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Effect of Quercetin Supplementation on Functional Membrane Integrity of Surti Buck Semen Preserved at Refrigerated Temperature

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ABSTRACT

A study was conducted to examine antioxidant effect of quercetin in tris egg yolk citrate extender on chilling quality of Surti buck semen. Total 72 semen ejaculates were collected from four Surti buck (18 ejaculate/buck) twice in a week by artificial vagina method. Semen samples were diluted with Tris egg yolk citrate extender and different quercetin concentration viz. C-0 µM, T1-15 µM, T2-25 µM, T3-50 µM, T4-75 µM, T5-100 µM and stored at refrigerated temperature (4-5ºC). Evaluation of semen parameters was done at 0, 24, 36 and 48 hours. The result showed that functional membrane integrity was well maintained at 15µM quercetin in tris egg yolk citrate extended buck semen maintained at refrigerated temperature up to 48 hours.

Introduction

Goats (*Capra hircus*) are one of the oldest domesticated species and one among the economically important livestock in India. Considering the poor production potential of goat, the need for genetic improvement *via* implementation of Artificial Insemination (AI) from superior sires through semen preservation is crucially required. Goat semen can be preserved either at room temperature temporarily, refrigerated temperature for 24-48 hours (Ferdinand *et al.,* 2012) or cryopreserved (Beltran *et al.,* 2013) for long term storage. In mammals, sperm protection against oxidative stress is provided mainly by seminal plasma which contains many antioxidants (Zini *et al.,* 2002), but, the protective capacity of endogenous antioxidants may be insufficient to prevent peroxidative damage during storage (Aurich *et al.,* 1997). Therefore, the harmful action of the free radicals can be blocked by exogenous antioxidant substances (Kumaran and Karunakaran, 2006). Natural most widely distributed dietary polyphenolic compounds antioxidant is quercetin, a non-enzymatic antioxidant (Nogueira *et al.,* 2013).

Supplementation of semen extender with quercetin has been reported to have beneficial antioxidant properties on post-thaw characteristics in sperm cells of bulls (Tvrda *et al.,* 2016), rams (Silva *et al.,* 2012) and bucks (Silva *et al.,* 2016). A functional membrane is requisite for the fertilizing ability of spermatozoa, as it plays an integral role in sperm capacitation, acrosome reaction, and binding of the spermatozoon to the egg surface (Ramu and Jeyendran, 2013). The hypo-osmotic swelling test (HOST) evaluates the functional integrity of the sperm's plasma membrane and also serves as a useful indicator of fertility potential of sperm (Ramu and Jeyendran, 2013).

Materials and Methods

Semen collection

Total four apparently healthy Surti bucks above 1 years of age maintained under All India Coordinated Research Project (AICRP) on Goat at Livestock Research Station, Navsari Agricultural University, Navsari were selected. The selected bucks were housed in a common covered pen and managed under uniform managemental and feeding conditions. The animals were allowed to graze between 2:30 PM to 4:30 PM and fed with good quality fodder *ad lib.* along with 500 g of concentrate per animal per day. After completion of the training period, semen was collected twice a week from each buck by artificial vagina up to 9 weeks .Total 72 semen ejaculates were collected. Semen was collected from all the selected bucks twice in a week at early morning between 6.00 am to 8.00 am using eight inch artificial vagina (AV) with 40 to 42°Cinner temperature and sufficient pressure.

Preparation of quercetin

To prepare stock solution (1mg/ml), 20mg of quercetin hydrate (Sigma-Aldrich, St.Louis, USA) was dissolved in 200µl of 1M NaOH with the help of vertex mixture. Thereafter, 18 ml of Mili Q water was added and pH of solution was adjusted to 8.0 with 70% orthophosphoric acid. Finally, make volume to 20 ml by adding Mili Q water to get stock solution (1mg/ml) and stored at refrigerated temperature.To prepare 500µM of working solution of 1ml, 150 µl of stock solution was diluted with 850µl of Mili- Q water.

Preparation of tris egg yolk citrate diluter with different concentrations of Quercetin

The TEYC diluter was prepared on the day of experiment by adding 20% egg yolk in Tris-citric acid-fructose buffer in sterile flask. The mixture was thoroughly mixed with vigorous shaking of flask for five minutes followed by centrifugation for 4 minutes at 3000 rpm in centrifuge machine and the supernatant from each tube was obtained carefully in a sterile glass bottle.Afterward the extender was supplemented with one of the quercetin concentrations [0 µM (control), 15 µM (T1), 25 µM (T2), 50 µM (T3), 75 µM (T4) and 100 µM (T5)] and the observed pH was 6.6-6.8

Experimental groups

Immediately after semen collection, the samples were pooled. The experiment was repeated eighteen times. Only semen samples with $\geq$ 70 % motility were considered for further processing. The pooled semen was divided into six aliquots and each aliquot was diluted with extender containing Tris-egg yolk citrate diluter with 0 µM (control), 15 µM (T1), 25 µM (T2), 50 µM (T3), 75 µM (T4) and 100 µM (T5) quercetin separately to a final concentration of 200 x 10^6 sperm/ml. After grouping, the semen samples were examined for functional membrane integrity at 0 hour and periodically up to 48 hours for post-chilled functional membrane integrity at 24, 36 and 48 hours.

Evaluation of functional membrane integrity

To evaluate the functional membrane integrity of spermatozoa, Hypo Osmotic Swelling Test (HOST) was determined by mixing 0.1 ml of diluted semen with 1.0 ml of hypo-osmotic solution and incubated at 37°C for 30 minutes. To ascertain the percentage of functional membrane integrity of spermatozoa, smear was prepared on pre-warmed clean glass slide by gentle mixing of a drop of diluted sample with a drop of eosin-nigrosin stain. Total 200 spermatozoa were counted in different fields and percentage of spermatozoa exhibiting tail curling (reacted) was calculated by following formula.

Reacted sperm (%) =

$$\frac{\text{No. of HOS reacted sperms}}{\text{Total no.of sperm counted}} \times 100$$

Statistical analysis

The data pertaining to various aspects were suitably tabulated and analysed using R-3.3.2 software. The differences among the parameter means were performed using appropriate statistical methods *viz.,* ANOVA, DNMRT (Duncan's New Multiple Range Test). The mean differences were considered significant at p<0.05 and p<0.01.

Results and Discussion

The Functional Membrane Integrity (%) at 0 hour differed non-significantly between control (65.33±0.90), T1 (64.72±0.85), T2 (64.33±0.88), T3 (64.61±0.90) and T4 (65.50 ±1.07) and T5 (64.94±0.81) groups.

Similarly, Functional Membrane Integrity (%) at 24 hours differed non-significantly between control (61.61±0.78), T1 (61.56±0.88), T2 (60.11±0.69), T3 (59.06±0.79) and T4 (60.00 ±0.98) groups, however, T5 (62.94±0.81) group differed significantly (p<0.01). At 36 hours, functional membrane integrity (%) in control (57.50 ±0.99), T1 (58.17±0.78), T2 (56.33±0.65) and T4 (56.11±0.90) groups differed non-significantly, whereas, T3 (53.22±0.78) and T5 (51.83±0.44) groups were significantly (p<0.01) lower as compared to control, T1, T2 and T4 groups. The functional membrane integrity (%) at 48 hours was significantly (p<0.01) lower in control (50.78±0.63) group as compared to T1 (53.50 ±0.76) group, and significantly (p<0.01) higher when compared to T5 (48.44±0.61) group. Furthermore, there were no significant difference between control and T2 (50.67±0.62), T3 (48.89±0.69) and T4 (51.33±0.62) groups. Additionally, T1 differed significantly (p<0.01) as compared to T2, T3, T4 and T5 groups, whereas, T2, T3; T2, T4 and T3, T5 groups differed non-significantly with each other.

The corresponding pooled mean of functional membrane integrity (%)irrespective of treatment and control groups were reduced with increasing preservation time at 0 (64.90 ±0.36) hour followed by 24 (60.88 ±0.35), 36 (55.53±0.38) and 48 (50.60 ±0.31) hours.

Furthermore, the functional membrane integrity (%) in control, T1, T2, T3 and T4 groups were significantly (p<0.01) higher at 0 (65.33±0.90, 64.72±0.85, 64.33±0.88, 64.61±0.90 and 65.50 ±1.07) hour as compared to 24 (61.61±0.78, 61.56±0.88, 60.11±0.69, 59.06±0.79 and 60.00 ±0.98) hours, 36 (57.50 ±0.99, 58.17±0.78, 56.33±0.65, 53.22±0.78 and 56.11±0.90) hours and 48 (50.78±0.63, 53.50 ±0.76, 50.67±0.62, 48.89±0.69 and 51.33±0.62) hours. Moreover, the functional membrane integrity (%) at 24, 36 and 48 hours differed significantly (p<0.01) among each other (Table 1).

Table.1 Effect of different concentrations of quercetin and storage duration on functional membrane Integrity (%) of Surti buck semen preserved at refrigerated temperature (Mean±SE)

Groups	HOST reacted- spermatozoa (%) (n=18)				Overall (n=72)	F value	P value
	0 hr	24 hr	36 hr	48 hr			
C	$65.33\pm0.90_{w}$	$61.61\pm0.78^{b}_{x}$	$57.50\pm0.99^{a}_{y}$	$50.78\pm0.63^{bc}_{z}$	58.81 ± 0.76^{ab}	55.59**	0.00
T1	$64.72\pm0.85_{w}$	$61.56\pm0.88^{b}_{x}$	$58.17\pm0.78^{a}_{y}$	$53.50\pm0.76^{a}_{z}$	59.49 ± 0.64^{a}	34.34**	0.00
T2	$64.33\pm0.88_{w}$	$60.11\pm0.69^{b}_{x}$	$56.33\pm0.65^{a}_{y}$	$50.67\pm0.62^{bc}_{z}$	57.86 ± 0.69^{abc}	65.67**	0.00
T3	$64.61\pm0.90_{w}$	$59.06\pm0.79^{b}_{x}$	$53.22\pm0.78^{b}_{y}$	$48.89\pm0.69^{cd}_{z}$	56.44 ± 0.80^{c}	74.69**	0.00
T4	$65.50\pm1.07_{w}$	$60.00\pm0.98^{b}_{x}$	$56.11\pm0.90^{a}_{y}$	$51.33\pm0.62^{b}_{z}$	58.24 ± 0.76^{abc}	43.72**	0.00
T5	$64.94\pm0.81_{w}$	$62.94\pm0.81^{a}_{w}$	$51.83\pm0.44^{b}_{x}$	$48.44\pm0.61^{d}_{y}$	57.04 ± 0.90^{bc}	140.82**	0.00
Overall (n=108)	$64.90\pm0.36_{w}$	$60.88\pm0.35_{x}$	$55.53\pm0.38_{y}$	$50.60\pm0.31_{z}$	--	--	--
F value	0.42	4.37**	10.26**	7.68**	--	--	--
P value	0.83	0.00	0.00	0.00	--	--	--

$^{a-d}$Means with different superscript within a column (between the groups) differs significantly at $p<0.05$; $p<0.01$.
$^{w-z}$Means with different subscript between a column (between time intervals) differs significantly at $p<0.05$; $p<0.01$.
** $p<0.01$; * $p<0.05$.
C - Control; **T1** - Quercetin 15µM; **T2** - Quercetin 25µM; **T3** - Quercetin 50µM; **T4** - Quercetin 75µM; **T5** - Quercetin 100µM

In T5 group, functional membrane integrity (%) differed non-significantly at 0 (64.94±0.81) hour and 24 (62.94±0.81) hours, whereas, at 36 (51.83±0.44) and 48 (48.44±0.61) hours there were significantly (p<0.01) lower HOST reacted sperm percentages as compared to 0 and 24 hours. However, the functional membrane integrity (%) differed significantly (p<0.01) between 24, 36 and 48 hours.

In this study highest post chilled HOST reacted sperm was observed in T1 group as 58.17±0.78 and 53.50 ±0.76 at 36 and 48 hours, respectively, as compared to other groups.

In accordance to our present findings, Seifi-Jamadi *et al.,* (2017) demonstrated that significantly (p<0.05) higher freeze-thaw plasma membrane integrity in 10μM (55.00±1.65) quercetin group when compared with 20μM (49.60 ± 1.16) quercetin and non-significantly higher than control (51.48 ± 1.69) group. Functional Membrane Integrity in present study remained non-significant between control and 15 μM up to 36 hours, although, they differed significantly at 48 hours. Equally, Avdatek *et al.,* (2018) in their study found no significant difference between control and quercetin at 25 μg/ml (34.37±1.71) group in post-thaw bull semen, however, membrane integrity was marginally reduced at 50 μg/ml and significantly (p<0.05) reduced at 100 μg/ml quercetin concentration. In another similar study, Silva *et al.,* (2016) found no significant difference among quercetin treated groups when compared to control at 0 and 1 hour, though, plasma membrane integrity was significantly lower (p<0.05) after 1 hour of incubationat34˚C.

Conflicting to our present data, 100μM quercetin group had significantly (p<0.01) lower functional membrane integrity than control group. Similarly, Seifi-Jamadi *et al.,* (2016) also observed that 0.1 mM (42.92±2.39%) quercetin was not significantly different than control (42.91±0.96 %) groups. But, at higher quercetin concentration (0.2 and 0.3 mM) sperms were adversely affected. They declared that at higher concentrations a partial prooxidant effect of quercetin on membrane integrity could limit its usage.

In addition, Filho *et al.,* (2017) and Kim *et al.,* (2014) found no significant difference between control and any of the quercetin treatment groups in equine and boar semen, respectively. Thus, similarly to our findings these experiments concluded that quercetin supplementation did not affect the membrane integrity at any concentration.

Furthermore, when quercetin was administered *In vivo* by Yelumalai *et al.,* (2019), they found that the percentage of HOS tail coiled sperm was highest in normal, non-diabetic rats, which was not affected by quercetin treatment. Similarly, when quercetin was co-administered with H_2O_2 at 40 μmol quercetin, it gave non-significantly higher result than other groups Ghaniei *et al.,* (2019). They also declared that per cent of sperm membrane functionality of rooster semen was reduced (p<0.05) in a time dependency manner for 0, 24 and 48 hours of freezing.

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Conflict of interest statement

Authors declare that they have no conflict of interest.

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