EFFECTS OF QUERCETIN AS AN EXTENDER-ADDITIVE ON CRYOPRESERVATION OF GIR BULL SEMEN

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ABSTRACT

The present investigation was undertaken on semen of four mature healthy Gir bulls (4-6 years) maintained identically on University farm, Junagadh (India) during hot summer months. Semen collected using AV, soon after routine evaluation, was diluted with extender Andromed containing Quercetin as an antioxidant, *viz.* 0 µM (control), 50 µM, 100 µM and 200 µM level, filled in French medium straws and frozen with conventional system. The samples were evaluated for sperm quality parameters and oxidative markers, *viz.,* LPO/MDA and GST activity in seminal plasma at per-freeze (post-equilibrium) and post-thaw stage of cryopreservation. The mean pre-freeze values of individual sperm motility, viability, HOS reactive sperm and acrosomal integrity (75.58±1.07, 77.88±0.99, 65.88±0.31 and 81.46±0.29 %, respectively) in 100 µM Quercetin group were significantly ($p<0.05$) higher with lower sperm abnormality, malondialdehyde (MDA) level and GST activity (15.21±0.66%, 6.99±0.12 µM and 20.23±2.29 nmol/ml/min) as compared to those of 50 µM and 200 µM Quercetin and control groups. At post-thaw stage also 100 µM Quercetin group showed similar trend with significantly higher sperm motility, viability, HOS reactivity and acrosomal integrity (59.58±0.48, 62.13±0.89, 59.04±0.88 and 73.04±0.45 %, resp.) and lower sperm abnormality, malondialdehyde (MDA) level and GST activity (20.96±0.48%, 7.25±0.57 µM and 38.70±3.65 nmol/ml/min) as compared to those of 50 µM and 200 µM Quercetin and control groups. All the sperm quality and oxidative stress parameters were better in 100 µM Quercetin group at both pre-freeze and post-thaw stage of cryopreservation. It is thus concluded that 100 µM is the most appropriate concentration of Quercetin as an additive to improve the quality parameters of cryopreserved semen of Gir bulls.

Key words: Cryopreservation, Gir bull semen, Oxidative markers, Quercetin, Sperm quality parameters.

INTRODUCTION

Artificial insemination (AI) in the production animals is a way to increase their reproductive efficiency and production. Sperm cryopreservation contributes to the expansion of this reproductive technique (Medeiros et al., 2002), however, it involves several steps which affect sperm structure and function (Bailey et al., 2003). Generally, sperm viability decreased by 50%, whereas fertilizing capacity is affected by factor of sevenfold after cryopreservation (Lessard et al., 2000). The process of cryopreservation is a damaging phenomenon resulting in deterioration of sperm quality due to excess production of oxygen free radicals during sperm freeze-thawing cycles (Amidi et al., 2016). The high content of polyunsaturated fatty acids.

(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 is not capable to scavenge these excess ROS generated from dead sperm that leads to loss of structural and functional integrity of membranes, increasing membrane permeability, DNA structural damage and cell death (Aitken and Baker, 2004; Dowling and Simmons, 2009).

Quercetin, a flavonoid polyphenol, is the most potent scavenger of ROS (Gibb *et al.,* 2013). It increases the body's antioxidant capacity by regulating levels of GSH (Glutathione). This is because, once oxygen free radicals are generated in the body, superoxide dismutase (SOD) quickly captures O^{2-} and transforms it into H_2O_2. This enzyme further catalyzes the decomposition of H_2O_2 to the non-toxic H_2O (Xu *et al*., 2019). The addition of Quercetin to bovine (Tironi *et al.,* 2019) and ovine sperm (Banday *et al*., 2017) has been shown benecial on fresh and post-thawed sperm quality parameters. The presence and location of hydroxyl (-OH) substitutions and catechol type B ring make Quercetin an effective antioxidant that possesses more intensive ROS scavenger activity than vitamin E & vitamin C (Ozgen *et al*., 2016). Quercetin may play important roles in ROS absorption and neutralization because of its oxidoreduction properties. Keeping all these facts in view, the present study was undertaken to evaluate the effect of different levels of Quercetin in terms of sperm quality parameters and oxidative stress markers in Gir bull

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semen during cryopreservation.

MATERIALS AND METHODS

Semen Collection

For this study four sexually mature healthy Gir bulls, aged 4 to 6 years, maintained under identical nutritional and managerial conditions at Cattle Breeding Farm, JAU, Junagadh (India) were utilized. The study was conducted during hot summer months from March to July 2021. The climate of Junagadh located at 107 meters above the mean sea level is tropically hot and humid with three distinct seasons. The bulls were fed as per the Minimum Standard Protocol of Government of India (2000), *i.e.,* with concentrate, dry fodder and green fodder @ 0.8 kg, 1.6 kg and 4.16 kg per 100 kg body weight, and mineral mixture supplement @ 60 gm/ day/head, and had free access to clean drinking water throughout the day. As a routine, bathing and grooming were carried out daily and exercise in rotatory bull exerciser was practiced a day before semen collection. Semen was collected in the morning hours once a week from each bull by using sterile artificial vagina (IMV, Danish model). Immediately after collection, the ejaculates were kept in water bath at 34°C and assessed for routine quality parameters adopting standard procedures (Tomar, 1984). Soon after evaluation, the freshly collected semen samples were diluted @ 80 million sperm/ml with extender AndroMed® (Minitube, Germany).

Extender Additive and Dilution

A stock solution (1.0 mM) was prepared of Quercetin procured from Sigma Aldrich, USA and was stored in refrigerator. Just before use, it was diluted and added in the extender to get 50 µM, 100 µM, 200 µM of final concentration. Egg yolk free concentrated AndroMed® (Minitube, Germany) was used as a semen extender.

The selected semen ejaculates >70% initial sperm motility were diluted with the final volume of extender as per dilution rate (80 million sperm/ml determined using Bovine Accucell Photometer, IMV) and were divided into four equal aliquots. Subsequently, Aliquot-1 was kept as control without any additive (0 mM Quercetin), while Aliquots - 2, 3 and 4 were added with Quercetin from stock solution to get final concentration of 50 µM, 100 µM, 200 µM Quercetin.

Processing and Evaluation of Semen

After final dilution and incorporation of different concentrations of Quercetin, the semen aliquots were filled and sealed in French medium straws (0.5 ml) by automatic filling and sealing machine (MRS1 Dual, IMV, France) and were equilibrated at 4 ºC for 4 hours in cold handling cabinet (Macro Scientific Pvt. Ltd, New Delhi). The contents of six-seven straws from each aliquot were collected after equilibration, and were evaluated for pre-freeze sperm motility, viability, abnormality, plasma membrane integrity (HOST) and acrosomal integrity. Seminal plasma was collected from these aliquots by centrifugation at 3000 rpm for 10 min and stored at -20º C for assay of lipid peroxidation and glutathione-S-transferases activities. The remaining straws were vapor freeze using the conventional method. The straws were thawed at 37 ºC for 30 seconds in a water bath and were again evaluated for post-thaw sperm motility, viability, abnormality (eosin-nigrosin stain), plasma membrane integrity (HOST), and acrosomal integrity (Giemsa stain) adopting standard procedures, and the seminal plasma samples collected as above from frozen-thawed semen were stored at -20°C till further analysis.

Seminal Plasma Biochemical Assays

The stored seminal plasma samples were analyzed for lipid peroxidation and glutathione-S-transferase activities. The peroxidative membrane damage was determined in terms of malondialdehyde (MDA) produced by using the standard kit procured from Sigma Aldrich (Saint Louis, USA), and Glutathione-S-Transferases (GSTs) was estimated by using kits supplied by HiMedia Lab. Pvt. Ltd., Mumbai employing CDNB substrate as per the procedure and instructions of manufacturer (Hayes *et al.,* 2005).

The data obtained for various sperm and plasma parameters were analyzed statistically and expressed as Mean ± SEs for pre-freeze and post-thawed values. The data were analyzed by one-way ANOVA and Duncan's multiple range test using SPSS software version 20.00 to determine significant differences between Quercetin levels at $P<0.05$.

RESULTS AND DISCUSSION

The findings on the effect of various levels of Quercetin in Andromed extender during cryopreservation of Gir bull semen on various sperm quality parameters and oxidative stress markers are presented in Tables 1-3.

Sperm Motility, Viability and Morphology

The mean values of sperm motility and viability in extender having 100 µM Quercetin were significantly ($p<0.05$) higher both at pre-freeze and post-thaw stages as compared to those of the control and 200 µM Quercetin, but statistically similar to those of 50 µM Quercetin group. The post-thaw motility in particular was at par with all three levels and significantly ($p<0.05$) higher as compared to the control group. The mean sperm abnormality % in 100 µM and 50 µM Quercetin groups were significantly ($p<0.05$) lower at pre-freeze and post-

thaw stages as compared to those of 200 µM Quercetin group and the later was statistically at par with the control (Table 1). Very similar observations have been reports earlier in buffalo and Holstein bull semen (Tvrda *et al.,* 2016; Ahmed *et al.,* 2019), while Avdatek *et al.* (2018) reported relatively lower sperm motility and sperm abnormality at 100 µM Quercetin. Furthermore, El-Khawagah *et al.* (2020) and Ardeshirnia *et al.* (2017) in buffalo semen and Silva *et al.* (2012) and Banday *et al.* (2017) in ram semen reported high progressive sperm motility and viability at lower concentration of Quercetin.

In the present study lower sperm motility and viability at higher concentration (200 µM) of Quercetin may be explained by Quercetin's pro-oxidant ability. Quercetin has to be converted to *ortho*-semiquinone before exerting its ROS scavenger effects. This free radical is converted to *ortho*-quinone accompanied by the production of ROS, such as hydrogen peroxide (H_2O_2). H_2O_2 has been shown to oxidize Quercetin to its reactive intermediates, *ortho*-quinone/quinone mediates, in several *in vitro* studies (Kaldas *et al*., 2005). Consequently, these reactive species lead to DNA damage (including single and double strand DNA breaks), lipid peroxidation and auto-oxidation of this flavonoid (Harwood *et al*., 2007).

Differences in the effects of Quercetin on sperm motility/viability observed in present study and in the literature, might be due to species difference, semen collection technique, semen additive composition, preservation protocol, concentration of Quercetin used, thawing time, time taken by the observer, disorder of axonemal proteins functioning etc. (Mughal *et al*., 2013). Beneficial effects of the Quercetin on semen quality parameters in the present study are confirmed by significant and positive correlations among important sperm quality parameters. Quercetin's beneficial effect on sperm motility might be related to the mechanism proposed by Nass-Arden and Breibat (1990) that sperm motility was inhibited in the first 2 hours of incubation with Quercetin due to sperm H+ and lactate secretion or acidification of medium, but during the next 3-4 hours it was stimulated due to increasing respiratory rate and ATP content of sperm cells.

The correlation study revealed that the individual sperm motility was highly significantly ($p<0.01$) and positively correlated with the viability, HOST reactive sperm and acrosome integrity in pre-freeze and/or post-thawed semen. The sperm viability was significantly ($p<0.05$; $p<0.01$), and positively correlated with the motility, HOST reactive sperm and acrosome integrity pre-freeze and/or post-thawed semen. The sperm abnormality had significant negative ($p<0.05$) correlations with sperm viability, acrosome integrity and HOST reactive sperm (Table 2). Similar correlations were reported by earlier workers in cattle and buffalo semen (Shelke and Dhami, 2001; Chaudhary *et al.,* 2017; Dhami *et al.,* 2018; Chaturvedi *et al.,* 2021)

Table 1: Individual sperm motility, viability and morphology at pre- and post-freezing of Gir bulls semen with different concentration of Quercetin in Andromed

(Mean ± SE)

Quercetin level	Sperm motility (%)		Sperm viability (%)		Sperm abnormality (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Control	70.08±1.48^{a}	54.08±1.01^{a}	73.63±0.77^{a}	58.00±0.91^{a}	18.08±0.44^{b}	24.04±0.50^{b}
Q50	73.00±1.48^{ab}	58.83±1.27^{b}	75.96±0.96^{ab}	60.58±0.82^{ab}	15.67±0.38^{a}	21.96±0.42^{a}
Q100	75.58±1.07^{b}	59.58±0.48^{b}	77.88±0.99^{b}	62.13±0.89^{b}	15.21±0.66^{a}	20.96±0.48^{a}
Q200	70.17±1.54^{a}	56.92±0.78^{ab}	74.04 ±0.77^{a}	58.17±0.80^{a}	17.13±0.35^{ab}	23.04±0.66^{ab}
P value	0.0101	0.0058	0.0184	0.0137	0.0037	0.0073

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

Table 2: Correlation among the seminal attributes at pre- and post-freeze stage in Gir

Sperm attributes	Individual motility	Sperm viability	Sperm abnormality	Plasma membrane integrity	Acrosomal integrity
Correlations: Post-thawed sperm					
Individual motility	1	0.596*	-0.155	0.406**	0.360**
Sperm viability	0.645**	1	-0.139	0.407*	0.269**
Sperm abnormality	-0.113	-0.231*	1	-0.313	-0.296**
Sperm plasma membrane integrity	0.316**	0.225*	-0.329**	1	0.331
Acrosomal integrity	0.012	0.064	-0.365**	0.152	1
Correlations: Pre-freeze sperm					

bulls semen

* Significant at $p<0.05$ level, ** Significant at $p<0.01$ level

Sperm Acrosomal and Plasma Membrane Integrity

The mean values of sperm plasma membrane integrity (HOST reacted sperm) and acrosomal integrity in Gir bull semen at post-thaw stage of semen cryopreservation in presence of 100 µM Quercetin were significantly ($p<0.05$) higher as compared to those in control, 50, and 200 µM Quercetin, which were statistically similar. However at pre-freeze stage the values of HOST reacted sperm and acrosomal integrity were at par in 50 µM and 100 µM Quercetin, but significantly higher than in 200 µM Quercetin and/or control (Table 3).

These findings concurred well with the earlier reports (Silva *et al.,* 2016; Ahmed *et al.,* 2019; El-Khawagah *et al.,* 2020). However, Banday *et al.* (2017) reported that the addition of Quercetin in tris extender showed a non-significant difference in the acrosomal integrity % compared to the control group in ram semen, while Avdatek *et al.* (2018) reported insignificant variation in post-thawed plasma membrane integrity in Quercetin supplemented semen. Positive effect of Quercetin supplementation in present study may be due to its specific inhibitory action on plasma membrane calcium ATPase, followed by an increase in intracellular calcium and modulatory effects on sperm capacitation (Cordoba *et al.,* 2005). Variable effect of the Quercetin observed in different literatures, might be due to species variation,

freezing extender composition, preservation protocol and concentration of Quercetin used. The improved post-thaw sperm membrane integrity may be because of ability of Quercetin to interact with & penetrate through lipid bilayers (Ben *et al*., 2011).

Sperm plasma membrane integrity had highly significant ($p<0.01$) and positive correlations with the individual sperm motility and viability, and negative correlations with sperm abnormality in pre-freeze and post-thawed semen. The acrosomal integrity showed highly significant negative ($p<0.01$) correlation with the sperm abnormality, and positive correlations with sperm motility and viability in post-thawed semen (Table 2).

Table 3: Sperm plasma membrane and acrosome integrity at pre- and post-freezing of Gir bulls semen with different concentration of Quercetin in Andromed (Mean±SE)

Quercetin level	Plasma membrane integrity (%)		Acrosome integrity (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Control	63.29±0.08^{a}	54.13±0.55^{a}	78.21±0.38^{a}	69.96±0.63^{a}
Q50	64.79±0.32^{b}	57.25±0.82^{a}	80.38±0.37^{bc}	71.08±1.03^{a}
Q100	65.88±0.31^{b}	59.04±0.88^{b}	81.46±0.29^{c}	73.04±0.45^{b}
Q200	63.13±0.28^{a}	55.75±0.95^{a}	79.79±0.36^{b}	70.33±0.51^{a}
p value	0.0001	0.0068	0.0002	0.0364

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

Lipid Peroxidation and Glutathione-S-Transferases

The mean malondialdehyde (MDA) values at pre-freeze and post-thaw stages in extender fortified with 100 µM Quercetin were significantly ($p<0.05$) lower as compared to those of the 50 µM, 200 µM Quercetin and control groups. The mean glutathione-s-transferases (GSTs) activity both at pre-freeze and post-thaw stages were significantly ($p<0.05$) lower with 100 µM and 50 µM Quercetin as compared to those of 200 µM Quercetin and control groups (Table 4). These findings concurred well with Tvrda *et al.* (2016) in Holstein-Friesian bull semen. Banday *et al.* (2017), Avdatek *et al.* (2018) and El-Khawagah *et al.* (2020) also found similar results but at lower concentration of Quercetin in ram, cattle and buffalo bulls, respectively.

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 ROS (Perumal *et al.,* 2009). Quercetin suppresses lipid peroxidation (Nass-Arden and Breitbart, 1990) which is an event coupled with motility loss (Aitken *et al*. 2006). Quercetin administration almost completely inhibited lipid peroxidation and enhanced the longevity of bovine sperm in the presence of an oxidative challenge (Mcniven and Richardson, 2002). Boots *et al.* (2008) hypothesized that Quercetin suppressed free radical formation at several levels via inhibiting the formation of the superoxide radical, by chelating iron (limiting the formation of hydroxyl radicals) and by inhibiting the formation of lipid peroxidative radicals.

In present study, post-thaw level of GST activity was increased in control group than Quercetin treated groups. Kumar *et al.* (2014) reported lower GST activity in freshly ejaculated spermatozoa compared to cryopreserved buffalo spermatozoa. The increased GST activity in the control group in present study clearly indicates the effective participation of GST or glutathione dependent defense mechanism against cryopreservation induced oxidative stress. The plausible reason for this may be explained as by increased production of thiobarbituric acid reactive substance (TBARS) under increased oxidative stress, which stimulates the sperm cells glutathione to reduce the ROS, including H_2O_2 to H_2O and itself gets oxidized to oxidized glutathione (GSSG). So in present study, prevention of sperm damage, reduction of oxidative stress and ROS level by Quercetin probably due to declined lipid peroxidation and oxidative damage resulted into reduction in GST activity.

Table 4: Lipid peroxidation and GST activity (nmol/ml/min) at pre- and post-freezing of Gir bulls semen with different concentration of Quercetin in Andromed (Mean±SE)

Quercetin level	Lipid peroxidation (µmol/ml)		GST activity (nmol/ml/min)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Control	6.99±0.12^{b}	9.40±0.36^{b}	31.10±2.56^{b}	68.95±7.7^{b}
Q50	6.34±0.40^{b}	8.45±0.15^{ab}	23.90±2.89^{a}	47.00±2.67^{a}
Q100	5.74±0.31^{a}	7.25±0.57^{a}	20.23±2.29^{a}	38.70±3.65^{a}
Q200	6.94±0.34^{b}	8.73±0.07^{b}	28.48±3.08^{ab}	56.93±5.37^{ab}
p value	0.0321	0.0026	0.0480	0.0036

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

CONCLUSION

The findings of the present investigation conclude that all the sperm quality and oxidative stress parameters were better in Andromed extender fortified with 100 µM or 50 µM Quercetin compared to 200 µM Quercetin or unadded control extender both at pre-freeze and post-thaw stage of cryopreservation of Gir bull semen. 100 µM Quercetin was the most appropriate concentration as an additive to improve the quality parameters with reduced oxidative stress in cryopreserved semen of Gir bulls.

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