Effect of adding the Quercetin (FQ) and Quercetin-loaded-Niosomes (QLN) Nanocarriers to the tris extender on the viability and motility of goat Epididymal sperms under cryopreservation conditions

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Abstract

This study aims to Evaluated the effect of both free quercetin (**FQ**) and quercetin–loaded-niosomes (**QLN**) in concentrations of **(10%, 15% and 20%)** on the motility and viability of goat epididymal sperm. The results shows that the use of (**QLN**) in the cryopreservation extender Tris-Citrate-Fructose yolk (**TCFY**) at a concentration of 10% led to significant increasing ($p<0.01$) in the sperm viability (**SV**) (87.65) and motility of progressive motility (**PR**) (29.67), non-progressive motility (**NP**) (24.09), or immotile sperm (**IM**) (24.09) . This improvement is relate to the concentrations used, as high concentrations lead to a decrease in sperm motility and viability.

According to based on the results of this study, we can conclude that **(QLN)** enhances its antioxidant activity, contributes to improve the sperm characteristics under cryopreservation conditions, to increase the fertility rates in male goats.

.**Keywords:** Quercetin-loaded-Niosomes, Epididymal Sperm, Cryopreservation.

Introduction

Quercetin is a flavonoid compound found naturally in many foods, such as fruits and vegetables. This compound exhibits several biological activities, most notably its antioxidant activity **[1]**. Quercetin may also inhibit the release of extracellular enzymes that contribute to sperm damage during cryopreservation **[2،3]**. This protective role makes quercetin a promising antioxidant that can improve and maintain sperm quality under all the stress conditions associated with semen preservation. Several studies have recommended the use of Quercetin in semen thinners in various animal species, including sheep, to improve sperm quality during preservation **[4]**.

Previous studies have indicate that Quercetin has positive Effects on fresh sperm as well as after thawing following cryopreservation in several types of farm animals **[1]**. Also, the addition of Quercetin leads to a significant improvement in sperm viability by reduce damage caused by oxidative stress and reactive oxygen species (ROS) **[5]**. Several studies have shown that the addition of Quercetin improves sperm motility and viability or reduces lipid peroxidation in various species, including humans **[6]**, rabbits **[7]** and buffalo **[3]**.

However, Ranawat et al **[8]** reported contrasting results, finding that quercetin increased (**ROS**) production and lipid peroxidation in rat testicular membranes **[9]**. In in vitro studies,

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quercetin was show to improve sperm motility and zona Pellucid (boundary) in cryopreservation equine sperm **[10]**. It also demonstrated a protective effect in maintaining human sperm motility and viability **[11]**, and studies have shown that quercetin reduces oxidative stress by neutralizing free radicals, thus protecting sperm from (ROS)-induced damage **[12]**.

Despite the potential health benefits of flavonoids, some studies have questioned their in vivo efficacy. This is attribute to their low water solubility, poor absorption, and rapid metabolism, all of which limit their bioavailability **[13]**. A promising strategy to overcome this problem is loading polyphenols into water-soluble carriers, such as niosomes **[13،14]**. Loading these compounds into niosomes increases their solubility, allowing for easy addition to inorganic media and offering clear advantages in in vivo therapeutic applications, as well as in vitro applications.

Therefore, this study aime to elucidate the effect of adding free or Quercetin–loaded-niosomes (**QLN**) to Tris extender (**TCFY**) on certain semen characteristics of goat.

Material and Methods

Preparation of Quercetin and Quercetin-Loaded-niosomes:

Quercetin was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) and used directly without any further Purification steps. Quercetin-loaded niosomes were prepared at the University of Baghdad/College of Science/Department ofBiotechnology/NanotechnologyLaboratory using the thin-film hydration method according to the method of Najafi **[15]**, with minor modifications to suit the experimental context.

Testicle and Sperm Collection:

Testicles Were obtained from healthy, adult male Goats (1.5 to 3 years old) weighing between (40 and 50 kg) immediately after slaughter at the local abattoir. Animal selection was based on external examination to ensure good health, the absence of visible lesions, normal scrotal structure, and the absence of any reproductive tract abnormalities.

The testicles were transported to the laboratory within (30 minutes) of slaughter in an insulated container filled with (0.9 NaCl) physiological saline supplemented with penicillin (100 IU/mL) and streptomycin (100 g/mL), and stored at (35–37$^{\circ C}$). Upon arrival at the laboratory, the testicles were briefly washed with fresh, warm saline before total sperm was collected from the epididymis **[16]**. The tail of the epididymis was then removed from the testicle using sterile surgical instruments under sterile conditions in an airflow chamber. The epididymal tail was isolated from the testis using a lamellar (hood) technique.

Careful dissection and isolation of the tail region were performed to avoid contamination of the sample with blood or other tissue fluids. After isolation, the epididymal tail was placed in a sterile (100 mm) Petri dish. Approximately (15–20) small incisions were made on the surface of the epididymal tail using a sterile scalpel blade to allow passive diffusion of sperm into the diluted (**TCFY**) medium. To enhance diffusion, the epididymal tail was incubated in (**TCFY**) medium at (37°C for 10 minutes). Following incubation, a sterile pipette was used to collect the sperm suspension Evaluation of Sperm Motility and Viability After Treatment with Quercetin and Quercetin-Loaded niosomes Transfections

Sperm were cryopreserved with different concentrations of free quercetin or quercetin-loaded niosomes transfections

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(10%, 15%, 20%). All treatments were diluted with Tris-Citrate-Fructose Yolk (**TCFY**) extender at a (1:15) ratio. Samples were then incubated at (37°C for 10 minutes). Sperm motility was evaluated using a light microscope, and sperm were classified according to their motility patterns into three main categories: progressive motility (PR), non-progressive motility (NP), and immotile (IM), according to the procedures and criteria outlined in the WHO Guidelines for Semen Examination and Treatment, 6th Edition **[17].**

Sperm viability was assessed using eosin-nigrosin staining, based on the principle of dye exclusion by the intact sperm membrane, according to the method described in the World Health Organization **[17]** guidelines, with modifications to suit the nature of goat epididymal sperm. Eosin-nigrosin staining was prepared by dissolving 0.67 g of Eosin Y and 0.90 g of sodium chloride (NaCl) in 100 mL of purified water with gentle heating. Ten g of nigrosin was then added to the solution, the mixture was boiled, and then allowed to cool to room temperature. It was filtered using filter paper and stored in a dark, airtight glass bottle.

After obtaining the epididymal sperm sample, (50 µL) of the sample was taken and mixed with (50 µL) of the Eosin-nigrosin staining. The mixture was then left to stand for 30 seconds. A drop of the mixture was then placed on a clean glass slide, a thin smear was prepared, and the smear was allowed to air dry **[17]**

The samples were then examined under a microscope, and unstained cells were counted as live, while stained cells were considered dead. At least (200 sperm) were counted per sample, and all experiments were performed in triplicate. The results were presented in graphs for ease of understanding and visualization.from the (**TCFY**) medium **[18]**, and initial sperm motility (more than 85%) was assessed.

Experimental Design:

Each sample was divided into several parts, including:

- T1 (**CONTROL**) sperm treated with TCFY (at a 1:15 dilution ratio) with 0% (**FQ**) and (**QLN)**
- Sperm treated with **TCFY** (at a 1:15 dilution) with different concentrations of free quercetin:

1. T2: (10% **FQ)**
2. T3: (15% **FQ)**
3. T4: (20% **FQ)**

- Sperm treated with **TCFY** (at a 1:15 dilution) with different concentrations of quercetin Loaded to niosomes:

1. T5: **(**10% **QLN)**
2. T6: (15% **QLN)**
3. T7: (20% **QLN)**

Statistical Analysis

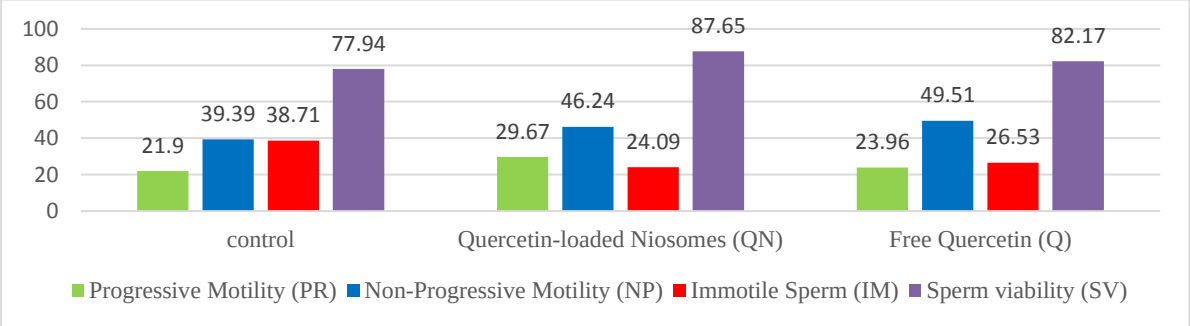
The SAS statistical software, version 9.4 (SAS Institute Inc., Cary, NC, USA), was used for all statistical analyses. Data from three experimental replicates were collected and entered into the statistical analysis. The fixed effect of the treatment on each outcome variable was analyzed using one-way ANOVA at each time point. When ANOVA showed significant differences between the means at a significance level of ($p < 0.01$), post-hoc comparisons were performed using Duncan's Multiple Range Test (DMRT). The results were presented as means for all studied variables. These means were converted into graphs and text to facilitate interpretation and clarity of presentation. A ($p < 0.01$) was used as the criterion for statistical significance.

Results and Discussion

The results showed a significant increasing ($p<0.01$) in the percentage of

(**PR**) type movement as shown in Figure (1), (**NP**) type movement as shown in Figure (2), and (**IM**) type movement as shown in Figure (3) in the quercetin group loaded with niosomes (**QLN**) compared to the free quercetin group (FQ) and the control group. The results also showed a clear significant improvement in the free quercetin group compared to the control group after preservation under cryopreservation conditions.

Figure 1. Effect of adding 10% of Quercetin-loaded Niosomes (QN) or Free Quercetin (FQ) on the percentage of progressive Motility (PR) ،Non-Progressive Motility (NP) ،Immotile Sperm (IM) and Sperm Viability (SV) of goat Epididymis sperm under cryopreservation conditions.



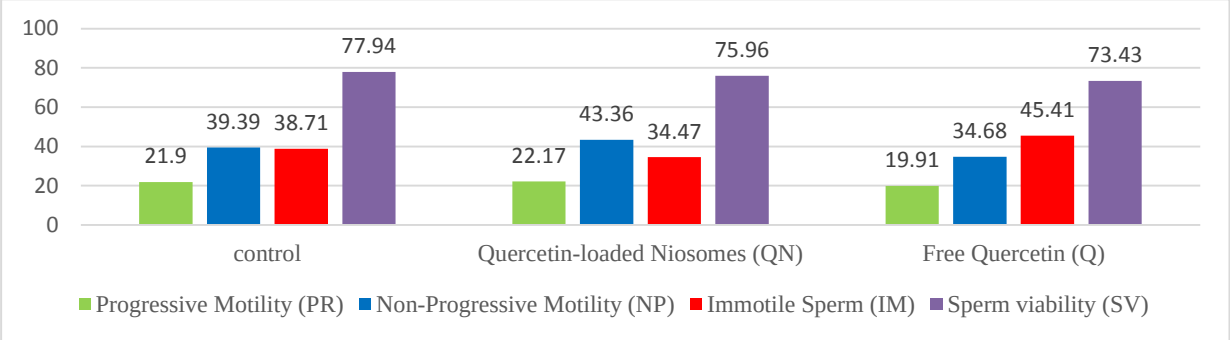
The results showed significant increasing ($p<0.01$) in sperm motility after Cryopreservation and thawing, depending on the concentration of free or Niosomes - bound quercetin. The highest percentage of (**PR**), (**NP**), and (**IM**) motility was obtained when using a 10% concentration of liposome-bound quercetin, with motility reaching 29.67, 46.24, and 24.09 respectively. This effect did not appear when using liposome-bound quercetin or free quercetin at concentrations of 0%, 15%, or 20%.

This may be attributed to quercetin's ability to maintain sperm motility and protect mitochondria from damage caused by (**ROS**). This effect may also be due to quercetin's interaction with the Ca^{2+} - ATPase enzyme, which is responsible for regulating sperm motility **[19]**. Intracellular calcium ion (**Ca^{2+}**) concentrations play a crucial role in regulating sperm motility by stimulating the production of cyclic adenosine monophosphate (**cAMP**), which in turn influences the production of adenosine triphosphate (**ATP**), the energy required for sperm movement. Mitochondria are the primary energy source in sperm and the main site of reactive oxygen species (**ROS**) production **[20]**. Therefore, adding quercetin to seminal fluid diluent helps protect mitochondria and maintain energy production, as well as reduce oxidative damage. This is due to quercetin's ability to penetrate cells and accumulate within mitochondria, thus helping to regulate ROS production **[21]**.

Quercetin is also a phytoestrogen, possessing stimulating and inhibitory effects on estrogen receptors, and it binds to these receptors located in the sperm tail **[22]**. Accordingly, quercetin is likely to influence the regulation of sperm motility **[23]**. Sperm motility was also reduced in samples treated with free quercetin or neosome-loaded at a concentration of 20% compared to the control group, as shown in Figure 3. Quercetin is an antioxidant, but its effect is dose- or concentration-dependent. At low and medium concentrations, quercetin can reduce

excess (**ROS**) in sperm, thus protecting sperm motility. However, at high concentrations, quercetin may convert from an antioxidant to a pro-oxidant under certain conditions, potentially increasing oxidative stress rather than reducing it [**24**].

Figure 2. Effect of adding 15% of Quercetin-loaded Niosomes (QN) or Free Quercetin (FQ) on the percentage of progressive motility (PR) ،Non-Progressive Motility (NP) ،Immotile Sperm (IM) and Sperm viability (SV) of goat epididymis sperm under cryopreservation conditions.



The low bioavailability of polyphenols, including quercetin, is known to be a major obstacle to their use. Therefore, loading these compounds into liposomes is a promising strategy for improving their solubility and stability, given liposomes' properties as efficient drug delivery systems for faster cell delivery. Mignet et al**. [13]**, in their review of published studies, indicated that liposome-loaded polyphenols were more effective in vitro than free polyphenols. This finding was confirmed by the results of our current experiment.

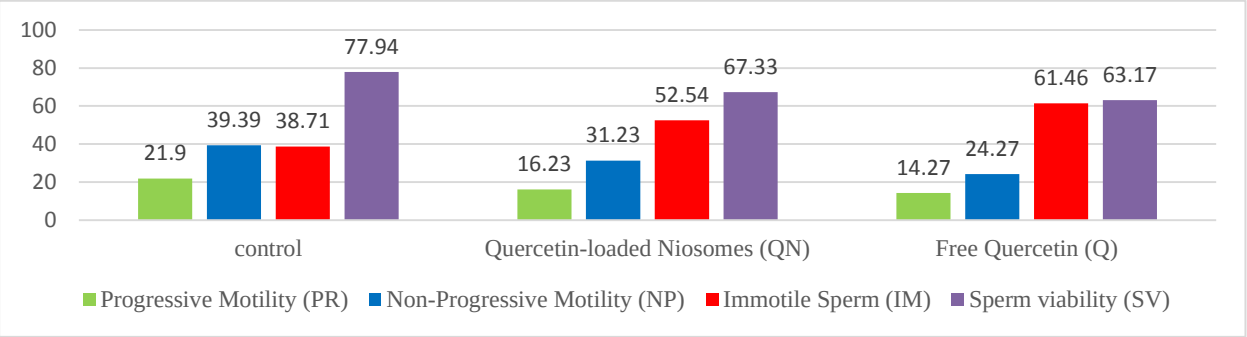
When comparing sperm viability between groups, the percentage of live sperm was found to be significantly higher ($P < 0.001$) in samples treated with liposome-bound quercetin compared to samples treated with free quercetin and the control treatment, as shown in Figures (1), (2) and (3). The results showed that there is an effect on sperm viability that depends on the concentration of free or Niosomes-bound quercetin. The highest percentage of sperm viability was obtained when using 10% liposome-bound quercetin, where sperm viability reached 87.65% when using this concentration, while this effect did not appear when using liposome-bound quercetin or free quercetin at a concentration of 0%, 15%, or 20%.

These results show that the addition of quercetin contributed to improving sperm viability in goats, possibly as a result of its role in improving seminal fluid qualities by reducing damage caused by oxidative stress and lowering levels of reactive oxygen species **[5]**. It is known that exposure of sperm to oxidative stress during the period of cryopreservation, particularly due to the oxidation of polyunsaturated fatty acids present in the plasma membrane, leads to an increase in the formation of lipid peroxides,

which causes changes in the structure of the sperm membrane, especially in the head region, and this is reflected negatively on sperm viability **[25]**.

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Figure 3. Effect of adding 20% of Quercetin-loaded Niosomes (QN) or Free Quercetin (FQ) on the percentage of progressive motility (PR) ،Non-Progressive Motility (NP) ،Immotile Sperm (IM) and Sperm viability (SV) of goat Epididymis epididymis sperm under cryopreservation conditions.



Conclusion

The results of the current study confirm the promising potential of (QLN) in protecting goat sperm from damage caused by oxidative stress and maintaining sperm structural integrity. Adding 10% (QLN) to seminal fluid diluent improved the viability and motility of epididymal sperm in frozen goats compared to the (FQ) group and the control group. This suggests that loading quercetin onto neosomes may contribute to improving its efficiency and availability in the preservation medium. Furthermore, the study results indicate that the effect of QLN is concentration-dependent. Low and medium concentrations showed a more favorable effect in maintaining the motility and viability of goat epididymal sperm, while high concentrations led to a decrease in some of the studied traits. This suggests that increasing the concentration of antioxidants does not necessarily equate to increasing their protective effect.

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