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Highlights

- Q loaded in liposomes shows lower toxicity than Q for sperm motility and viability
- Q loaded in liposomes may damage sperm DNA
- Q loaded in liposomes shows less antioxidant activity vs Q on sperm induced LPO

AbstracT

A strategy to circumvent the poor polyphenols bioavailability is to load these compounds into liposomes. We evaluated the in vitro effects of quercetin (Q) and Q-loaded liposomes (QLL, 30, 50, 100 μM) on motility, viability and chromatin integrity of swim-up selected human sperm. Antioxidant power was assayed against tert-butylhydroperoxide induced lipid peroxidation (LPO) using C11-BODIPY581/591 fluorescent probe and transmission electron microscopy. QLL showed decreased toxicity for sperm motility and viability and increased DNA damage compared to Q. The percentage of sperm with fluorescence, marker of LPO, was decreased in samples incubated with Q vs QLL ($P<0.001$). The ultrastructure of acrosomes and membranes was preserved with Q 30/100 μM, whereas QLL did not prevent membrane injury.

Q alone appeared more effective than Q incorporated into liposomes; however liposomes could be considered as carriers that may convey different compounds inside sperm; they may therefore represent a field of research rich of many applications.

Abbreviations:

BWW: Biggers Whitten Whittingham; C11-BODIPY581/5914: 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4-adiaza-s-indacene-3-undecanoic acid; DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine); DOPE (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine); DOPA (1,2-dioleoyl-sn-glycero-3-phosphate); DOTAP (1,2-dioleoyl-3-trimethylammonium-propane); Lactosyl PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lactosyl); LPO: lipid peroxidation; ROS: reactive oxygen species; Q: quercetin; TBHP: tert-butylhydroperoxide; TEM: transmission electron microscopy; WHO: World Health Organization.

Key words: lipid peroxidation, quercetin-loaded liposomes, sperm motility, sperm viability, sperm chromatin, TEM.

1. INTRODUCTION

Quercetin (Q) is a bioflavonoid, daily consumed in foods, widely distributed in fruits, vegetables and beverages, such as red wine, red onions, apple peel and tea and may exert positive effects on human health preventing lipid peroxidation (LPO), DNA oxidative damage and lowering blood pressure [1]. As for other flavonoids, a large body of evidence indicates that Q can be considered as a pharmacological agent for its ability to prevent several degenerative and chronic diseases, including cardiovascular problems and cancer, and for its antioxidant properties [2-4]. However, flavonoids show a sort of dichotomy: at a low concentration they exert antioxidant activity improving cell survival and at high doses they may increase cell death, inducing cytotoxicity, DNA strand breaks and apoptosis [5]. The antioxidant properties of Q have been extensively explored in the field of reproductive health and sperm production [6]. Oxidative stress hampers the normal sperm function, resulting in LPO of the sperm membrane and DNA fragmentation, all of which culminate in sperm dysfunction, decreased sperm motility and infertility [7]. Sperm are particularly vulnerable to the attack of the reactive oxygen species (ROS), since their membranes are rich in polyunsaturated fatty acid [8, 9].
The study of natural compounds with antioxidant properties is appealing since it can offer the possibility of therapeutic options for male infertility and the development of new strategies of media supplementation in vitro used for semen handling [7].

A comprehensive review of recent literature agrees in a protective effect of Q on testicular damage induced by several toxicants in animal models [10-18]; however, Ranawat et al. [19] observed that Q increased ROS generation and membrane LPO in the mouse testis. As regards to in vitro studies, Q improves sperm motility and zona binding ability of cryopreserved stallion sperm [20]. Q also shows a protective effect on sperm motility and viability in human sperm [21] and in rat epididymal sperm [22] after oxidative stress induction. In addition, a recent study reported that Q is effective against oxidative stress in rat spermatozoa stored at 15 °C [23].

Despite the putative health promoting properties of flavonoids, some studies have questioned the real in vivo efficacy of these compounds. In vivo, flavonoids seem to exhibit a small antioxidant activity, due to their scarce water solubility, weak absorption and rapid metabolism, which are all factors that determine their limited bioavailability [24]. An interesting strategy to circumvent the above-mentioned limitations consists in loading polyphenols into water-soluble carriers such as liposomes [24, 25]. The enhanced solubility of these compounds loaded into liposomes allows injecting of the active polyphenols into non-organic solvents with indubitable benefits in in vivo administration and in vitro applications. Specifically, regarding Q-loaded liposomes, Jangde and Singh [26] prepared a formulation aimed at achieving sustained release of the drug in wound areas.

In addition, liposomal Q may have a potential application for the treatment of tumor, since it reduced the proliferation in vitro of human MCF-7 carcinoma cells [27]. The present study was designed to compare, in vitro, the antioxidant properties of Q and Q-loaded liposomes on LPO and DNA damage induced in human ejaculated sperm by tert-butylhydroperoxide (TBHP). The oxidative damage was evaluated in samples incubated with and without Q and Q-loaded liposomes, using C11-BODIPY581/591 probe that highlights LPO, and acridine orange (AO) test that enables the evaluation of chromatin integrity. To validate the quoted methods, the specimens were examined by transmission electron microscopy (TEM). Finally, sperm motility and viability of samples treated with free Q were compared with those evaluated in samples incubated with Q-loaded liposomes.

2. MATERIALS AND METHODS

2.1 Quercetin and quercetin-loaded liposome preparation

Quercetin was purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland) and used without further purification. Stock solutions were stored at -20 °C.

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPE (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine), DOPA (1,2-dioleoyl-sn-glycero-3-phosphate), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and Lactosyl PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lactosyl) were purchased from Avanti Polar Lipids Inc., Alabaster, AL, USA.

2.1.1 Liposome preparation

DOPC/DOPE, DOPE/DOPA, DOTAP/DOPE and DOPE/Lactosyl PE liposomes were prepared at 1:1 mol ratio with a total lipid concentration of 1.0×10^{-2} M. Q-loaded liposomes were prepared to obtain the final molar ratio lipid/Q of (1:0.5).

Liposomes were prepared in a round bottom vial by mixing the appropriate amounts of stock solutions, which were 4x10-2 M in chloroform for lipids and 2x10-2 M in ethanol for Q. A dry lipid film (with or without Q) was obtained by evaporating the solvent under vacuum overnight. Rehydrating with Milli-Q grade H2O yielded multilamellar dispersion. At the moment of vortex, multilamellar vesicles were obtained, which were then submitted to eight freeze/thaw cycles. This method improved the homogeneity of the size distribution in the final suspension. Liposomes were subsequently reduced in size and converted to unilamellar vesicles by extrusion through 100 nm polycarbonate membranes. Twenty-seven extrusions were performed with the LiposoFast apparatus (Avestin, Ottawa, Canada). All liposomes were stored at 4 °C.

2.1.2 Size and surface charge of liposomes

The size and surface charge of the plain, functional loaded liposomes were measured by Dynamic Light Scattering, DLS, (Coulter Sub-Micron Particle Analyzer N4SD, equipped with a 4 mW helium-neon laser and 90u detector) and Zeta potential (Coulter DELSA 440 SX), respectively.

Liposome dispersions were diluted with TBS pH 7.40 and ζ-potential values were measured at 25 °C. As the radii of liposomes were always large enough compared with the Debye–Huckel parameters, the ζ-potentials were calculated directly by means of the Helmoholtz–Smolowkovski equation (by the zetasizer) [28]. Size was also calculated in the same experiments, according to the procedure described by Langley [29].

2.1.3 Encapsulation efficiency (EE)

The EE data was obtained by UV-VISIBLE spectra, recorded at 25 °C with a Perkin-Elmer Lamda 25 spectrophotometer (10 mm cuvettes). Prior to spectra recording, liposome disruption was carried

out in order to get rid of the scattering background (scaling as λ-4), due to large aggregates in solution, which can affect precise intensity evaluation. To disrupt liposomes and release the entrapped Q, samples have undergone several cycles of freezing (-32°C).
A calibration curve was built by measuring the absorbance of solutions with known Q concentration at 375 nm.

2.2 Semen analysis

Semen samples were obtained from healthy men (age 20-35 years) recruited at Department of Molecular and Developmental Medicine, University of Siena. The subjects were informed of and gave a written consent for the procedures related to the study. Semen specimens were collected by masturbation after 3–5 days of sexual abstinence and, after liquefaction at 37 °C for 30 min, they were examined. Volume, pH, concentration and motility were evaluated according to World Health Organization guidelines [30].

2.3 Sperm selection: swim-up

Swim-up technique has been used to obtain motile sperm fraction: 1 ml of each semen sample was loaded in a sterile conical centrifuge tube and gently layered with 1.2 ml of Biggers Whitten Whittingham media [BWW;30]. The tubes, inclined at a 45° angle, were incubated at 37°C in an atmosphere containing 5% of CO2 for 45 min. One ml of the uppermost media, which contained highly motile sperm, was then recovered.

2.4 Sperm motility and viability and after Q and Q-loaded liposomes incubation

Swim-up selected sperm were incubated with different concentration of Q and Q-loaded liposomes (DOPC/DOPE, DOPE/DOPA, DOTAP/DOPE and DOPE/Lactosyl PE liposomes: 20, 30, 50, 100 μM) diluted in BWW media. Mixtures were incubated at 37 °C in atmosphere with 5% of CO2 for 1 h.

Sperm motility was assessed using a Burker counting chamber. The spermatozoa were classified as: sperm with progressive motility, sperm with non-progressive motility and immotile spermatozoa [30].

For viability evaluation, the sperm were stained with 0.5% Eosin Y (CI 45380) in 0.9% sodium chloride aqueous solution and observed by light microscope (Leica, Wetzlar, Germany). Unstained (viable) and stained (dead) cells were scored. Aliquots of sperm without Q and Q-loaded liposomes treated in the same conditions were used as controls. All experiments were carried out in triplicate and results were reported as mean values and standard deviation (SD).

2.5 Evaluation of chromatin quality: Acridine Orange (AO) Test

Acridine orange (AO) test measures the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). AO tests were performed as previously reported by Tejada et al. [31].

A stock solution of 0.1% AO (3, 6-bis [dimethylamino] acridine, hemi [zinc chloride] salt, BDH Chemicals Ltd, Poole, England) was prepared and stored in the dark at 4 °C until use. The working solution was prepared by mixing 4 parts of AO stock solution with 16 parts of 0.1 M citrate and 1 part 0.3 M Na2HPO4 7H2O. A drop of each treated sample was smeared onto a glass slide. The slides were fixed overnight in Carnoy fixative (methanol acetic acid 3:1) then were washed in distilled water, allowed to air-dry for few minutes and stained for 5 min. Then the slides were gently rinsed with distilled water, mounted and immediately evaluated with a Leitz Aristoplan fluorescence Microscope (Leica, Wetzlar, Germany) equipped with a 490 nm excitation light and 530 nm barrier filter. Nuclei from 300 spermatozoa were examined and scored as green or red (sometimes orange-yellow) depending on fluorescence. When the head showed green fluorescence, sperm were considered normal (double-stranded DNA); if the head displayed a spectrum of yellow-orange to red fluorescence, the DNA was considered denatured (single-stranded DNA). Results were expressed as the percentage of the sperm that showed normal DNA (green fluorescence). These experiments were performed in triplicate and the results were reported as mean and standard deviation.

2.6 LPO induction and Q and Q-loaded liposomes treatment

Selected human sperm were incubated as previously reported [21] with a fluorescent fatty acid probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4-adiaza-s-indacene-3-undecanoic acid (C11-BODIPY581/591, Molecular Probes, Eugene, USA) able to intercalate within the phospholipid bilayer. The intact probe fluoresces red when it is intercalated in the membrane and it shifts to green after oxidative radical attack.

After C11-BODIPY581/591 treatment, each sample was divided into aliquots, which were formed by sperm treated with TBHP (45 µM) and Q (30, 50, 100 µM) or Q-loaded liposomes (30, 50 100 µM) and sperm treated with TBHP alone. The specimens were incubated at 37 °C in an atmosphere with 5% of CO2 for 1 h. The stained specimens were examined by means of Leitz Aristoplan fluorescence Microscope (Leica, Wetzlar, Germany). The presence of the green signal, a marker of LPO, was scored as high and low fluorescence. For each sperm sample, a minimum of 300 cells

were examined. All experiments were carried out in triplicate and results were reported as mean values and SD.

2.7 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed in the following specimens: 1- sperm and TBHP; 2- sperm, TBHP and Q (30 µM); 3- sperm, TBHP and Q-loaded liposomes (30 µM); 4- sperm TBHP and Q (100 µM); 5-sperm Q-loaded liposomes (100 µM). Sperm samples were fixed and processed as previously reported [21]. Ultra-thin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate and observed and photographed with a Philips EM208 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands). A minimum of 300 different sperm sections were analysed for each sample and the anomalies related to the acrosome (reacted, swollen), the chromatin (disrupted), the axoneme (disorganized) and the plasma membrane (broken) were quantified. These experiments were performed in triplicate and the results were reported as mean and SD.

2.8 Statistical analysis

The correlations between Q and Q-loaded liposome concentrations vs. the percentages of sperm viability and motility were evaluated by Spearman correlation coefficient (r). The comparisons between the variables, such as sperm viability, sperm motility and percentages of sperm with green fluorescence obtained after Q and Q-loaded liposome treatment at different concentrations and controls (sperm samples without Q and Q-loaded liposomes and processed in the same experimental conditions), were calculated by means of Friedman test [32, 33]. When its results were worthy of further consideration, Dunn’s post hoc test was accomplished [34].
The comparison of sperm viability, sperm motility and percentages of sperm with green fluorescence (AO test) between samples treated with Q and Q-loaded liposomes for each concentration was performed by Mann-Whitney U test [35].
The comparisons between the percentage of sperm with high fluorescence (C11-BODIPY581/591 assay) after treatment with TBHP alone and TBHP with Q and Q-loaded liposomes were performed with Kruskal-Wallis test [36]; when the results of this test were significant, Mann-Whitney U post hoc test was used to compare the values related to the different treatments with those obtained by incubating sperm with TBHP alone. The same statistical procedure was applied to compare the values obtained by TEM and related to the alterations of sperm organelles after incubation with TBHP alone and TBHP with both Q formulations. For all applied tests, a P value < 0.05 (two-tailed) was considered statistically significant. All the analyses were performed using SPSS statistical software version 20 (SPSS Inc., Chicago, IL, USA) or GraphPad software version 5 Inc. for Windows.

3. RESULTS

3.1 Liposome size and surface charge

Results of the size distribution and surface charge values of Q-free liposomes and Q loaded liposomes are presented in Table 1. In all cases, there was a slight increase in liposome mean diameter when Q was incorporated. The difference in the mean diameter of Q-free liposomes and Q loaded liposomes was small indicating that Q can be well loaded in the lipid bilayers. The low polydispersity indexes (P.I.) showed that the liposomes were not altered by the interactions with Q. The Q-loaded liposomes remained monodisperse. Since the aggregation may be used as a measure of liposome physical stability, we may conclude that the incorporation of Q in all different liposome bilayers does not modify their physical stability. Analyzing the data reported in Table 1 we can observe that empty DOPC/DOPE liposomes had a small negative Zeta potential, though the net polar head charge of zwitterionic phospholipids was zero. The incorporation of Q did not change the net surface charge of the liposomes. The same behaviour was evident for all the liposome preparations. This data demonstrated the incorporation of Q in the lipid bilayers of all three types of liposomes: zwitterionic, cationic and anionic.

3.2 Encapsulation efficiency (EE)

In order to study the effect of the loading on the activity of Q, UV-visible analysis was carried out after the encapsulation step (as reported in materials and methods section). This experimental data is essential in order to calculate the EE (Table 2). The EE values were not high for all formulations. We observed non-significant differences in EE of Q between zwitterionic, cationic and anionic liposomes.

3.3 Experimental studies

First of all, swim up selected sperm were treated with four liposomes: DOPC/DOPE, DOPE/DOPA, DOTAP/DOPE and DOPE/Lactosyl PE liposomes. Since DOPE/DOPA, DOTAP/DOPE and DOPE/Lactosyl PE liposomes negatively influenced sperm motility and viability, for this reason we performed our experiments using only DOPC/DOPE liposomes.

Then the effects of different concentrations of Q and Q-loaded liposomes (DOPC/DOPE) on sperm viability, motility and chromatin integrity were assessed in swim-up selected sperm. We then studied, using C11-BODIPY581/591 and TEM, the antioxidant activity of these two formulations in the same sperm samples treated with TBHP, which is able to induce the oxidative stress.

3.3.1 Effect of Q and Q-loaded liposomes on sperm viability and motility

The percentages of viable sperm after treatment with different concentrations of Q and Q-loaded liposomes are showed in Figure 1. A significant dose dependent effect on sperm viability was observed for Q ($r= -0.95$, $P<0.001$), but not for Q-loaded liposomes ($r= -0.443$, $P=0.149$). In addition, the sperm viability in samples incubated with Q at 100 μM were significantly reduced (Friedman test: $P<0.01$; Dunn test: $P<0.05$) compared to the control sample; no significant differences in sperm viability were detected between untreated samples and samples treated with different concentrations of Q-loaded liposomes (Friedmann test: $P= 0.0747$). When we compared the viability percentage between sperm treated with Q and with Q-loaded liposomes, we observed that this parameter was significantly higher in specimens treated with Q-loaded liposomes that in those incubated with Q (30 μM: $P<0.01$; 50 μM and 100 μM: $P<0.001$; Figure 1).

The effects of Q and Q-loaded liposomes used at different concentrations on the motility of swim-up selected sperm are reported in Figure 2.

A dose dependent effect on sperm motility was observed for Q ($r= -0.972$, $P<0.001$), but not for Q- loaded liposomes ($r= -0.479$, $P=0.115$). As observed for the sperm viability, the sperm progressive motility was significantly reduced (Friedman test: $P<0.01$; Dunn test: $P<0.05$) in specimens treated with Q at 100 μM vs controls, whereas no differences were observed between samples treated with different concentrations of Q-loaded liposomes and controls (Friedman test: $P=0.0747$).

At each concentration used the motility percentage of sperm treated with Q-loaded liposomes was increased in respect to that observed in samples incubated with Q (30 μM and 50 μM: $P<0.01$; 100 μM: $P<0.001$; Figure 2). These results were in agreement with those obtained with Eosin Y staining that assessed the sperm viability.

3.3.2 Chromatin integrity assessment

The results were expressed as the percentage of the sperm that showed normal DNA (double stranded DNA, green fluorescence, Figure 3). The percentage of sperm with double stranded DNA decreased in a dose dependent manner in specimens treated with Q-loaded liposomes ($r= - 0.95$: $P<0.001$).

A significant decrease of sperm with green fluorescence was observed in samples treated with 100 μM Q-loaded liposomes (Friedman test: $P<0.001$; Dunn test: $P<0.01$) vs controls (Figure 3), whereas Q at the tested concentrations did not show any effect on DNA integrity. A significant decrease in percentage of sperm with green fluorescence was detected in samples treated with Q-

loaded liposomes at each tested concentration ($P<0.05$) vs. the percentage of sperm incubated with Q.

3.3.3 Effect of Q and Q-loaded liposomes on induced LPO detected by C11-BODIPY581/591 and TEM

As reported in other papers, the LPO localization identified by C11-BODIPY581/591 probe is mainly evident in the mid-piece region. Compared to samples treated with TBHP only, the percentage of sperm with highly intense fluorescence was significantly decreased in specimens incubated with Q 30 μM, 50 μM and 100 μM ($P<0.001$) and with 100 μM Q-loaded liposomes ($P<0.05$; Figure 4).

It is noteworthy that for each concentration examined, the percentage of sperm with high fluorescence was significantly decreased in samples treated with Q vs those incubated with Q-loaded liposomes ($P<0.001$; Figure 4).

TEM analysis enables to explore the ultrastructural characteristics of sperm samples treated with TBHP (Figure 5a), TBHP+ Q (30 μM and 100 μM) and TBHP + Q-loaded liposomes (30 μM and 100 μM). Means and standard deviations of the percentages of the most frequent alterations, such as reacted or swollen acrosome, disrupted chromatin, broken plasma membrane and disorganized axoneme are reported in Table 3 where statistics and P values are also shown.

The percentage of reacted acrosome was significantly reduced in sperm treated with all Q combinations (except for 30 μM Q-loaded liposomes) compared to that observed in samples incubated with TBHP only; the characteristic of reacted acrosome was significantly reduced in sperm treated with Q 30 μM vs sperm treated with Q-loaded liposomes at each concentration (Figure 5 b, c) and in sperm treated with Q 100 μM (Figure 5 d) vs sperm incubated with Q 30 μM and with Q-loaded liposomes (Figure 5 b, c). One hundred μM Q-loaded liposomes showed a better protective effect on the acrosome in respect to that exerted by 30 μM Q-loaded liposomes (Table 3). The percentage of swollen acrosome was significantly increased in samples treated with Q-loaded liposomes at 100 μM (Figure 5 c, Table 3).

The percentage of disrupted chromatin was significantly higher in sperm treated with TBHP compared to that observed in sperm incubated with all Q formulations (Table 3).

The percentage of broken plasma membrane was significantly higher in samples treated with TBHP vs all the Q formulations and in sperm treated with 30 and 100 μM Q-loaded liposomes vs those incubated with Q 30 μM and 100 μM.

The percentage of disorganized axoneme was similar in the two Q-loaded formulations and was significantly higher in samples treated with TBHP compared to that observed in specimens treated with all the Q formulations (Table 3).

4 DISCUSSION

Among different flavonoids, those important compounds involved in the prevention of several diseases, Q is, perhaps, the most studied molecule and is known for its ability of counteracting oxidative stress in the spermatogenetic process and in spermatozoa both in in vivo and in vitro systems.

As regards the relationship between Q and the male reproductive system, this flavonoid has been administered in animal models with induced testis damage [10, 12-18]; it was also used as a supplement in in vitro studies in human and animal sperm models to explore its antioxidant properties [21-23]. In addition, other researches focused on the protective effect of Q during cryopreservation and thawing processes of spermatozoa from several mammals including man [20, 37, 38]. In our previous work, we observed the real efficacy of Q against the TBHP-induced LPO on human spermatozoa and we suggested its potential use as supplementation of sperm culture media that could preserve sperm from ROS damage during mechanical procedures in vitro [21].

The poor bioavailability of polyphenols, including Q is well known, and a strategy to overcome solubility and stability limitations may consist in loading these compounds in liposomes, excellent candidates for drug delivery purposes. Mignet et al. [24], reviewing the literature, reported that in vitro polyphenols loaded in liposomes were more effective than those used alone.

Many researches highlighted particularly the role of Q-loaded liposomes as a potential new therapeutic approach in treating different pathologies. [26, 27]. Taking into consideration these observations, we designed our research aiming to test the ability of Q-loaded liposomes compared to Q administered alone in preventing oxidative stress induced in vitro in swim up selected human sperm. We have already tested the effect of Q and other polyphenols on induced LPO in human sperm cells and the results of the present study, concerning the use of Q, confirmed those observations [21]. However, the results related to Q-loaded liposomes are novel as they were described for the first time in human spermatozoa.

Firstly, we tested the effect of different unloaded liposomes on sperm motility and viability and the experiments performed with DOPC/DOPE liposomes, the only model that did not affect the studied parameters. As far as we know until now there was very little data available on the relationship between surface charge of liposomes and human spermatozoa; however it is well known that this liposomal characteristic influences many biological processes, including the liposome uptake in blood vessels affected by angiogenic solid tumors [39] and their binding and endocytosis in human ovarian carcinoma cell and murine macrophage cell lines [40]. Studies showed that negatively

charged liposomes are endocytosed faster and to a greater extent than neutral liposomes by phagocytotic cells. Negative surface charges can be recognized by receptors found on a variety of cells, including macrophages [40]. Cationic liposomes showed cytotoxic effects, they induced apoptosis in different cell lines [41, 42] and influenced ROS generation [43, 44].

In the present study, we observed a dose dependent effect of Q on sperm viability and motility as reported also by other studies [21, 45], but the mechanism of action was not completely clarified. Williams and Ford [46] proposed a possible interaction between Q and a Ca2+-ATPase, an important enzyme involved in sperm motility regulation. Q-loaded liposomes showed a lower toxicity for sperm in term of motility and viability than that observed in samples treated with Q.

Q is a phytoestrogen with both estrogen-agonist and antagonist effects and it binds estrogen receptors (ERs) [47] that are located in human sperm tail. Based on these observations, it is reasonable to postulate that Q may regulate sperm motility [48]. Different is the case of Q-loaded liposomes, since liposomes plausibly deliver Q inside the cell, avoiding the direct contact of Q with ER receptor located on the plasma membrane; consequently, the motility is not affected by increased Q concentrations. The release of Q inside the sperm by liposomes may explain also the observation that double stranded DNA decreases in a dose dependent manner in specimens treated with Q-loaded liposomes, whereas the DNA integrity in the presence of free Q was preserved, since Q acts outside the sperm and it does not reach DNA. Q shows a weak mutagen activity in in vitro assays and possesses both anti- and pro-oxidant activity depending on the dose at which Q is utilized [49, 50]. Probably, the direct contact between Q delivered by liposomes and sperm DNA may cause the DNA alterations highlighted by AO test.

Although Q-loaded liposomes showed no toxicity for sperm viability and motility even at high concentrations, they were definitely less efficient than Q alone to protect sperm against LPO induced by TBHP. One hundred μM Q-loaded liposomes only showed a weak protective effect compared to the activity of free Q, but we observed that this concentration has genotoxic effect on sperm DNA. Q, also at the lowest concentration, was absolutely the most powerful formulation in contrasting oxidative damage induced by TBHP, as reported in a previous research [21]. The observed phenomena were confirmed by TEM that enables to perform the ultrastructural analysis of different sperm organelles and to determine the protective effects of free Q and Q-loaded liposomes. All the tested Q formulations were active in protecting axonemal and periaxonemal structures; flagellum is molded by proteic rods that confer stability and resistance and probably it is the most resistant sperm structure towards the damage induced by TBHP.

Acrosome and plasma membrane were equally preserved in the presence of 30 μM and 100 μM Q, whereas in the presence of Q-loaded liposomes they were damaged in high percentage. Chromatin integrity was protected by all the tested formulations; it is then evident that DNA denaturation, as detected by AO test in sperm treated with 100 μM Q-loaded liposomes, is not detectable by TEM analysis. It is noteworthy the observation that 100 μM Q-loaded liposomes showed less powerful activity in contrasting LPO than that showed by 30 μM Q alone, confirming that Q was the most effective formulation against oxidative stress induced in human ejaculated sperm. In particular, the extent of damage such as acrosome-reacted sperm and broken plasma membrane were partially reduced with 100 μM Q-loaded liposomes, even if the structure of the acrosome was not preserved, as shown by the high percentage of swollen acrosomes.

5. CONCLUSIONS

What is the reason to study the antioxidant ability of flavonoids and different ways of their administration to prevent or reduce the oxidative stress induced in vitro in human spermatozoa? Semen handling, semen cryopreservation, centrifugation are all procedures that induce the production of free radicals. For this reason our interest was oriented at the study of natural compounds that can be added to media in order to counteract the effect of free radicals, in particular during the protocols related to assisted fertilization.

Q and Q-loaded into liposomes showed different in vitro effects on sperm motility, viability, DNA integrity and in their ability to counteract LPO induced by TBHP. Respect to free Q, Q-loaded liposomes showed decreased toxicity for sperm motility and viability, increased genotoxicity, especially if used at high concentrations, and they were not active against induced LPO in human sperm. The different behavior of Q and Q-loaded liposomes could be explained by a different mechanism of action: Q may act outside the cell modulating sperm motility and counteracting LPO at plasma membrane level, the liposomes plausibly deliver Q inside the sperm cell for this reason the antioxidant effect of Q on induced LPO is decreased and the negative effect on DNA is evident.

For the purposes of the present study, Q appeared more effective than liposome formulation. However, these biocompatible carriers with tunable physic-chemical properties and loaded with substances of different nature should be taken into account in carrying different compounds inside the human sperm cells, as it could be a field of research with a large amount of perspective.

Conflict of interest

The authors declare that there were no conflicts of interest.

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Figure captions

Figure 1. Mean and SD of the percentages of viable sperm treated with Q-loaded liposomes and Q at different concentrations. 0 µM = controls, same conditions without flavonoid. Q 30µM vs Q-loaded 30µM: **P<0.01; Q 50-100 µM vs Q-loaded 50- 100 µM: ***P<0.001.

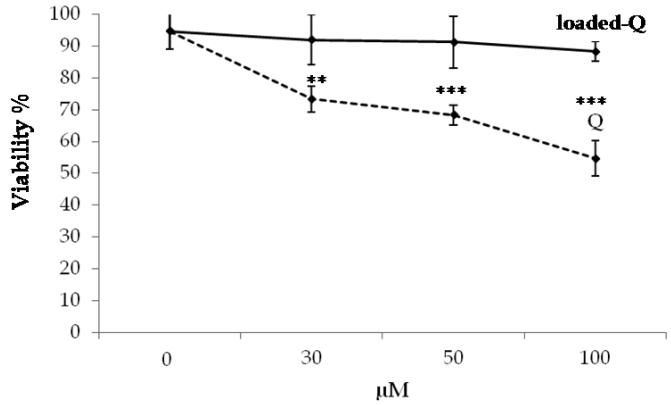

Figure 2. Mean and SD of the percentages of progressive motility in sperm treated with Q-loaded liposomes and Q at different concentrations. 0 µM = controls, same conditions without flavonoid. Q 30-50µM vs Q-loaded 30-50µM: **P<0.01; Q 100µM vs Q-loaded 100µM: ***P<0.001.

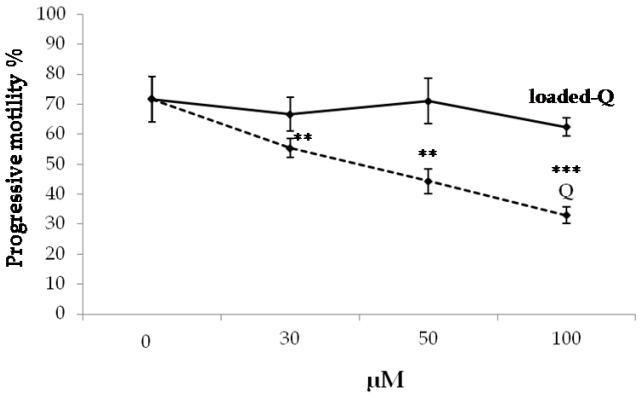

Figure 3. Mean and SD of the percentages of human sperm labeled with green fluorescence (AO test), expressing double stranded DNA, in samples treated with Q-loaded liposomes and Q at different concentrations. 0 µM = control, same conditions without flavonoid. Q 30-100 µM vs Q-loaded 30-100 µM: *P<0.05.

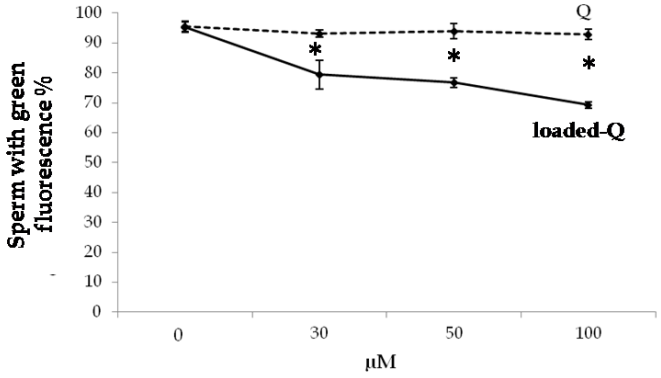

Figure 4. Mean and SD of the percentages of sperm showing high level fluorescence (C11-BODIPY581/591probe) indicating LPO induced by TBHP. Sperm were treated with TBHP only, TBHP with 30 µM, 50 µM and 100 µM Q-loaded liposomes and TBHP with 30 µM, 50 µM and 100 µM Q alone. P<0.05: Q loaded 100 µM vs TBHP; P<0.001: Q 30-100µM vs TBHP and vs Q-loaded liposomes.

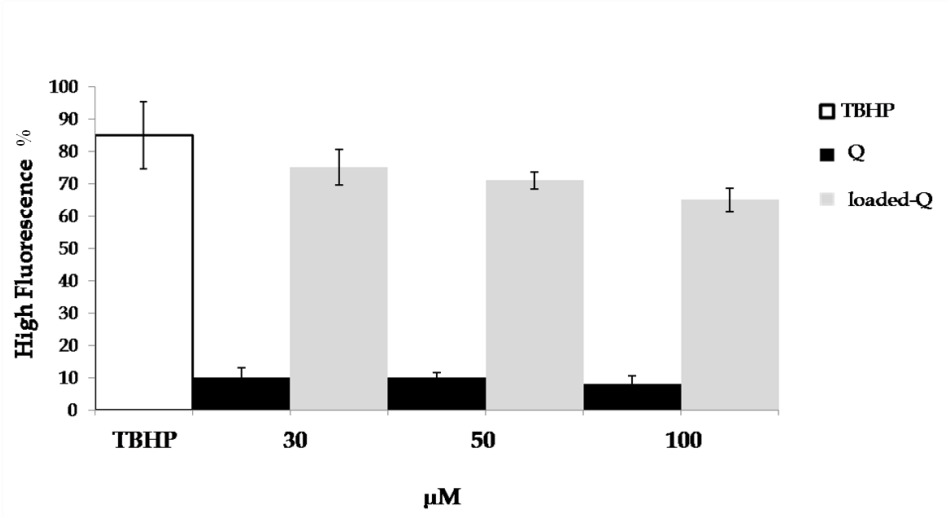

Figure 5. (a, b, c, d) TEM micrographs of human sperm treated with TBHP alone, TBHP with 30 µM and 100 µM Q-loaded liposomes, 30 µM and 100 µM Q alone. (a) Sperm incubated with TBHP; (b) sperm incubated with TBHP+30 µM Q loaded liposomes; (c) sperm incubated with TBHP+100 µM Q-loaded liposomes; (d) sperm incubated with TBHP+100 µM Q. A: normal acrosome; aA: absent acrosome; sA: swollen acrosome; rA: reacted acrosome; N: nucleus; dCh: disrupted chromatin. Bars: a, c) 1µm; b, d) 2µm

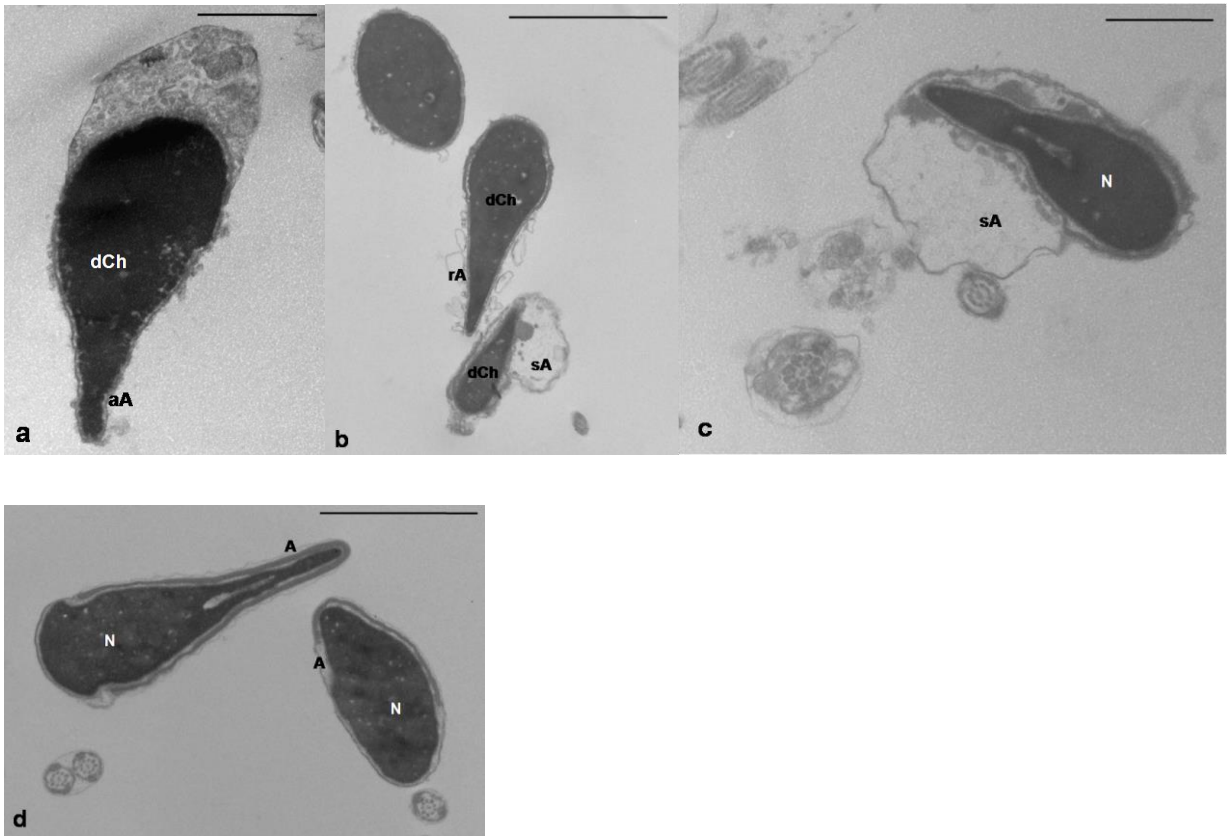

Table 1 Size and surface charge of Q-free liposomes and Q-loaded liposomes obtained by extrusion through 100 nm polycarbonate membranes

Liposome Composition	Size (nm)± SD	P.I.	Zeta potential (mV)± SD
DOPC/DOPE	105 ± 15	0.16	-11 ± 5
DOPC/DOPE + Q (1:0.5)	113 ± 10	0.20	-19 ± 7
DOPE/DOPA	109 ± 13	0.17	-37 ± 2
DOPE/DOPA + Q (1:0.5)	125 ± 21	0.21	- 32 ± 5
DOPE/DOTAP	115 ± 12	0.15	48 ± 5
DOPE/DOTAP + Q (1:0.5)	124 ± 15	0.19	50 ± 9
DOPE/L-PE	120 ± 17	0.18	-23 ± 5
DOPE/L-PE + Q (1:0.5)	138 ± 23	0.19	- 27 ± 6

PI: polydispersity index

Table 2. Encapsulation efficiency (EE) of Q in liposomal formulation (mean ± SD, n=3 experiments)

Liposome Composition	Encapsulation Efficiency (EE) %
DOPC/DOPE + Q (1: 0.5)	18.9 ± 5.3
DOPE/DOPA + Q (1: 0.5)	14.2 ± 1.9
DOPE/DOTAP + Q (1: 0.5)	15.6 ± 1.5
DOPE/L-PE + Q (1: 0.5)	13.8 ± 2.0

Table 3

Mean and SD of ultrastructural alterations detected by TEM in spermatozoa treated with TBHP alone and TBHP with Q 30 µM and 100 µM and Q-loaded liposomes 30 µM and 100 µM. Statistics are also showed. The significant values reported in the last column were obtained by applying the Mann Whitney U-test among the pairs of groups.

						Statistics
Variables	TBHP (1)	TBHP Q30 (2)	TBHP LQ30 (3)	TBHP Q100 (4)	TBHP LQ100 (5)	Kruskal-Wallis Test (p-value)
Reacted acrosome	85.33±5.24	7.33±2.06	79.67±7.23	4.17±2.4	40±4.19	0.0000
Swollen acrosome	12.33±2.94	13±3.03	12.66±3.01	12.66±2.42	46.67±6.02	0.0070
Disrupted chromatin	58.33±11.93	16±2.83	20.33±8.26	14.66±4.67	19±6.89	0.0001
Broken membrane	83±6.55	20.33±2.08	40.23±6.43	18.33±4.16	39±7.94	0.0147
Disorganized axoneme	54±3	23.33±3.51	28±3.6	21±4.58	28.66±5.68	0.0396

- *P< 0.05; ** P< 0.01
- Legend: TBPH: tert-butylhydroperoxide; Q: quercetin; L: liposome