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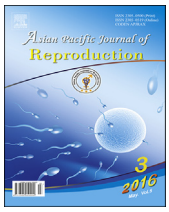

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Ameliorative potentials of quercetin against cotinine-induced toxic effects on human spermatozoa

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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-