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Effect of Quercetin Supplementation on Motility of Surti Buck Spermatozoa Preserved at Refrigerated Temperature

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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 from four Surti buck (18 ejaculate/buck) were collected by artificial vagina method twice in a week from each buck upto 9 weeks. Semen samples were diluted with Tris egg yolk citrate extender and quercetin at different 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 Addition of 15µM and 25 µM quercetin in tris egg yolk citrate extender maintained motility above 50 % till 36 hours at refrigerated temperature (4-5^{0}C).

Introduction

Goats (*Capra hircus*) are one of the oldest domesticated species and one among the economically important livestock in India. 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. For successful fertilization motile sperm is most essential. Therefore, the evaluation of sperm motility is an essential criterion in the evaluation of the quality of a semen sample prior to its use for AI (Salamon and Maxwell, 2000). Reactive Oxidative Stress produced during the cryopreservation of spermatozoa exerts some physical and chemical changes in the sperm membrane. 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) belonging to aglycone flavonoid of the flavonols subclass (Kelly, 2011), that is found in plants, with the ability to scavenge reactive species and hydroxyl radicals (Boots *et al.*, 2008) in the treatment of male infertility (Johinke *et al.*, 2014). 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).

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 $^{\circ}$C inner temperature and sufficient pressure.

Preparation of Quercetin

To prepare stock solution (1mg/ml), 20 mg of quercetin hydrate (Sigma-Aldrich, St. Louis, USA) was dissolved in 200 µl 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 500 µM of working solution of 1 ml, 150 µl of stock solution was diluted with 850 µl 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 10^6sperm/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 Motility

Individual sperm motility from the extended samples was determined at 0, 24, 36 and 48 hours by placing a small drop of the diluted sample on a warmed glass slide covered with cover slip using phase contrast microscope with warm stage (37˚C). The proportion of progressively motile spermatozoa was expressed in percentage from 0 to 100 with

intervals of 5 %. To decide motility more accurately, minimum four to five different microscopic fields were assessed.

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

Motility

The initial motility differed non-significantly at 0 hour between control (79.44 ± 0.89), T1 (80.00 ± 0.81), T2 (80.00 ± 0.81), T3 (79.72 ± 0.75), T4 (79.44 ± 0.80) and T5 (79.72 ± 0.75) groups. Post-chilled individual sperm motility (%) at 24 hours was significantly ($p<0.01$) lower in control (59.17 ± 0.83) group as compared to T1 (70.28 ± 0.75), T2 (65.56 ± 0.98) and T3 (62.22 ± 1.35), whereas, it was non-significantly lower than T4 group and non-significantly higher than T5 group.

Post-chilled individual sperm motility (%) at 36 hours was significantly ($p<0.01$) lower in control (47.78 ± 0.83) group in comparison with T1 (59.72 ± 0.75) and T2 (54.17 ± 1.01) groups and significantly ($p<0.01$) higher as compared to T5 group. Alike observations were also noted at 48 hours. Highest post chilled individual motility was found at 24, 36 and 48 hours in T1 group followed by T2, T3 and T4 groups at refrigerated temperature. While, lowest motility was maintained in control group followed by T5 group at 24, 36 and 48 hours, respectively. The sperm motility was reduced with increasing preservation time (table.1).

In accordance to these findings Silva *et al.,* (2016), Seifi-Jamadi *et al.,* (2017) in buck; Avdatek *et al.,* (2018) in bull; Kim *et al.,* (2014) in boar; Moretti *et al.,* (2012); Ardeshirnia *et al.,* (2017) in ram; Yoshimoto *et al.,* (2017) in mouse; Khaki *et al.,* (2010) in rat; Gibb *et al.,* (2013), Seifi-Jamadi *et al.,* (2016) in stallion also reported higher sperm motility in lower concentration quercetin groups as compared to control group when evaluated after post thaw/incubation/post chilling.

Highest post-chilled individual motility was observed in T1 group (15 µM quercetin) followed by T2 (25 µM quercetin), which was in agreement with Silva *et al.,* (2016), who also found significantly ($p<0.05$) higher total motility in buck semen at 1 hour of post- thawed incubation in 15 µM quercetin added group as compared to control group.

Avdatek *et al.,* (2018) reported that total post thawed motility in 25 µg/ml quercetin tris based extender group was non-significantly higher than control group in bull semen. Besides, Kim *et al.,* (2014) suggested that quercetin in the freezing extender have positive effects on boar sperm motility at 1-50 µM upto 6 hours incubation period.

Furthermore, Moretti *et al.,* (2012) concluded that quercetin is most effective and if used at a low concentration in extender, has a limited effect on sperm motility in humans. Similarly, Khanduja *et al.,* (2001) agreed that post-thaw sperm motility at 50 µM quercetin in the freezing extender exhibited better result but when added at higher concentration (100 and 200 mM) rendered the sperm immobile post 6 hours of incubation.

The freezing extender supplemented with quercetin may be able to reduce H_2O_2-mediated oxidative damage in spermatozoa during chilled storage (Johinke *et al.,* 2014). Moreover, Yelumalai *et al.,* (2019) observed administration of 10, 25 and 50 mg/kg/b.w. quercetin for 28 days to normal, non-diabetic rats did not cause significant changes to the percentage of motile sperm as compared to control group.

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

Groups	Motility (%) (n=18)				Overall (n=72)	F value	P value
	0 hr	24 hr	36 hr	48 hr			
C	$79.44\pm0.89_{w}$	$59.17\pm0.83^{d}_{x}$	$47.78\pm0.83^{c}_{y}$	$35.28\pm0.85^{cd}_{z}$	55.42 ± 1.97^{bc}	482.88**	0.00
T1	$80.00\pm0.81_{w}$	$70.28\pm0.75^{a}_{x}$	$59.72\pm0.75^{a}_{y}$	$49.17\pm0.83^{a}_{z}$	64.79 ± 1.42^{a}	285.29**	0.00
T2	$80.00\pm0.81_{w}$	$65.56\pm0.98^{b}_{x}$	$54.17\pm1.01^{b}_{y}$	$43.06\pm1.08^{b}_{z}$	60.69 ± 1.69^{ab}	262.94**	0.00
T3	$79.72\pm0.75_{w}$	$62.22\pm1.35^{c}_{x}$	$48.06\pm1.08^{c}_{y}$	$38.06\pm0.72^{c}_{z}$	57.01 ± 1.92^{bc}	321.03**	0.00
T4	$79.44\pm0.80_{w}$	$59.72\pm1.10^{cd}_{x}$	$49.17\pm1.16^{c}_{y}$	$35.56\pm1.51^{cd}_{z}$	55.97 ± 1.99^{bc}	250.44**	0.00
T5	$79.72\pm0.75_{w}$	$57.50\pm0.93^{d}_{x}$	$44.44\pm0.89^{d}_{y}$	$32.78\pm0.83^{d}_{z}$	53.61 ± 2.11^{c}	555.93**	0.00
Overall (n=108)	$79.72\pm0.32_{w}$	$62.41\pm0.58_{x}$	$50.56\pm0.62_{y}$	$38.98\pm0.67_{z}$	--	--	--
F value	0.10	22.23**	32.26**	36.64**	--	--	--
P value	0.99	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 context to our study post-chilled sperm motility at higher concentrations (100 µM) of quercetin in freezing extender, was severely impaired in comparison to control and other treatments, which was in agreement with Liang *et al.,* (2016) where total sperm motility was significantly (p<0.01) inhibited at 100, 200 and 400 µM quercetin compared to the controls at 6 and 12 hours. Thus, they inferred that quercetin inhibiting the sperm functions may be due to the decrease in the sperm [Ca^{2+}] *in vitro* and suppressing tyrosine phosphorylation. The decreased activity of key Ca^{2} + -ATPase – key enzyme related to sperm motility and male fertility, results in Ca^{2}+ accumulation in the spermatozoa, which in turn causes blockade of the motility apparatus and a concomitant fall in motility (Khanduja *et al.*, 2001).

Contrary to our findings at 100 µM of quercetin group, Gibb *et al.*, (2013) and Seifi-Jamadi *et al.*, (2016) showed that quercetin at concentration above 100µM in freezing extender showed significantly (p<0.05) higher total post-thaw motility compared to other treatments in stallion. Equally, Seifi-Jamadi *et al.*, (2017) reported that supplementation of extender with 10µM of quercetin in combination with DMA showed significantly (p<0.05) higher post-thawed sperm motility compared to control group in goat. Meanwhile, Filho *et al.*, (2017) reported that quercetin supplementation (0.25, 0.5, 0.75 or 1 mM) to equine freezing extender did not affect progressive motility. The divergent results observed in present experiment with quercetin can be attributed to many factors such as the different species, dosages, extenders and methods of administration used, which may all affects its antioxidant action and ultimately various seminal parameters.

Unlike ejaculated semen, Ardeshirnia *et al.*, (2017) evaluated motility in frozen-thawed ram epididymal spermatozoa and found that motility decreased significantly (p<0.05) at higher concentrations of quercetin (≥20 µg/mL) but concentrations of 5 and 10 µg/mL quercetin appeared beneficial. In lab animal study conducted by Khaki *et al.*, (2010) in Wistar rats, observed that motility was non-significantly higher in treatment (35.42 ± 6.88) group as compared to control (33.75 ± 6.88) group in *in-vivo* administration. Nevertheless, co-

combination of quercetin with DMSO (Yoshimoto *et al.,* 2017) produced the highest rates of motile sperm and progressive motile sperm in mice after 7 and 11 days of storage at refrigerated temperature.

Contrasting to our findings, higher sperm motility was observed by Ben Abdallah *et al.,* (2011) in 200 µM (90.12 ± 0.12) quercetin added extender as compared to control, 10µM (80.00 ± 0.12) and 100µM (85.32 ± 0.43) quercetin groups in rats. Similarly, Johinke *et al.,* (2014) demonstrated that post freezing progressive motility of rabbit spermatozoa supplemented with higher concentrations of quercetin (50, 100 and 200 µM) in extender was greater than those diluted in 25 µM of quercetin.

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Conflict of interest statement

Authors declare that they have no conflict of interest.

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