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Cryoprotective Assessment of Lycopene in Tris-based Extender on Freezability of Bull Semen

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ABSTRACT

Background: Intensive use of artificial insemination is the demand of the present era, it deals with cryopreservation. During this process many changes, especially oxidative damages, which affect fertility and can be neutralized by supplementing some antioxidants.

Methods: To conduct this study, a total of thirty-two ejaculates collected from four bulls were preliminary evaluated. Based on progressive motility and viability, qualified samples were processed for cryopreservation. Diluted ejaculates with Egg yolk tris glycerol (EYTG) were split into four aliquots and grouped as Group I: control (without lycopene), Group II, Group III and Group IV as treatment group with 0.25 mM, 0.5 mM and 1 mM lycopene respectively. Post-thaw evaluation for progressive motility, viability, plasma membrane integrity, capacitation status, motility and kinematics, *in vitro* cervical mucus penetration and antioxidative enzymes activity was performed.

Result: The treatment group containing 0.25 mM lycopene showed significant ($P<0.05$) improvement in post-thaw sperm progressive motility (%), viability (%), HOS response (%), capacitation status (%), motility and kinematics (CASA), bovine cervical mucus penetration and antioxidant activity (SOD, GST, MDA). This study concluded that lycopene supplementation in bull semen protects spermatozoa from cryo-injury and oxidative stress during the freezing-thawing process.

Key words: Antioxidant, Cryopreservation, Hariana bull, Lycopene, Spermatozoa.

INTRODUCTION

Advancements in cryopreservation solved the problem of long-term storage of semen which plays a vital role in first-generation reproductive biotechnology *i.e.* artificial insemination. Cryopreservation entails different chemicals and falls in temperature at various rates that place sperm under many biochemical, mechanical and ultra-structural stresses, resulting in detrimental impacts on post-thaw quality and fertility potential (Sharafi *et al.,* 2022). The abundance of polyunsaturated fatty acids (PUFAs) in the sperm membrane structures causes susceptibility to lipid peroxidation (LPO) and reactive oxygen species (ROS) generated during the cryopreservation process lead to cellular damage (Halt, 2000). The addition of antioxidant compounds to the semen extender before bull semen cryopreservation may decrease ROS levels and hence their deleterious effects on spermatozoa (Bilodeau *et al.,* 2001). Previous researchers used various antioxidants *e.g.* resveratrol (Kumar *et al.,* 2022), glutathione (Yadav *et al.,* 2019), sericin (Yadav *et al.,* 2018) and melatonin (Thumar *et al.,* 2017) to protect the spermatozoa from such cryodamages.

Lycopene ($C_{40}H_{56}$) is a carotenoid which can be found in tomatoes, watermelon, papaya, red grapefruits, apricots and guava *etc* (Mozos *et al.,* 2017). It is a highly unsaturated straight-chain hydrocarbon with 13 double bonds, 11 of which are conjugated, which makes it a very powerful antioxidant. It has been shown to quench singlet oxygen twice as efficiently as β-carotene and ten times faster in comparison to α-tocopherol (Rao *et al.,* 2003). Protective effects of lycopene against oxidative damage induced by cryopreservation previously have been reported in several species, ram (Al-Sarray *et al.,* 2019), dog (Sheikholeslami *et al.,* 2020) and turkey (Rosato *et al.,* 2012) *etc*. It may efficiently reduce the detrimental effects of oxidative stress on spermatozoa by allaying singlet oxygen and trapping peroxy radicals (Tvrda *et al.,* 2016).

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MATERIALS AND METHODS

Semen collection and preliminary evaluation

A total of 32 semen ejaculates were collected from four healthy *Hariana cattle* bulls aged 5-6 years maintained in a semi-intensive management system at Semen Biology Lab of the University at a frequency of twice a week from

each bull using an artificial vagina (AV). Immediately ejaculates were passed into the laboratory for further evaluation and processing. Semen samples were evaluated primarily for mass motility, initial progressive motility and viability by using a phase-contrast microscope (Leica DFC295). Ejaculates having ≥ 3.0 mass motility (0-5 scale), ≥ 70% progressive motility and > 80% viability was processed further for cryopreservation.

Design of experiment for *Invitro* study and semen evaluation

After preliminary evaluation, semen was extended with EYTG diluter. Treatment groups were supplemented with lycopene (Sigma-Aldrich, PHR1770) which dissolved in Dimethyl sulfoxide (DMSO). The dilution rate was calculated to keep the final sperm concentration 80×10^6 spermatozoa per ml and divided into four equal aliquots: Group I (control) whereas Group II, III and IV contained lycopene in concentration of 0.25 mM, 0.5 mM and 1 mM respectively. All four group samples were filled and sealed in French mini straws, computerized auto printing through Domino Ink Jet Printer A200 Pinpoint (Domino UK Ltd, Bar Hill, Cambridge, CB3 8TU, England) and kept for equilibration at 4°C for 4 hrs. Equilibrated semen straws were kept in an automatic programmable biofreezer (Biological cell freezer, IMV technology, France) till the temperature of the straw reached -140°C with the freezing rates: 4 to - 10°C @ 10°C/ min, -10°C to -100°C @ 40°C/min and -100 to -140°C @ 20°C/min and subsequently stored into liquid nitrogen (-196°C) for storage. Semen samples were evaluated for progressive motility, viability, HOS response, capacitation status and motility and kinematics at pre-freeze and post-thaw stages. Antioxidant enzyme activity evaluation and vanguard sperm travel distance in bovine cervical mucus were assessed at post thaw stage.

After 24 hours of storage frozen straw were thawed at 37°C for 45 sec for post-thaw evaluation. The live percentage of spermatozoa was estimated using an eosin-nigrosin stain, as described by Sachan *et al.* (2023). The functional integrity of spermatozoa was assessed by hypo-osmotic swelling test (HOST) was performed according to the method described by Jayendran *et al* (1984) using with HOST solution of 150 mOsmol/litre previously. The assessment of sperm capacitation and acrosome reaction were assessed using Chlortetracycline (CTC) staining as described by Rathi *et al* (2001). For motility and kinematics, CASA (Computer Assisted Semen Analyzer), Minitube, Tifenberg, Germany, supported with AndroVision® software was used. A minimum of 2000 spermatozoa were counted in different 10 fields as previously described (Gupta *et al.,* 2022).The microscope was equipped with a thermostatic stage to maintain a constant temperature of 37°C**.**

Seminal plasma superoxide dismutase (SOD) activity was measured using the method as described by Madesh and Balasubramanian (1997) with some modifications previously performed (Sharma *et al.,* 2017). Glutathione - S- transferase (GST) and Malondialdehyde (MDA) assay was done by the TBARS (Thiobarbituric acid reactive substance) method described by Kumar *et al.* (2023).

To perform the BCMPT test, cervical mucus was collected from the cattle having estrus phase and then the test was performed as per the method described by Gupta *et al.* (2022).

RESULTS AND DISCUSSION

The maximum percentage of progressive motile spermatozoa($P<0.05$) was found in the group treated with 0.25 mM than control as well as the group treated with a higher concentration of lycopene in the pre-freeze stage as well as at the post-thaw stage. Not only the greater number of progressive motile spermatozoa but also significantly higher ($P<0.05$) percentage of viable spermatozoa and HOS test responsive spermatozoa were observed in group II both at pre-freeze as well as post-thaw stage (Table 1). Previously lycopene was used in various species like in bull semen (Bintara *et al.,* 2023; Tvrda *et al.,* 2017), ram semen (Ren *et al.,* 2018), rabbit semen (Rasoto *et al.,* 2012) and fowl semen (Mangiagalli *et al.,* 2007) and found improvement in viability and progressive motility as well as its significant protective effect on functional integrity by performing HOS test. Similarly, Uysal and Bucak (2007) also find a decline in seminal attributes at higher concentration of lycopene.

On performing the CTC assay, a significantly higher percentage ($P<0.05$) of uncapacitated spermatozoa was obtained in the group treated with 0.25 mM at pre-freeze and post-thaw stage in comparison to the control group and other treatment groups. Similarly, a significantly reduced number ($P<0.05$) of acrosome-reacted spermatozoa were found in group II than in control and treatment groups (Table 2). Shah *et al* (2017) also reports

Table 1: Effect of different concentrations of lycopene on spermatozoa motility, viability and Hos test responsiveness (Mean±SE=32).

Parameters	Freezing stages	Group I	Group II	Group III	Group IV
Progressive	Pre-freeze	69.84±20.93^b	72.97±14.11^a	49.84±19.65^c	39.53±33.97^d
motility (%)	Post-thaw	43.44±23.21^b	50.16±29.17^a	32.97±23.67^c	23.6±21.8^d
Viability (%)	Pre-freeze	74.58±0.37^b	76.92±0.37^a	64.81±0.73^c	54.59±0.98^d
	Post-thaw	66.08±0.61^b	68.88±0.39^a	55.25±0.94^c	40.31±0.99^d
HOS test	Pre-freeze	70.17±0.66^b	72.06±0.55^a	51.50±0.71^c	44.38±0.52^d
responsive (%)	Post-thaw	61.65±0.75^b	63.90±0.54^a	43.72±0.64^c	34.88±0.93^d

Different small letter superscripts in the same row indicate a significant difference ($p<0.05$).

antioxidant supplementation in hariana bull semen improve the capacitation status but very meagre studies may be there revealing the effect of lycopene on capacitation during cryopreservation.

On observing the spermatozoa kinematics parameter, VCL, VSL, VAP and DAP were found significant increments ($P<0.05$) at both stages. However, VCL, VSL, VAP and DAP were significantly reduced ($P<0.05$) in comparison to the control group. Beat cross frequency was also higher significantly ($P<0.05$) in group II at the pre-freeze stage. However, BCF at the post-thaw stage was non significantly different between group II and the control but significantly higher ($P<0.05$) than other treatment groups (Table 3). In earlier studies, different workers have used lycopene with different concentrations in different species of animals and have reported different velocity parameters. Tvrda *et al* (2017) used bull semen-supplemented lycopene and reported higher VAP, VSL, VCL, ALH, BCF, STR and LIN values after freezing and thawing. Similar patterns of spermatozoa motion kinematic were observed in our study. However, the kinematic and path velocities reported values were different compared to the values of the present study and this may be due to the different concentrations of lycopene used or due to the different breeds of bull used. In another study, in Holstein bulls sperm velocity parameters were evaluated (Tuncer *et al.,* 2014). All sperm kinematic parameters in lycopene treated group compared to the control had higher values except, BCF. Bucak *et al* (2015) bulls also reported higher VAP, VSL, VCL, ALH and LIN values as compared to control in Holistein. Although, these studies indicated the positive role of lycopene on sperm motion parameters, further detailed investigation is required for validation.

For evaluating the antioxidant effect of lycopene, the activity of various enzymes was observed. GST and MDA activity was found significantly lower ($P<0.05$) in the seminal plasma of the sample treated with 0.25 mM lycopene in comparison to the control and other treatment groups.

Table 2: Effect of different concentrations of lycopene on capacitation and acrosome reacted spermatozoa (Mean±SE=32).

Parameters	Freezing stages	Group I	Group II	Group III	Group IV
"F" pattern (%)	Pre-freeze	74.70±0.41^{b}	78.53±0.32^{a}	71.36±0.44^{c}	64.20±0.45^{d}
	Post-thaw	47.86±0.98^{b}	56.20±0.87^{a}	49.80±0.76^{b}	37.70±0.94^{c}
"B" pattern (%)	Pre-freeze	16.71±0.26^{c}	13.18±0.50^{d}	18.50±0.48^{b}	22.24±0.56^{a}
	Post-thaw	42.55±0.79^{a}	35.00±0.75^{b}	36.71±0.91^{b}	44.61±0.90^{a}
"AR" pattern (%)	Pre-freeze	8.59±0.42^{c}	8.30±0.64^{c}	10.13±0.59^{b}	13.55±0.48^{a}
	Post-thaw	9.60±0.58^{c}	8.79±0.63^{c}	13.49±0.87^{b}	17.68±0.98^{a}

*Different small letter superscripts in the same row indicate a significant difference ($p<0.05$).

Table 3: Effect of different concentrations of lycopene on sperm motility and kinematic parameters of Hariana bull semen (Mean±SE=32)

Parameters	Freezing stages	Group I	Group II	Group III	Group IV
TM (%)	Pre-freeze	73.7±1.35^{b}	81.85±0.94^{a}	57.87±1.30^{c}	46.89±1.06^{d}
	Post-thaw	55.44±1.18^{b}	58.95±1.21^{a}	46.74±1.00^{c}	35.70±0.82^{d}
PM (%)	Pre-freeze	66.80±1.41^{b}	76.22±1.22^{a}	49.99±1.36^{c}	38.32±1.02^{d}
	Post-thaw	47.18±1.27^{b}	51.39±1.22^{a}	38.29±0.87^{c}	28.11±0.76^{d}
VCL (µm/s)	Pre-freeze	123.06±1.96^{b}	142.97±3.96^{a}	95.27±2.68^{c}	77.5±2.08^{d}
	Post-thaw	90.58±2.57^{b}	99.10±2.39^{a}	77.08±1.72^{c}	56.32±1.78^{d}
VSL (µm/s)	Pre-freeze	42.93±0.80^{b}	50.80±1.70^{a}	34.31±1.12^{c}	28.80±0.80^{d}
	Post-thaw	33.30±1.14^{b}	35.86±0.95^{a}	28.76±0.71^{c}	20.55±0.70^{d}
VAP (µm/s)	Pre-freeze	59.88±1.05^{b}	69.63±1.81^{a}	46.79±1.32^{c}	39.35±1.08^{d}
	Post-thaw	45.12±1.28^{b}	48.23±1.13^{a}	39.04±0.92^{c}	28.15±0.80^{d}
DAP (µm)	Pre-freeze	17.30±0.60^{b}	19.43±0.49^{a}	14.18±0.43^{c}	11.63±0.36^{d}
	Post-thaw	13.63±0.41^{a}	14.12±0.43^{a}	11.50±0.28^{b}	8.55±0.20^{c}
BCF (Hz)	Pre-freeze	8.63±0.16^{b}	9.64±0.13^{a}	7.50±0.21^{c}	5.88±0.15^{d}
	Post-thaw	7.09±0.23^{a}	7.58±0.22^{a}	5.88±0.14^{b}	4.70±0.13^{c}
LIN (VSL/VCL)	Pre-freeze	0.34±0.00^{c}	0.36±0.00^{b}	0.36±0.00^{b}	0.37±0.00^{a}
	Post-thaw	0.36±0.00^{a}	0.35±0.00^{b}	0.37±0.00^{a}	0.36±0.00^{a}
STR (VSL/VAP)	Pre-freeze	0.71±0.00^{b}	0.72±0.00^{ab}	0.73±0.00^{ab}	0.73±0.00^{a}
	Post-thaw	0.73±0.00	0.73±0.00	0.74±0.00	0.73±0.00

*Different small letter superscripts in the same row indicate a significant difference ($p<0.05$).

Table 4: Effect of different concentrations of lycopene on seminal plasma enzymatic activity at post-thaw stage of Hariana bull semen (Mean±SE=32).

Parameters	Group I	Group II	Group III	Group IV
SOD (U/ml)	804.07±20.51	770.51±19.12	782.41±21.09	787.91±21.69
GST (nM/min/ ml)	65.09±1.63^{a}	46.17±4.72^{b}	59.18±1.85^{a}	66.99±4.99^{a}
MDA (nM/µl)	0.13±0.01^{a}	0.07±0.01^{c}	0.09±0.01^{bc}	0.10±0.01^{b}

*Different small letter superscripts in the same row indicate a significant difference (p<0.05).

Table 5: Effect of different concentrations of lycopene on Venguard distance (mm) at post-thaw stage of Hariana bull semen (Mean±SE=32).

Parameters	Group I	Group II	Group III	Group IV
Vanguard distance (mm)	23.53±0.60^{c}	34.75±0.68^{a}	28.75±0.75^{b}	22.56±0.47^{c}

*Different small letter superscripts in the same row indicate a significant difference (p<0.05).

However, there was no significant difference (P>0.05) observed in all the groups provided the lower numerical value was in group II (Table 4). Similarly, Tvrda *et al* (2016) reported no significant effect of supplementation of lycopene in the bull semen on SOD activity, which is in confirmation to our results. However, Tvrda *et al* (2016) did not find any significant effect of MDA in bull semen supplemented with lycopene which is contrary to our results. Perusal of available literature did not reveal any report where a Glutathione-S-Transferase (GST) activity study was conducted in bull seminal plasma with the use of lycopene but in the present study, GST activity was found significantly lower in the group treated with lycopene. Overall, we found the protective effect of lycopene against oxidative stress when used at the concentration of 0.25 mM.

At the post-thaw stage, the mean Vanguard distance (mm) was significantly higher (P<0.05) in group II. However, no significant difference was observed among groups I and IV, being highest in group II and lowest in group IV (Table 5).To assess the capability of frozen-thawed spermatozoa to penetrate through cervical mucus, a bovine cervical mucus penetration test was conducted. Singh *et al* (2016) observed a stronger correlation between sperm motility and cervical mucus penetration by spermatozoa also found in this study but no previous study reports using lycopene.

In the present study, the maximum protective effect of lycopene at the concentration of 0.25 mM on various spermatozoa parameters was recorded. Whereas this protective effect was found diminished when lycopene was used in higher concentrations. Further, to our observation, Uysal and Bucak (2007) also find a decline in seminal attributes following the increase in concentration of lycopene. It may be because of increasing reductive stress due to excess levels of antioxidant (Shanmugam *et al.,* 2020). It is needed to further more investigate the reason behind the reduction in the protective effect of antioxidants on their excess use.

CONCLUSION

The finding of this study indicated that supplementation of lycopene @ 0.25 mM to egg yolk-based extender improved the freezability of Hariana bull spermatozoa in terms of motility, viability, membrane integrity, capacitation status, sperm motion *and* kinematics. It effectively controls the oxidative stress of semen during cryopreservation and improves the post-thaw quality of spermatozoa. Lycopene at a higher concentration (≥0.5 mM) was found to have an inhibitory effect on post-thaw parameters of bull spermatozoa.

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Conflict of interest

All authors declare that they have no conflict of interest.

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