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Research Article

EFFECT OF QUERCETIN SUPPLEMENTATION ON OXIDATIVE STRESS PARAMETERS AND MEMBRANE CHARACTERISTICS IN GOAT SEMEN STORED AT 4º C

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ABSTRACT

During the experiment, Quercetin (Q) with strong antioxidative property was evaluated for its effect on sperm characters in diluted semen stored at 4º C. Six healthy breeding bucks aged between 2-3 years were selected for semen collection. With a collection frequency of two ejaculates per buck/semen was collected using artificial vagina. A total of eight ejaculates were collected from each buck. The collected semen was immediately transferred to the laboratory and later pooled to reduce variation among the samples. The pooled sample was divided into four equal parts and diluted with Tris based semen extender containing different concentration of Q as per the experimental design, *viz.*, T-1 (25 mM), T-II (50 mM), T-III (75 mM) while control (C) was diluted with extender without Q. The diluted samples were stored at 4º C for 48 hours and later evaluated for seminal attributes using flow cytometry, CASA and antioxidative enzyme assay. The proportion of viable sperm was significantly (P 0.01) higher in T-II and those with active mitochondrial function were significantly (P 0.01) higher in T-II and T-I compared to other group studied during experiment. Acrosomal integrity, HOST and ROS unaffected sperm were significantly (P 0.01) higher in T-II. Antioxidative enzyme SOD was significantly (P 0.01) higher in T-II while catalase was significantly (P 0.01) higher in both T-II and T-I. The concentration of MDA was significantly (P 0.01) lower in T-II. Motility characters exhibited non-significant difference in VCL (µm) and ALH. The values for VAP (µm), VSL (µm), Str (%) and Lin (%) were significantly (P 0.01) higher in the T-II while the values of BCF (hz) was significantly (P 0.01) higher in T-II and T-I. The result recorded during the experiment indicated that Q in T-II group supplemented at 50mM exhibited a better response in regulation of oxidative stress, restored the sperm characters and protected the mitochondria energy production system with better motility characters. It may be concluded that Q (50 mM) can be an effective supplement to protect the sperm against oxidative damage during its short-term storage.

Keywords: Buck semen, Membrane characteristics, Oxidative stress, Quercetin

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Goat is an important livestock species mostly reared by landless, marginal and small farm families in India. With limitation of resources and scientific inputs, the goat rearers still follow traditional rearing practices (Vahoniya *et al.*, 2022). Majority of goats are maintained on field residues and agricultural waste under extensive feeding system. This practice not only affects the animal health but promote indiscriminate breeding (Giger-Reverdin *et al.*, 2013). In addition to this, practice of selling out superior buck and allowing doe to be mated by inferior bucks has intensified the situation in last few decades. Today, majority of Indian goats are categorized as low producers. Genetic improvement of Indian goat population is urgently required to conserve native breeds and improve their production potential.

Artificial Insemination (AI) is considered as the most efficient technological intervention to disseminate and improve the animal genetics (Watson, 2000). Storage of semen at 4º C and its cryopreservation at -196º C are two different modes to utilize semen for artificial insemination. But with wide variability in goat breeds, limited goat frozen semen station and unavailability of cryopreserved doses, the use of short term stored liquid semen at 4º C seems to be an immediate practical solutionto make available the superior germplasm for genetic improvement through AI.

Maintaining metabolically active and motile sperm with intact sperm characters and fertilizing ability is a challenge during semen storage. During short term storage, semen is subjected to low temperature that induces stress leading to over production of reactive oxygen species (ROS). ROS denature the structural integrity and affect the function of sperm through membrane denaturation (Sabeti *et al.*, 2016). Unlike other domestic species, goat semen exhibits higher degree of sperm damage due to presence of phospholipase A2 in bulbourethral secretions that interact with egg yolk of semen extender and stimulates efflux of membrane constituent increasing sperm susceptibility to low temperature (Anand *et al.*, 2017). Interactive damages together with oxidative stress aggravate time dependent sperm damages in stored goat semen (Anand *et al.*, 2023). Developing strategies to minimize sperm damage with prolonged storage time during short term storage is urgently required for its optimum use.

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To regulate oxidative stress and protect sperm against cold damages, extenders are supplemented with antioxidative agents. These antioxidants are thought to increase antioxidative capacity of seminal fluid and prolong semen storage time. Quercetin (Q) is a natural antioxidant that has been well documented to regulate reactive oxygen species (ROS) level in body (Qamar *et al.*, 2023). But in addition to it, Q has peculiar character to cross plasma membrane that can be helpful in regulating sperm internal mechanism and mitochondrial ROS production imparting protection against DNA damage and apoptosis (Tamma *et al.*, 2018). Evaluation of Q as supplement in goat semen extender for its role in regulating the oxidative stress and capacity to maintain sperm characters can be helpful in developing strategies for better use of stored semen at 4º C. Hence, the experiment was designed to evaluate the effect of Q supplementation on sperm protection and antioxidative regulation in semen stored at 4º C for short term.

MATERIAL AND METHODS

The experiment was designed to evaluate the effect of Q supplementation in regulating oxidative stress and restore sperm characters during its storage at 4º C. A total of six healthy Barbari bucks maintained at frozen semen production station, Department of Veterinary Physiology, DUVASU Mathura were selected for the experiment. The bucks were maintained on stall feeding under intensive feeding system. The selected bucks were well trained to donate semen using artificial vagina. With a frequency of two collections per week, 8 ejaculates (total 48 ejaculates) were collected from each of six bucks during the experiment. The immediately after collection semen was transferred to the semen analytical laboratory and initially evaluated for concentration and quality. The collected semen from all bucks were pooled to establish similarity and later divided into 4 equal parts. One part of semen that was diluted with Tris glycerol semen extender containing 15% egg yolk was designated as control while other three parts were diluted with semen extender containing different concentration of Q as per experimental design and designated as treatment (T) viz. T-I (25 mM), T-II (50 mM), T-III (75 mM). The diluted semen with final concentration of 400×10^6 sperm/ml was stored at 4º C for 48 hours. The diluted stored semen was later analyzed to evaluate the effect of Q on sperm characters. To evaluate the semen through flow-cytometer, the semen concentration was reduced to 100×10^6 sperm/ml using easy buffer B (EBB) solution. The prepared semen stock solution was utilized further for evaluating different seminal attributes. The viability, mitochondrial activity, ROS affected sperm and acrosomal integrity of viable sperm was evaluated using specially designed kits (Easy kits I, II, III, V; IMV, France) for seminal attributes with specific gating as indicated by manufacturer. Hypo osmotic swelling test (HOST) was performed to evaluate the function of sperm plasma membrane (Jeyendran *et al.*, 1984). To evaluate the sperm kinematics and motion characters in sperm, 2µl of stock solution of semen was place on 8 chambered Leza slide maintained at 37º C and observed under phase contrast microscope with 10-X objective using appropriate setting for motion detection of sperm. Activity of antioxidative enzymes were evaluate in seminal plasma that was separated by centrifugation (4000 rpm, for 15 min) and stored at -20º C until analysis. The concentration of superoxide dismutase (SOD) and catalase in seminal plasma was evaluated by spectrophotometric methods described by Madesh and Balasubramanian (1998) and Bergmeyer (1983), respectively. TBARS (thio-barbituric acids described reactive substance) was used to evaluate Malondialdehyde (MDA) through assay a described by the Ohkawa *et al.* (1979).

Statistical analysis: The data recorded in different treatment groups were evaluated for the effect of Q supplementation using one-way ANOVA (version 20 of the IBM SPSS analysis program (2011)). Differences in group were tested using Multiple range test of Duncan (1955) results were presented as means ± SE significant among means at $p<0.01$.

RESULTS AND DISCUSSION

During the study, effect of Q with strong antioxidative property was evaluated for oxidative regulation and sperm characteristics in semen stored at 4º C. The values recorded for viable sperm were significantly ($p<0.01$) higher in T-II as compared to other groups evaluated during the experiment (Fig. 1). The possible reason for the improved values recorded in T-II may be attributed to better capacity of Q to regulate ROS at 50 µM concentration while the lower values may be attributed to either decreased antioxidative capacity of Q at lower concentration while lower values at higher Q concentration may have resulted from ortho-quinone over production as an end product after metabolism of quercine that changed the nature of Q from antioxidant to prooxidant (Rietjens *et al.*, 2005) increasing oxidative load in semen leading to sperm damage and loss of viability. Further, the protection incurred by Q at different levels was evaluated on plasma membrane through assessment of sperm with normal plasma membrane fluidity, acrosomal integrity and HOST response which exhibited significantly ($p<0.01$) higher values in the T-II (Fig. 2). This differential response of Q in different treatment group may be attributed to concentration dependent ROS regulation in semen diluter and also the fate of Q intermediate product formed during its action as antioxidant (Tvrdá *et al.*, 2020). Processing of semen for short term storage involves various steps that include semen collection, its dilution and storage at low temperature which induces stress on sperm. The dilution changes the sperm external environment leading to osmotic stress while low temperature during sperm

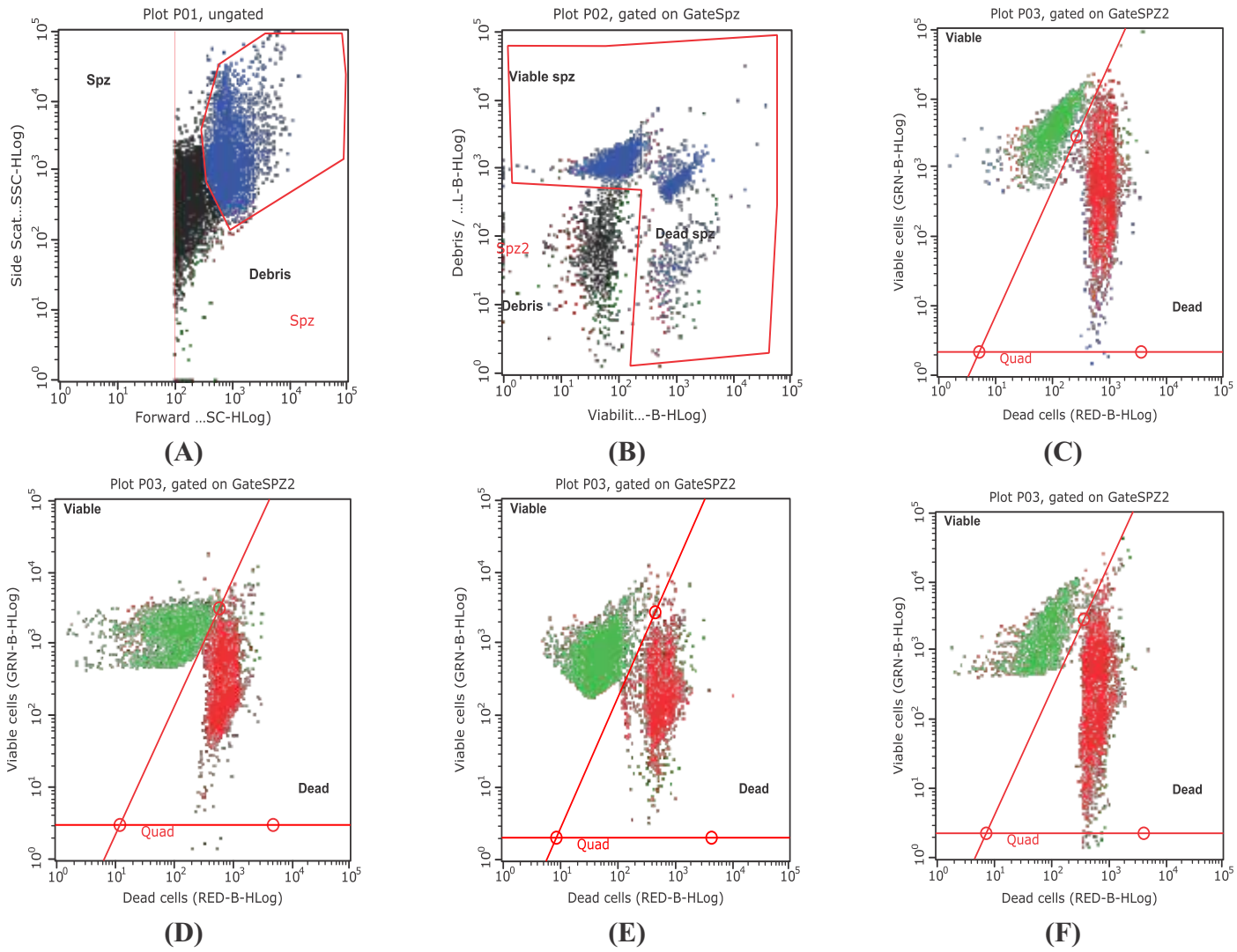

Fig. 1. Gating and flowcytometric evaluation of percent viable sperm in different treatment group during the experiment.
Green- Viable sperm, Red- Dead Sperm, A- Forward and side scattering, B- gating to separate debris and sperm, C- control, D- Treatment-1 (25 µM Q), E- Treatment II- (50 µM Q), F- Treatment III ((75µM Q)

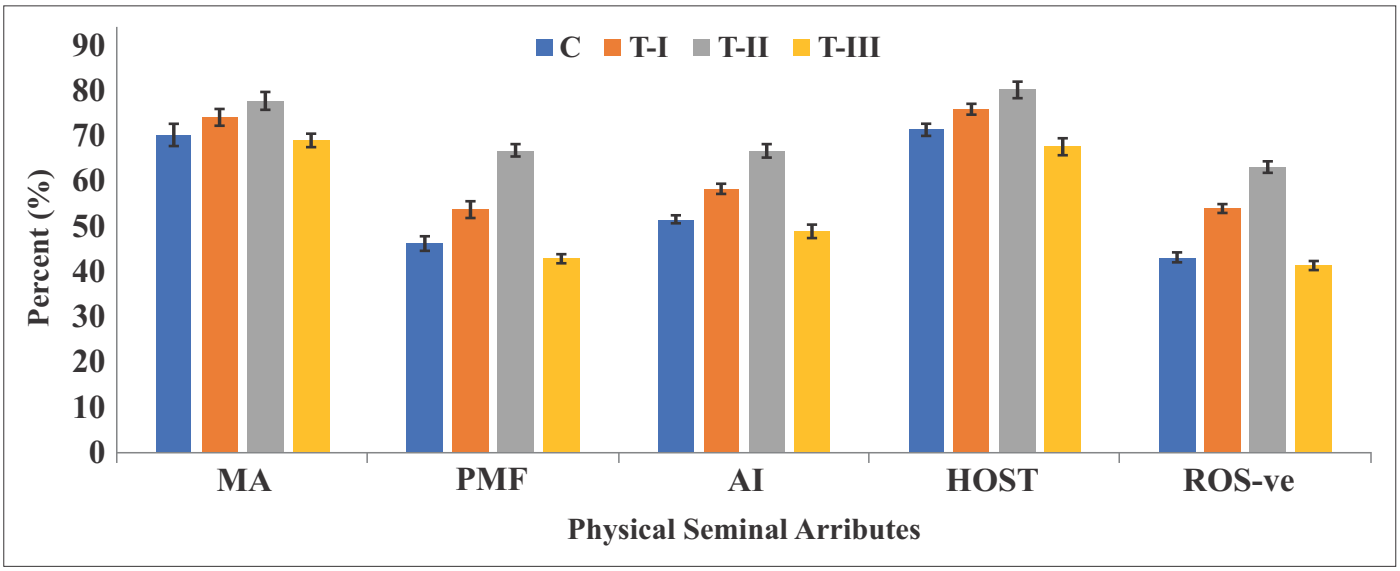

Fig. 2. Physical seminal attributes exhibited by sperm in semen supplemented with different concentration of Quercetin during short-term storage at 4° C
MA- Mitochondrial activity, PMF- Plasma membrane fluidity of viable cell, AI- Acrosomal Integrity of viable cell, HOST- Hypo osmotic swelling test, ROS-ve - Viable sperm unaffected with reactive oxygen species, Significance ($P < 0.01$)

Table 1. Effect of Quercetin on antioxidative enzyme and MDA level in seminal fluid of different group during its short-term storage at 4° C

Parameter / Group	Control	Treatment-I	Treatment-II	Treatment-III
Superoxide dismutase (Units /mg protein)	25.18^{A}±0.186	26.58^{B}± 0.215	27.76^{C}± 0.202	25.01^{A}± 0.182
Catalase (CAT) (mMH_2O_2 utilized/min/mg.protein)	15.55^{A}± 0.205	17.09^{B}± 0.202	17.86^{B}± 0.175	15.28^{A}± 0.213
MDA (nmol/ml seminal plasma)	0.65^{C}± 0.017	0.57^{B}±0.019	0.46^{A}±0.017	0.65^{C}± 0.014

Mean with different superscripts within a row differ significantly (P<0.01)

Table 2. Effect of Quercetin on Motility, path velocity and kinematic characters exhibited by sperm in different group during its short-term storage at 4° C

Parameter / Group	Control	Treatment-I	Treatment-II	Treatment-III
Total motile sperm (%)	66.43^{AB}± 0.896	70.14^{B}± 0.738	75.28^{C}± 1.017	63.71^{A}±1.409
Progressive motile sperm (%)	26.57^{A}± 1.702	33.08^{A}± 3.089	43.82^{B}± 2.960	27.22^{A}± 1.870
Curvilinear velocity (VCL) µm/sec	162.69± 4.223	163.60± 2.777	169.93± 3.936	157.44± 3.471
Average path velocity (VAP) µm/sec	80.11^{A}± 2.331	83.33^{A}± 1.069	92.10^{B}± 2.489	78.12^{A}± 1.728
Straight line velocity (VSL) µm/sec	55.95^{A}± 1.415	60.31^{A}± 2.254	70.21^{B}± 2.617	55.71^{A}± 1.505
Straightness (Str) %	51.74^{A}± 0.789	55.30^{A}± 1.431	62.04^{B}± 1.532	50.76^{A}± 1.588
Linearity (Lin) %	26.59^{A}±0.659	29.28^{A}± 1.342	34.81^{B}± 1.176	26.22^{A}± 0.895
Amplitude lateral head displacement (ALH) %	7.44±0.288	7.77± 0.259	7.82± 0.216	7.72± 0.197
Beat Cross frequency (BCF) hz	25.18^{AB}± 0.576	26.63^{BC}± 0.644	28.83^{C}± 0.576	24.19^{A}± 0.595

Mean with different superscripts within a row differ significantly (P<0.01)

storage result in cold shock and ROS over production leading to oxidative stress (Rizkallah *et al.*, 2022). Further, ROS act on sperm membrane and cause efflux of membrane constituents that destabilize the plasma membrane. ROS not only cause membrane damage but initiates a cascade of reactions that intensifies ROS generation with increase in proportion of affected sperm (Agarwal *et al.*, 2014). The extent of ROS protection and production depend upon the seminal capacity to regulate the ROS level which was best exhibited in T-II with significantly (p<0.01) higher values for membrane associated seminal attributes. Loss of fluidity destabilizes the sperm membrane leading to damage and loss of semipermeable nature that was evaluated through assessment of acrosomal integrity and HOST. During the experiment, Q in T-II which exhibited significantly (p<0.01) higher values for sperm with intact acrosomal and HOST response might be the result of better membrane protection against ROS that prevented the efflux of membrane constituent and maintained membrane fluidity within normal range thus preventing the membrane destabilization, low degree of acrosomal damage and higher degree of HOST response. Q has also been reported to induce depolarization in sperm that may enhance CatSper which are non-selective cation channels together with the voltage gated proton channel (Hv1) that stimulates calcium influx leading to sperm capacitation and thereafter acrosome reaction (Wijerathne *et al.*, 2019) which may be another reason to variation in different treatment groups. To confirm the Q action through ROS regulation, the proportion of ROS unaffected sperm in different treatment and control was evaluated through flow cytometry. The proportion of viable sperm that remained unaffected by ROS (ROS-ve) exhibited significantly (p<0.01) higher values in T-II further supporting ROS regulation as a regulatory mechanism for Q action (Fig. 2). Change in the sperm structure has a positive correlation with sperm internal environment. Denaturation of sperm membrane leads to mitochondrial dysfunction. Since mitochondria is the major site for ROS production (Chianese and Pierantoni, 2021), so Q with a capacity to pass the cell membrane was evaluated for its effect in regulating the sperm mitochondrial function. Abdelnour *et al.* (2023) reported that Q contributes well to sustain the endogenous cellular antioxidant defense system thus protect the DNA and cellular organelle from oxidative damage which well defines the reason for higher values in T-II. SOD, catalase and MDA that depicts the antioxidative capacity and extent of lipid peroxidation (Layali *et al.*, 2015) were evaluated in seminal plasma to ascertain the role of Q with its best suited concentration for regulating oxidative stress (Table 1). SOD was significantly (p<0.01) higher in T-II while the catalase was significantly (p<0.01) higher in both T-II and T-I which may be result of selective action of Q to neutralize the ROS. The lower values of MDA in T-II may be attributed to better antioxidative regulation that protected the lipid peroxidation of sperm membrane. Effect of Q supplementation on path velocity and kinematic characters was evaluated using computer assisted semen analyzer (CASA, IVOS II, IMV) (Table 2). The total motile sperms and those exhibiting progressive motion were significantly

($p<0.01$) higher in T-II. El-Khawagah *et al.* (2020) also reported similar results with improved values in buffalo semen characteristics after Q supplementation.VCL (µm) and ALH (%) were non-significant among all the test and control samples. The values for VAP (µm), VSL (µm), Str (%) and Lin (%) were significantly ($p<0.01$) higher in the T-II while the values of BCF (hz) was significantly ($p<0.01$) higher in T-II and T-I. The probable reason for the change in motility character with best resulted recorded in T-II may have resulted in better antioxidative capacity of Q to regulate ROS when compared to other treatment groups which might have protected the sperm flagellar proteins (Yelumalai *et al.*, 2019) thus restoring the kinematic characters of sperm in T-II. The finding of the present study indicates a dose dependent variation in the response of Q to regulate the oxidative stress and protect sperm during the short-term storage.

CONCLUSION

It may be concluded that Q as a strong antioxidative supplement in semen extender regulates the oxidative load in seminal fluid. Q supplementation protect the plasma membrane form ROS induced sperm damages to maintain the sperm structure and maintains the function of sperm membrane thus promotes the sperm survival and maintains the motion characters of sperm during its storage at 4º C with optimum response at 50 µM of Q. Further, study may be conducted to evaluate the effect of Q at ultra- low temperature and understand the mechanism of Q action to protect sperm during semen processing at low and ultra-low temperature.

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