) with >70% initial motility were included in the study. Soon after initial evaluation, the semen was extended in Andromed extender @ 80 million sperm per mL and was divided into four equal aliquots. The Curcumin stock solution (10 mM) was added in these aliquots to get a final concentration of 0, 5, 10, and 15 µM and sperm motility was checked. It was soon filled and sealed in medium French straws (0.5 mL), equilibrated at 4°C for 4 h and frozen in LN2 vapour using conventional wide mouth freezer. Thawing of straws was done next day in water bath at 37°C for 30 sec, and various sperm quality parameters including oxidative markers were assessed. The results showed that supplementation of Curcumin to freezing extender improved semen quality and antioxidant capacity. The post-thaw sperm motility, sperm viability, HOST reactive sperm and acrosomal integrity in semen extended with 15 µM Curcumin concentration (57.33±0.67, 73.20±1.05, 64.77±1.56 and 72.43±1.38 %, respectively) were significantly ($p<0.05$) higher and sperm abnormality (18.00±0.41%) was significantly ($p<0.05$) lower as compared to those of control extender and the 5 µM Curcumin group. Mean malondialdehyde (MDA) level in post-thaw seminal plasma did not differ significantly ($p>0.05$) among the groups, while total antioxidants activity (TAC) with 15 µM Curcumin (476.36±16.52 µmol/L) was significantly ($p<0.05$) higher as compared to control and 5 µM Curcumin group. It is concluded that Curcumin @ 15 µM in semen extender improves post-thaw sperm quality and antioxidant capacity of cryopreserved Gir bull semen.

Keywords: Cryopreservation, Curcumin, Gir bull semen, Oxidative markers, Sperm quality parameters.

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Introduction

The production of high-quality frozen sperm is critical for AI in animals. However, the process of cryopreservation is a damaging phenomenon (Amidi *et al.*, 2016) resulting in deterioration of sperm quality due to excess production of oxygen free radicals during sperm freezing-thawing cycles (Seifi-Jamadi *et al.*, 2016). Basic cause to this damage is the presence of high content of polyunsaturated fatty acids (PUFA) in the plasma membrane of spermatozoa (Sarlos *et al.*, 2002). These PUFA, particularly of dead spermatozoa, bind with oxygen resulting in the production of high level of reactive oxygen species (ROS) (Sicherle *et al.*, 2011). The endogenous antioxidant system in sperm is not capable to scavenge the excess ROS generated mostly from dead sperm leading to an imbalance between the production and elimination of ROS resulting in oxidative stress (Dowling and Simmons, 2009). This leads to loss of structural and functional integrity of membranes, increasing membrane permeability, DNA structural damage and cell death (Aitken and Baker, 2004).

Cryopreservation decreases fertilizing ability of spermatozoa by causing damage to acrosomal membrane and mitochondria. However, the addition of Curcumin - [1,7 bis (4-hydroxy-3-methoxyphenyl) - 1,6 heptadiene-3,5 dione] a major phytochemical commonly found in turmeric (*Curcuma longa*) - maintains the sperm membrane integrity accompanied by decreasing MDA production, thus may maintain motility and decrease structural and functional alteration to the spermatozoa (Ishihara *et al.*, 2000;

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Salama *et al*., 2007; Tvrda *et al*., 2016). Curcumin has stronger antioxidant properties than vitamin E (Zhao *et al*., 1989). Curcumin is an effective ROS scavenger, a cryoprotective agent, and exhibits anti-oxidative functions (Rashid and Sil, 2015), although inconsistent evidence is available with respect to its exact role in male fertility. Curcumin has been used in a number of cell systems against ROS-induced damage mainly because of its well-defined cryoprotective and antioxidative roles against cold shock and oxidative damage (Mathuria and Verma, 2008). The addition of Curcumin has been shown beneficial on fresh and post-thaw sperm parameters of several mammalian species by many workers (Bucak *et al*., 2010; Omur and Coyan, 2016; Shah *et al*., 2017; Gupta *et al*., 2021). In view of the above facts, this study was planned to know the efficacy of different concentration of Curcumin supplementation in AndroMed® extender during cryopreservation of Gir bull semen.

Materials and Methods

The study was undertaken at the Department of Veterinary Gynaecology and Obstetrics of the College under Kamdhenu University, Junagadh (India) in collaboration with the Cattle Breeding Farm, Junagadh Agricultural University, Junagadh, during the period from January to June 2022 on semen of three Gir bulls kept under identical management and nutritional practices. All the bulls were in good health, under proper veterinary care and maintained in uniform sanitary conditions.

A total of thirty semen ejaculates were collected from three Gir bulls in artificial vagina at weekly interval and were evaluated. The sperm concentration and dilution rate were determined automatically by the photometer (Accucel, IMV, France). The semen was diluted with AndroMed extender (Minitube Germany) @ 80 million sperm per mL and was evaluated for motility. Semen ejaculates with more than 70% progressive motile spermatozoa on dilution were divided into four equal aliquots. Aliquots 1 to 3 were added with Curcumin (Sigma-Aldrich, USA) 10 mM stock solution in such a way to get 5 µM, 10 µM, and 15 µM final concentration in the extended semen, while the aliquot-4 was kept without additive and served as control. The diluted aliquots were filled and sealed in medium French straws (0.5 mL capacity, TBS™, IMV, France) by automatic machine (MRS1 Dual, IMV, France). Straws were then transferred to cold handling cabinet maintained at 4 °C and were equilibrated for 4 h prior to freezing. After equilibration, straws were vapour-freezed using conventional method in a wide mouth freezer and submerged in liquid nitrogen at -196 °C. After 24 h of cryopreservation, straws were thawed in a water bath at 37 °C for 30 sec and evaluated for post-thaw sperm motility, viability, abnormality, plasma membrane integrity (HOST) and acrosome integrity.

The contents of frozen-thawed semen straws of each aliquot (known volume and concentration) were centrifuged at 700 g for 15 min to separate out the sperm free seminal plasma, which was stored in sterile vials at -20 °C until assayed. The stored samples were thawed at room temperature before analyzing the lipid peroxidation (MDA produced) and total antioxidant capacity (TAC) using the standard kits of HiMedia Lab Pvt. Ltd., Mumbai as per the manufacturer's instructions. The data were expressed as Mean ± SEs and analyzed by one-way ANOVA and Duncan's post hoc test to determine significant differences at $p<0.05$ between the levels of additive Curcumin employing the SPSS software version 21.0 (Snedecor and Cochran, 1994).

Results and Discussion

The observations on post-thaw sperm progressive motility, viability, abnormality, hypo-osmotic swelling test, acrosome integrity and oxidative stress parameters like lipid peroxidation and total antioxidant capacity in Gir bull semen cryopreserved in Andromed extender without and with Curcumin 5 µM, 10 µM and 15 µM are presented in Tables 1 and 2.

Effect on Sperm Motility, Viability and Morphology

In the present study, the mean post-thaw percent sperm motility and viability were found significantly ($p<0.05$) higher in extender supplemented with 10 µM and 15 µM Curcumin compared to control and 5 µM Curcumin. However, the percent sperm abnormality was significantly ($p<0.05$) lower in extender supplemented with 15 µM Curcumin as compared to all other groups. Sperm in extender supplemented with 5 µM Curcumin showed significantly ($p<0.05$) higher post-thaw percent motility than control. However, there was no significant ($p>0.05$) difference in post-thaw percent viability and sperm abnormality with 5 µM Curcumin and control extender (Table 1).

Similar significantly improved sperm motility and viability of Hariana bull semen was observed by Gupta *et al.* (2021) with 10 µM Curcumin as compared to control group. Tvrda *et al.* (2016) recorded gradual increase in progressive sperm motility and viability in HF bull semen with addition of 5, 10, 25, 50 µmol/L Curcumin in the extender as compared to control group. Similarly in another study, Simmental-Fleckvieh bulls' semen treated with 50 µmol/L Curcumin led to significantly higher ($p<0.001$) sperm progressive motility and viability as compared to control group (Tvrda *et al.,* 2018). Interestingly, supplementation of Curcumin at a very higher concentrations (0.5 to 2.5 mM) have been reported to improve post-thaw motility and viability in Nili-Ravi buffalo bulls (Shah *et al.,* 2017), Holstein bull (Bucak *et al.,* 2012), ram (Omur and Coyan 2016), and Angora goats (Buck *et al*., 2010) spermatozoa.

The significance of sperm morphology has long been recognized. Several structural and ultrastructural sperm components have been affected by cryopreservation (Khalil *et al*., 2018). Cryopreservation has been associated to morphological alterations in sperm, including damage to mitochondria, the acrosome and the sperm tail when ROS

Table 1: Mean (± SE) post-thaw sperm quality parameters of Gir bull semen cryopreserved in AndroMed extender with different concentration of Curcumin

Groups (Curcumin, C-levels)	Post-thawed				
	Progressive sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
C - 0 µM	51.83±0.45^{a}	63.93±0.95^{a}	21.30±0.44^{c}	54.73±1.16^{a}	62.90±1.14^{a}
C - 5 µM	53.67±0.63^{b}	66.77±1.09^{a}	20.20±0.37^{bc}	59.17±1.37^{b}	66.80±1.33^{b}
C – 10 µM	55.17±0.61^{b}	70.63±1.00^{b}	19.47±0.38^{b}	62.90±1.44^{bc}	70.30±1.28^{bc}
C - 15 µM	57.33±0.67^{c}	73.20±1.05^{b}	18.00±0.41^{a}	64.77±1.56^{c}	72.43±1.38^{c}
p value	0.001	0.001	0.001	0.001	0.001

Means with different superscripts within column differ significantly at p<0.05 level.

exceeds the defense mechanisms of sperm (Woolley and Richardson, 1978). This damage can reduce post-thawed fertility of spermatozoa, and the zygotes or embryos often fail to be carried through to full-term pregnancy (Khan *et al.*, 2021). The acrosome and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP and these are responsible for motility of spermatozoa. The supplement might have displayed cryoprotective effect on the functional integrity of mitochondria by generating energy from ATP and may be responsible for improved post-thaw sperm motility (Reddy *et al.*, 2010). Findings of the present study suggested that Curcumin substantially improved post-thaw progressive sperm motility and viability in Gir bull semen at the concentration of 15 µM. In other studies, Curcumin offered improved progressive motility at lower or higher concentrations compared to the present one, which may be due to species and breed difference, semen collection technique, extender composition, additives used, preservation protocol, source and concentration of Curcumin used, thawing time and time taken by the observer etc. Several studies have revealed that Curcumin can improve sperm function by scavenging ROS (Tvrda *et al.*, 2018). This may be the probable reason for the beneficial effect of addition of Curcumin on sperm motility, viability and morphology in the present study.

Sperm Plasma Membrane and Acrosome Integrity

A biochemically active sperm membrane is required for sperm capacitation, acrosome reaction and spermatozoa binding to the egg surface, all of which entail changes in membrane characteristics. Acrosome is considered as limiting structure of fertilization. Intact acrosome is required for bringing out sperm-oocyte fusion and it is highly sensitive to freezing-thawing associated cryo-damage (Buck *et al.*, 2010). Spermatozoa with defective acrosome fail to bring out acrosomal exocytosis required during fertilization. Over production of ROS during freeze-thaw cycle results in rapid decrease in sperm motion behaviour, mitochondrial activity and plasma membrane and acrosome integrity (Ball, 2008; Bucak *et al.*, 2010) affecting their structural integrity and fertility potential. In the present study, the mean post-thaw sperm plasma membrane integrity (HOS reacted spermatozoa) and acrosome integrity were significantly (p<0.05) higher in all Curcumin supplemented groups as compared to control. Furthermore, the values of both the parameters were also significantly (p<0.05) higher in 15 µM Curcumin treated group as compared to 5 µM Curcumin treated group, and the values in 10 µM Curcumin group were intermediate of 5 and 15 µM Curcumin groups (Table 1).

Similar findings of higher sperm plasma membrane and acrosome integrity were also observed in Hariana bull semen by Gupta *et al.* (2021) with 10, 25 and 50 µM Curcumin supplementation as compared to control group, although the results were better with lower Curcumin concentration. However, Tvrda *et al.* (2018) found significantly higher (p<0.05) acrosome intact spermatozoa (82.70±0.52%) with higher concentration of Curcumin (50 µmol/l) in Simmental-Fleckvieh bulls' semen as compared to control group (70.80±0.47%), while Shah *et al.* (2017) reported significantly increased (p<0.05) percentage of post-thawed HOST reacted and acrosome intact spermatozoa with addition of 1.5 mM of Curcumin in Nili-Ravi buffalo bull semen as compared to the control group or 0.5 and 1.0 mM Curcumin supplements. Similarly, the plasma membrane integrity of sperm was also increased with inclusion of 0.5 mM Curcumin in Holstein bull semen (Bucak *et al.* (2012). Omur and Coyan (2016) reported significantly (p<0.05) increased post-thaw HOST reacted and acrosome intact spermatozoa in ram semen added with 1 and 2 mM Curcumin, and similar were the findings with 2.5, 5 and 10 mM Curcumin in Angora goat spermatozoa (Buck *et al.*, 2010).

Oxidative Stress Markers (LPO, TAC)

The mean malondialdehyde (MDA, µmol/L) concentration in post-thawed extracellular fluid (seminal plasma) of Gir bull semen cryopreserved in AndroMed extender using different concentration of Curcumin revealed that there were no significant (p>0.05) difference between control and other treatment groups. Lipid peroxidation however was found to be highest in control group as compared to Curcumin treated groups and the lowest in 5 µM Curcumin group. The

mean total antioxidant capacity (TAC) improved significantly ($p<0.05$) upon supplementation of Curcumin in AndroMed extender. The mean total antioxidant capacity (µmol/L) was significantly ($p<0.05$) higher in 15 µM Curcumin group as compared to control and 5 µM Curcumin group. However, the total antioxidant capacity of 10 µM Curcumin group did not differ significantly ($p>0.05$) from 5 and 15 µM Curcumin groups (Table 2).

Table 2: Mean (± SE) Lipid peroxidation and Total antioxidant capacity in the extracellular fluid (seminal plasma) of cryopreserved Gir bull semen in AndroMed extender with different concentration of Curcumin

Groups (Curcumin, C-levels)	Post-thaw stage	
	Lipid peroxidation (µmol/L)	Total antioxidant capacity (µmol/L)
Control - 0 µM	14.80±1.51	62.90±1.14^{a}
C - 5 µM	11.30±0.68	66.80±1.33^{b}
C – 10 µM	13.20±1.01	70.30±1.28^{bc}
C - 15 µM	13.70±0.80	72.43±1.38^{c}
p value	0.135	0.001

Means with different superscripts within column differ significantly at $p<0.001$ level.

Tvrda *et al.* (2016) reported a non-significantly decreased malondialdehyde level with all levels (5, 10, 25 and 50 µM) of Curcumin supplement in cryopreserved HF bulls' semen as compared to control group. However, in Simmental-Fleckvieh bulls' semen Curcumin administration at 50 µM significantly reduced malondialdehyde level as compared to the control group (Tvrda *et al.,* 2018), and in buffalo bull also MDA production was significantly lower with higher total antioxidant capacity in post-thawed semen with Curcumin levels 1.5 and 2.0 mM as compared to control and lower levels (0.5, 1.0 mM) (Shah *et al.,* 2017). In frozen-thawed rat semen, Soleimanzadeh and Saberivand (2013) reported significantly ($p<0.05$) higher total antioxidant capacity in 2.5 mM Curcumin treated group as compared to the control. However, contrary to present findings, the MDA values were reported to be higher with lower TAC at 0.5 and 5 mM Curcumin in Holstein bulls semen as compared to control group (Bucak *et al.*, 2012), and at 2.5, 5 and 10 mM Curcumin in Angora goat semen (Bucak *et al.*, 2010), suggesting that LPO could not be prevented when the samples were cryopreserved with Curcumin.

The susceptibility of mammalian spermatozoa to oxidative stress is because of their higher concentrations of unsaturated fatty acids and of limited repair mechanisms (Andrabi, 2009). Semen processing and cryopreservation decrease the antioxidant defense capacity of semen. Moreover, seminal antioxidant content is inadequate for the prevention of lipid peroxidation during freezing and thawing process (Storey, 1997). Consequently, fortification of cryodiluent with antioxidant is required (Andrabi *et al.,* 2008). During cryopreservation, the semen is exposed to cold shock at atmospheric oxygen which in turn increases the susceptibility to lipid peroxidation because of higher production of reactive oxygen species (Perumal *et al.*, 2009). Curcumin exhibits protective effects against cold shock and oxidative damage by inhibiting lipid peroxidation, as a result of powerful free radical scavenging activity (Bucak *et al.*, 2012). The addition of Curcumin maintains the sperm membrane integrity accompanied by decreasing MDA. As such, it may prevent peroxidative change to the sperm membrane structure, thus may maintain motility and decrease structural and functional alteration to the gamete (Ishihara *et al.*, 2000; Salema and EL Bahr, 2007; Tvrda *et al.*, 2016). At post-thaw stage, we found lower lipid peroxidation level with higher TAC at 5 and 15 µM Curcumin concentration than control group. This highlights that addition of Curcumin sustained the seminal total antioxidant capacity during cryopreservation. In the present study, the post-thawed sperm motility, viability, plasma membrane integrity, acrosome integrity and TAC were significantly and positively interrelated ($r= 0.33$ to 0.73) and all were negatively correlated with sperm abnormality and MDA production ($r= -0.33$ to -0.64).

Conclusions

The present findings using 0 µM, 5 µM, 10 µM and 15 µM Curcumin in Andromed extender during cryopreservation of Gir bull semen revealed that the 15 µM Curcumin concentration significantly ($p<0.05$) improves post-thaw sperm motility, sperm viability, HOST reactive sperm and acrosomal integrity with lower sperm abnormality as compared to control extender. Lipid peroxidation (MDA production) though did not vary significantly between Curcumin levels, it was highest in control group and lower at 5 µM Curcumin, while total antioxidants activity (TAC) was significantly ($p<0.05$) higher with 15 µM Curcumin as compared to control and 5 µM Curcumin, suggesting that Curcumin can be incorporated in semen extender at 15 µM concentration to improve post-thaw quality and antioxidant capacity of bull semen. Furthermore, *in vivo* fertility trials are warranted to validate the ultimate outcome.

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