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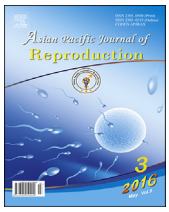

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Ameliorative potentials of quercetin against cotinine-induced toxic effects on human spermatozoa

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ABSTRACT

Objectives: Cotinine, the principal metabolite of nicotine found in smokers' seminal plasma, has been shown to adversely affect sperm functionality while quercetin, a flavonoid with diverse properties is associated with several *in vivo* and *in vitro* health benefits. The aim of this study was to investigate the potential benefits of quercetin supplementation against damage caused by the by-products of tobacco smoke in human sperm cells.

Methods: Washed human spermatozoa from 10 normozoospermic donors were treated with nutrient medium (control), quercetin (30 μmol/L) and cotinine (190 μg/mL, 300 ng/mL) with or without quercetin for 60 and 180 min incubation periods. Computer-aided sperm analysis was used to assess sperm motility while acrosome-reacted cells were identified under a fluorescent microscope using fluorescein isothiocyanate-labelled Pisum Sativum Agglutinin as a probe, viability was assessed by means of a dye exclusion staining technique (eosin/nigrosin) and oxidative stress by flow cytometry using dihydroethidium as a probe. Values were expressed as mean ± S.E.M. as compared by ANOVA.

Results: Higher cotinine concentrations reduced the number of viable cells after 60 and 180 min of exposure while viability of cells was increased in the cotinine aliquots supplemented with quercetin after 180 min of exposure when compared with cotinine only treated group.

Conclusion: This study indicates that the ameliorating ability of quercetin on cotinine-induced decline in sperm function is associated with increased number of viable cells.

1. Introduction

According to the World health organization, approximately 1/3 of the world's population over the age of 15 actively smoke tobacco [1]. The negative effect of cigarette smoking is widespread across populations due to the fact that not only active smokers (first hand smokers) but also second-hand smokers (passive smokers) display detrimental physiological effects due to the immense amount of harmful chemicals released as a result of tobacco combustion and inhalation. Sperm motility, capacitation and acrosome reaction are imperative in the successful fertilization of the female oocyte. The acrosome is a serine protease (acrosin) containing compartment which sheds when the sperm cell comes into contact with the zona pellucida, enabling the sperm cell to penetrate and fuse with the oocyte membrane [2,3]. This means that only acrosome-intact sperm cells are able to digest the zona pellucida and thus penetrate the oocyte, the appropriate timing of this reaction is required for fertilization to occur. It has been shown that toxins and chemicals have the ability to prematurely induce this reaction and subsequently reduce fertilizing capacity of these cells [4].

Cigarette smoke contains many dangerous compounds that are carcinogens and mutagens, which can directly affect spermatozoa, therefore decreasing male fertility [5]. Furthermore, it has been proven that exposure to first and second hand smoke causes measurable quantities of cotinine in seminal plasma [6].

Nicotine is the main component of cigarettes which is responsible for tobacco's addictive properties; it is an extremely toxic organic compound containing nitrogen and alkaloid and it is metabolized by humans into many other compounds in the body. Cotinine is the principal metabolite derived from nicotine and is usually found in higher volumes in the body when compared to nicotine [7]. Cotinine causes negative effects on

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sperm motility, membrane function, and fertilizing capacity [8,9]. It has a much greater half-life (10–37 h) than nicotine (1–2 h) and is present at approximately 15 times the concentration of nicotine in plasma [6,10,11]. Both substances are collectively attributed to increased levels of oxidative stress in seminal plasma, which is believed to be the leading cause of male infertility [12]. An increase in oxidative stress induces morphological deformations, DNA, membrane and protein damage. The increase in oxidative stress is caused by excessive productions of reactive oxygen species (ROS) and/or decreases in antioxidant defences mechanisms [13]. Antioxidants are produced by the body, nevertheless exogenous influence are a viable option when high levels of free radicals are present. Quercetin is a flavonoid that has been shown to have anti-carcinogenic, anti-inflammatory and anti-viral actions. Studies have also shown that quercetin can effectively decrease DNA damage, oxidative stress levels, inflammatory responses and lipid peroxidation caused by nicotine supplementation in circulatory systems of humans and rats [11,14].

Considering the fact that studies have shown that cotinine cause increases in oxidative stress and these subsequently have potential deleterious effects on spermatozoa, it is hypothesized that a known antioxidant will reduce these levels of oxidative stress and therefore reduce the potential damage to sperm cells. Therefore, the aim of this study was to investigate the potential benefits of quercetin supplementation against damage caused by cotinine in human sperm cells.

2. Materials and methods

2.1. Chemicals

Cotinine, fluorescein isothiocyanate-labelled Pisum Sativum Agglutinin (FITC-PSA), quercetin (isolated in its aglycon form), Hams F10 medium containing 3% bovine serum albumin (HAMS-BSA) and phosphate-buffered saline (PBS) were obtained from Sigma–Aldrich Pty. Ltd (St Louis, MO, USA). The cotinine and quercetin solutions were prepared and stored at 4 °C and kept in dark containers to prevent light exposure. Eosin and nigrosin were obtained from Fertipro (NV, Belgium). The dihydroethidium (DHE) was obtained from Molecular Probes, Invitrogen (Mount Waverley, Australia).

2.2. Sample preparation

Healthy donors between the ages of 19–25 were recruited and being an active smoker was the only exclusion criteria, they all provided informed consent for the research protocol that received IRB approval (Tygerberg, South Africa). Fresh semen samples were obtained by masturbation after 2–7 d of sexual abstinence. These samples were required to display functional parameters above the lower limits set forth by the WHO (2010) which are regarded as fertile, concentration ($\geq 15 \times 10^6$ cells /mL), volume ($\geq$ 1.5 mL) and total concentration ($\geq 39 \times 10^6$ cells per ejaculate).

Once the samples were acquired, they were incubated (37 °C, 5% CO_2, 95% humidity) for 30–45 min until liquefaction occurred. Total sperm fractions were obtained by double wash technique (2 000 r/min, 15 min) in Hams F10 medium containing bovine serum albumin (HAMS – BSA). The pellet was re-suspended in 7 mL HAMS – BSA medium. The sample was then divided into 1 mL treatment groups exposed to varying concentrations of cotinine with and without quercetin intervention as well as the control and quercetin control. Cotinine (300 ng/mL, 190 μg/mL) concentrations were chosen from previous studies which showed the average concentrations of cotinine in the seminal plasma of casual (1–15 cigarettes per day) and habitual (16–30 cigarettes per day) smokers [15,16]. The ideal concentration of quercetin for *in vitro* treatment was obtained from literature which showed that 30 μmol/L of quercetin displayed anti-oxidative effects on human sperm cells. Each 1 mL treatment group was further divided in half in order to allow for separate incubation times of 60 min and 180 min at 37 °C, 5% CO_2 and 95% humidity. The experimental groups were the control (CONT), quercetin treated (QU), low (300 ng/mL) cotinine treated (LC), high (190 μg/mL) cotinine treated (HC), low cotinine supplemented with quercetin (LC + QU) and high cotinine supplemented with quercetin (HC + QU). All aliquots were analysed independently after two incubation times, 60 min of incubation (T1) and 180 min of incubation (T2).

2.3. Assessment of motility parameters

Motility parameters were analysed by means of computer-aided sperm analysis (CASA), using the sperm class analyser® after exposure to cotinine with and without quercetin at 60 min and 180 min incubation times. This was performed by pipetting 2.5 μL of each treated sample into specialized Leja® 20 micron chamber slides for easy analysis using a light microscope paired with the CASA system. The system analysed WHO motility parameters: Fast progressive motility (Type A), slow progressive motility (Type B), non-progressive motility (Type C), Immotile (Type D) and a series of kinematic parameters including curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

2.4. Assessment of cell viability

Cell viability was determined using dye-exclusion staining technique (Eosin/Nigrosin) [17]. Treated samples were exposed to eosin and nigrosin stains and smeared onto slides then mounted. The red-stained cells (damaged membrane and non-viable) and unstained cells (membrane intact and viable) were counted at ×40 magnification using a light microscope. A minimum of 100 cells were analysed and the results were expressed as a percentage of viable cells versus non-viable cells.

2.5. Assessment of acrosome reaction

The extent of induction of premature acrosome reaction was assessed by creating spot smears of treated samples on slides and fixing in cold ethanol (4 °C, 30 min) once air-dried. The spots were then covered with fluorescein isothiocyanate-labelled Pisum Sativum Agglutinin (FITC-PSA) in phosphate-buffered saline for 45 min in a dark room, then rinsed with distilled water to remove excess FITC-PSA and left to air-dry. Once dried, the spots were mounted using Dako Fluorescent mounting medium and observed under a fluorescent microscope at ×100 magnification [18]. A minimum of 100 cells were analysed and the results were expressed as a percentage of cells with bright-

green fluorescing acrosomes (acrosomes intact) versus dull-green fluorescing acrosomes (acrosomes reacted).

2.6. Detection of reactive oxygen species (ROS)

Intracellular superoxide was chosen to measure the ROS levels in the treated sperm cells while dihydroethidium (DHE) was utilized as an oxidative fluorescent probe. Treatment groups were diluted with HAMS – BSA to reach a minimum concentration of 5×10^6 cells/mL. The dilution was subsequently exposed to DHE and incubated for 15 min (37 °C, 5% CO_2, 95% humidity). The probe-exposed samples were further diluted by PBS solution and then centrifuged (300 g, 5 min). The cells were re-suspended in PBS and analysed using flow cytometry which analysed a total of 20 000 cells per aliquot. The output data was then analysed using Flowjo© V10. As shown in Figure 1, the two peaks on the graph are gated as populations where DHE + populations represent cells positive for intracellular superoxide.

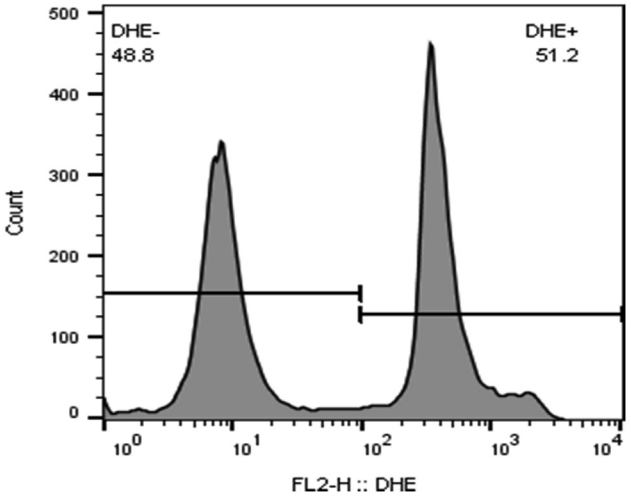

Figure 1. Histogram plot of DHE+ and DHE-sperm populations.

2.7. Statistical analysis

All data was expressed as mean ± SEM. One way analysis of variance (ANOVA) was performed using the Šídák. All statistical analysis was done using GraphPad Prism® Version 6 for windows. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of cotinine and quercetin on sperm motility parameters

Motility parameters were not significantly affected by the cotinine treatments with and without quercetin supplementation after both incubation periods (60 and 180 min) as shown in Figure 2.

3.2. Effect of cotinine and quercetin on cell viability

As shown in Figure 3, the percentage of viable cells was significantly decreased in aliquots treated with high concentrations of cotinine for both 60 and 180 min of exposure when compared to their respective controls ($P < 0.01$). Supplementing these samples with quercetin reduced the number of non-viable cells leading to no differences when compared to their respective controls, whilst showing a significant increase in the number of viable cells in the 180 min cotinine and quercetin supplemented group compared to the high cotinine group ($P < 0.001$).

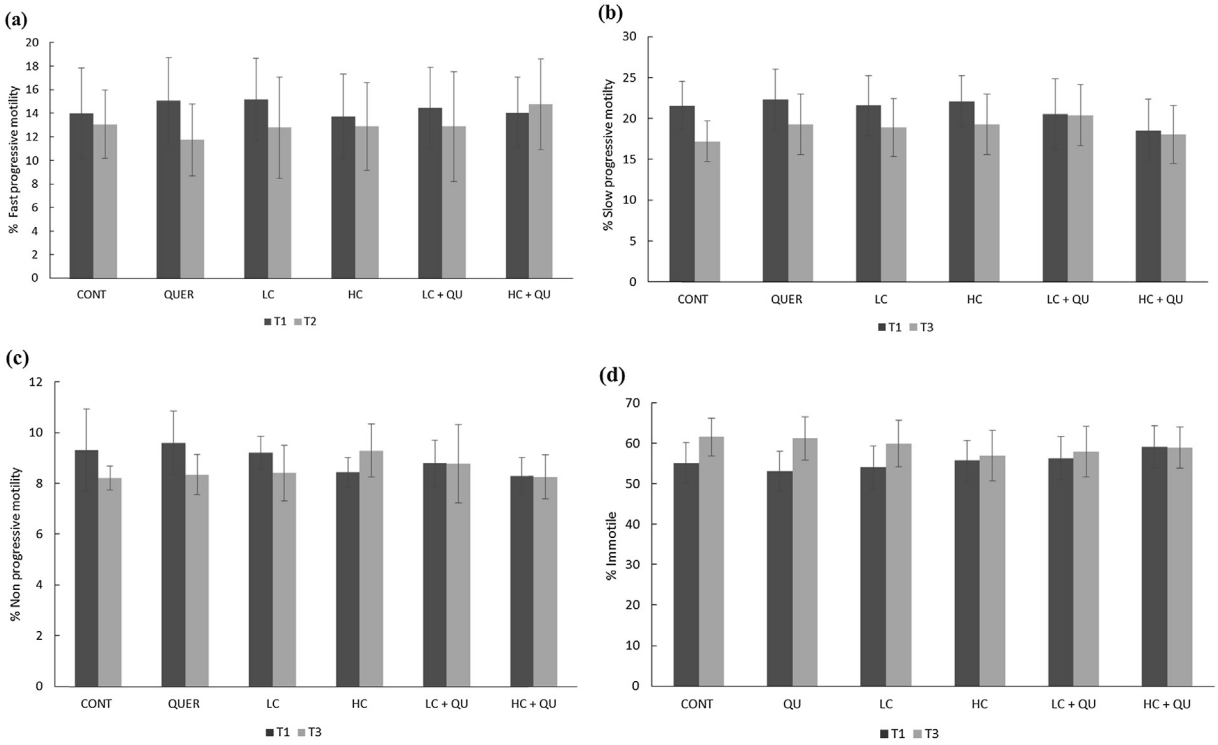

Figure 2. Bar graphs (a)–(d) represent the motility parameters for the cotinine and quercetin treatments.
Values are expressed as mean ± SEM of 10 human subjects. T1 = 60 min incubation, T2 = 180 min incubation, CONT = Control, QU = Quercetin, LC = Low cotinine, HC = High cotinine, LC+QU = Low cotinine plus quercetin, HC+QU = High cotinine plus quercetin.

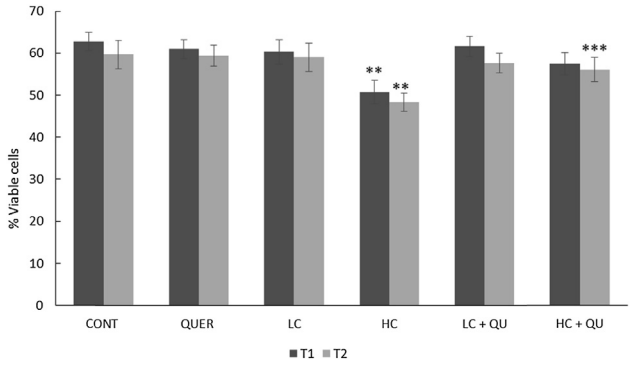

Figure 3. Bar graphs showing the percentage viable cells after cotinine and quercetin exposure at 60 and 180 min.
Values are expressed as mean ± SEM 10 human subjects. T1 = 60 min incubation, T2 = 180 min incubation, CONT = Control, QU = Quercetin, LC = Low cotinine, HC = High cotinine, LC+QU = Low cotinine plus quercetin, HC+QU = High cotinine plus quercetin. (**$P < 0.01$ vs. own control; ***$P < 0.001$ vs. HC T2).

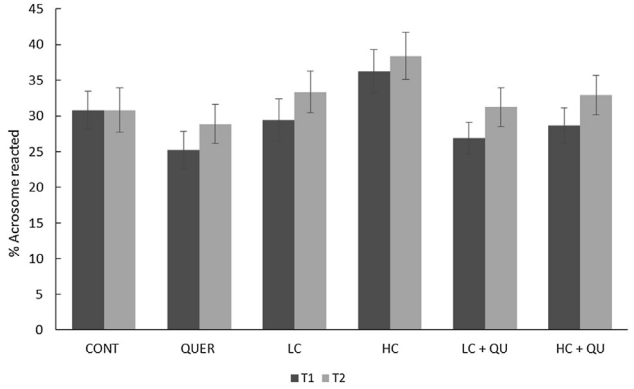

Figure 4. Bar graphs showing acrosomal status after cotinine and quercetin exposure at 60 and 180 min.
Values are expressed as mean ± SEM 10 human subjects. T1 = 60 min incubation, T2 = 180 min incubation, CONT = Control, QU = Quercetin, LC = Low cotinine, HC = High cotinine, LC+QU = Low cotinine plus quercetin, HC+QU = High cotinine plus quercetin.

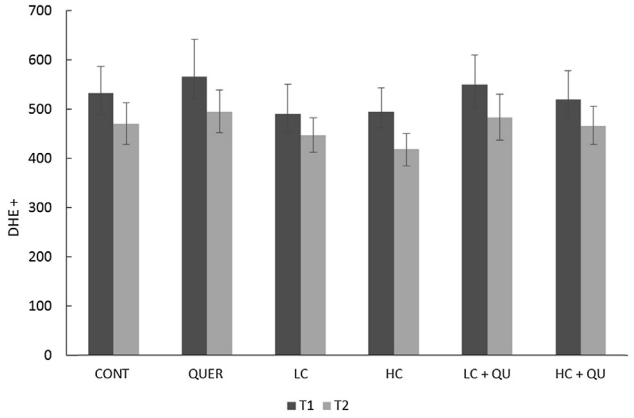

Figure 5. Bar graphs displaying the DHE + count for cotinine and quercetin treatments which correlates with the level of intracellular superoxide molecules present in sperm cells.
Values are expressed as mean ± SEM 10 human subjects. T1 = 60 min incubation, T2 = 180 min incubation, CONT = Control, QU = Quercetin, LC = Low cotinine, HC = High cotinine, LC+QU = Low cotinine plus quercetin, HC+QU = High cotinine plus quercetin.

3.3. Effect of cotinine and quercetin on acrosome reaction

There were no significant differences in the number of acrosome-reacted cells when compared with the different concentrations of cotinine treatment, with and without the quercetin supplementation (Figure 4).

3.4. Effect of cotinine and quercetin on ROS levels

The level of intracellular superoxide molecules did not significantly change in all treatments after both 60 and 180 min of exposure as shown in Figure 5. However, insignificant decreases in ROS levels in aliquots treated with high and low cotinine concentrations without the quercetin intervention was observed.

4. Discussion

In this study, the potential benefits of quercetin supplementation on washed healthy spermatozoa affected by the by-products of cigarette smoking *in vitro*, was assessed on the basis of functional and biochemical parameters. By assessing washed spermatozoa, it can only be speculated that the results of this experiment on isolated healthy sperm cells in an *in vitro* model will adequately represent the response of spermatozoa in a natural seminal environment. As hypothesized, quercetin supplementation was moderately effective in reducing certain detrimental effects of cotinine which according to previous studies, are primarily due to an increase in oxidative stress caused by these compounds [12,19,20]. This study has shown and confirmed that the quercetin treatment, as well as the low concentration of cotinine have negligible effects on all parameters measured including ROS levels.

In agreement with previous literature, extensive cotinine exposure caused significant decreases in the number of viable cells after both 60 and 180 min [19]. The non-viable cells were deduced by observing the uptake of the eosin/nigrosin stain by the sperm due to compromised membrane structure, implicating that the cell is no longer viable. Quercetin intervention was noticeably active in decreasing cotinine's deleterious effect on cell viability. Furthermore, quercetin was successful in significantly increasing the number of viable cells in the high cotinine supplemented with quercetin when compared to aliquots treated only with high cotinine concentration after 180 min.

There was no significant effect of quercetin on the premature induction of the acrosome reaction or any significant evidence of the ameliorative ability of quercetin in reducing the level of premature induction that amount in cotinine treated groups. However, in the high concentration of cotinine supplemented with quercetin aliquots, there was an improvement in the number of intact acrosomes when compared to the high concentration treatments only. High cotinine concentration supplemented with quercetin at 180 min had noticeably high percentage of reacted acrosomes when compared to the control group showing that the activity of quercetin at the concentration chosen (30 μmol/L) might not be adequate in counteracting the damage caused by high concentrations of cotinine treatment. The reduction of intact acrosomes has been associated with a compromised fertilizing capacity of sperm, by hampering the process of interaction with the zona pellucida of the female oocyte [21].

According to the literature, increased levels of oxidative stress in the semen is thought to be the leading mechanism in which the by-products of cigarette smoke, reduce the fertilizing capacity of spermatozoa by negatively affecting an array of functional parameters including the parameters measured in this experiment. Interestingly, according to the results of this particular study, the changes in ROS levels (intracellular superoxide) are not significant in any of the treatment cotinine group after both incubation times. The comparable level of ROS observed in this study may be the result of a variety of factors. Firstly, the probe used (DHE) in this study reacts with intracellular superoxide ions and proceeds to bind to DNA, emitting fluorescence. The fact that the probe solely reacts with superoxide may be the shortfall of the analysis, additionally the superoxide ions may not be the specific free radical which is associated with cotinine, and therefore measuring its flux is inadequate as an indication of the oxidative stress in this case. Secondly, there might be other unidentified factors and/or mechanisms involved in the process of cotinine induced spermatozoa toxicity aside of increased oxidative stress which could be elucidated in further studies. Hence, this present study can be viewed as a preliminary study whereby larger sample sizes as well as a broader spectrum of parameters are required to be analysed in future studies, for stronger evidence of these mechanisms.

To conclude, this study indicates that the ameliorating ability of quercetin on cotinine-induced decline in human sperm function is associated with improved viability of the cells. The exact mechanism in which these compounds act of sperm cells is debateable and requires extensive further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

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