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Effect of freezing–thawing process and quercetin on human sperm survival and DNA integrity ☆

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ABSTRACT

We aimed in the first part of our work to study the effect of cryopreservation on the human sperm DNA integrity and the activation of caspase 3, the main apoptosis indicator. In the second part, we were interested in testing the effect of quercetin, as an antioxidant, in preventing sperm damage during the freeze–thawing process. Seventeen semen samples were obtained from 17 men recruited for infertility investigations. Liquefied sperm was cryopreserved using spermfreeze®. Nine of the used samples were divided into two aliquots; the first one was cryopreserved with spermfreeze only (control) and the second one was cryopreserved with spermfreeze supplemented with quercetin to a final concentration of 50 μM. Sperm motility and viability were assessed according to WHO criteria. We used TUNEL assay and the Oxy DNA assay to assess sperm DNA integrity. Activated caspase 3 levels were measured in spermatozoa using fluorescein-labeled inhibitor of caspase (FLICA). Cryopreservation led to a significant increase in sperm DNA fragmentation, DNA oxidation and caspase 3 activation ($p < 0.01$). Supplementation of the cryopreservation medium with quercetrin induced a significant improvement in post thaw sperm parameters, compared to those of control, regarding sperm motility ($p = 0.007$), viability ($p = 0.008$) and DNA integrity ($p = 0.02$); however, it had no effect on caspase 3 activation ($p = 0.3$). We conclude that oxidative stress plays a major role in inducing sperm cryodamage but implication of apoptosis in this impairment requires further investigations. Quercetin could have protective effect during cryopreservation but further research is needed to confirm this effect.

Introduction

Despite the success of sperm cryopreservetion, this routinely used procedure in assisted reproduction technology (ART) causes cell damages and impairs sperm functions. It is widely reported that sperm freezing and thawing not only alters sperm motility and vitality but also causes an increase in sperm DNA damage [6,9,10,33,27,20,18]. The mechanism behind this damage may be related to cold shock, osmotic stress and intracellular ice crystal formation during cryopreservation [31,15]. These phenomena induce cell injury and affect organelle functions. In fact, it was shown that membranes and cytosqueletel elements were temperature-sensitive. Membrane fluidity and mitochondrial functions are affected during sperm cryopreservation [31]. Moreover, strong evidence suggests that freeze thawing of spermatozoa is associated with an increase in reactive oxygen species (ROS) [13,30,19]. These reactive species which are produced within the mitochondria are generated excessively during the process of cryopreservation/thawing [13,30,31] and could attack all cellular compounds including DNA [20,29].

Apoptosis represents another potential mechanism involved in the induction of sperm DNA damage during cryostorage [7,17,20]. Apoptosis occurs naturally during spermatogenesis, and is characterised by a decrease in cell volume, alterations in cell membranes, and DNA degradation. Caspases, which play a key role in the cellular apoptotic cascade, were found to be activated in fresh and freeze thawed spermatozoa and to correlate with some other apoptotic markers such as DNA fragmentation [17]. Caspases are a family of highly specific proteases. They are activated in a cascade manner and act as initiators (caspases 8, 9 and 10) or effectors (caspases 3, 6 and 7). Caspase 3 is the most important among them since it ensures the cleaving of many cellular structure proteins and the degradation of DNA [16,24]. Its activation marks a point-of- no- return in apoptosis.

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Many investigations have focused on the effects of antioxidants on female and male gametes, and embryo development. Indeed, numerous antioxidants have proven beneficial in treating male infertility [23]. Antioxidant species might act in vivo or in vitro to decrease oxidative damage to DNA, protein and lipids and many studies exist as regards the effect of antioxidants on the cryopreservation induced damage to sperm DNA [3,5,8,11,14,22,26–28]. In a previous study, we have noted a significant decline in sperm motility and viability after cryopreservation and an increase in oxidative DNA damage and DNA fragmentation following freeze–thawing process [33].

The present work further aimed to explore the implication of apoptosis in cryopreservation-induced human sperm damage by the detection of caspase 3 activation and to test the effect of quercetin as an antioxidant in preventing such damage during the freeze/thaw process.

Materials and methods

Semen sample collection

This study was approved by the Institutional Review Board of the Sfax Faculty of Medicine, Tunisia.

Seventeen semen samples were obtained from 17 men attending the Histology–Embryology laboratory of the Sfax faculty of Medicine for infertility investigations and with mean age (±SD) of 39.35 ± 1.9 years. Men with azoospermia, severe oligozoospermia or leucocytospermia were excluded from the study. Among the seventeen semen samples, nine ejaculates were used to test the effect of quercetin on post-thaw sperm motility, viability, DNA integrity and caspase 3 activation.

Semen analysis

Semen samples were obtained by masturbation into sterile containers after 3-5 days of sexual abstinence and left to liquefy at 37 °C. Basic semen analyses were performed within 1 h of collection and consisted in the measurement of semen volume and sperm concentration, motility, viability and morphology. All the parameters were carried out according to the World Health Organization (WHO) guidelines [32].

To determine the percentage of motile spermatozoa, a 10-μL drop of gently mixed semen was placed on a heated glass slide (37 °C) under a square cover glass (22 mm). The slide was placed on a heating stage (37 °C) and observed at 4000 magnification. The percentage of motile spermatozoa was evaluated according to WHO guidelines [32].

Viability was assessed using the Eosin/Nigrosin stain. Staining was performed by mixing 20 μl of semen with 20 μl of Eosine (1%). Afterwards, 20 μl of Nigrosin (10%) was added. A smear was made on a glass slide and allowed to dry. Unstained (intact) and pink-coloured (with damaged membranes) spermatozoa were counted under the microscope using 100 × object if and oil immersion. Sperm viability was defined as the percentage of intact cells.

Sperm morphology was assessed in Shoor-stained semen smears. All samples were examined using the same technique and by the same technician. Morphology assessment was performed according to WHO guidelines [32].

Semen cryopreservation

Briefly, after semen analysis, each sample was cryopreserved by a standard protocol using spermfreeze® (Fertipro, Belgium) as described previously [22]. Each of the nine semen samples used to test the effect of quercetin ((Sigma ChemicalCo. CAS-No 6151-25-3 (St Louis, France)) was divided into two equal aliquots. The first aliquot was cryopreserved with spermfreeze only (control) and the second one was cryopreserved with spermfreeze supplemented with quercetin to a final concentration of 50 μM. The choice of quercetin concentration was based on preliminary experiments that we performed to test the effects of different concentrations of this antioxidant on sperm motility and viability during cryopreservation (data not shown). Samples were diluted (1:0.7) with freezing medium in a drop-wise manner. After a 10-minute equilibration at room temperature, the mixture was frozen in liquid nitrogen vapour for 15 min and followed by the plunging of cryovials (Nunc International, Roskilde, Denmark) into liquid nitrogen (−196 °C) for storage. Samples were cryopreserved for an average period of 7 days.

After cryostorage duration, the specimens were thawed at room temperature for 15 min; then they were analysed for motility, viability, DNA fragmentation and oxidation assessment and caspase 3 activation.

TUNEL assay

For the evaluation of DNA fragmentation, a commercial kit (In situ Cell Death Detection Kit, Fluorescein, Roche, Germany) based on an enzymatic reaction of labelling free 3'-OH termini was used. We followed the manufacturer's instructions with little modifications [22]. In brief, 3.10^6 cells were washed with phosphate-buffered saline (1xPBS, pH 7.4) then fixed with 200 μl of 4% paraformaldehyde for 1 h at room temperature in the dark. Afterwards, sperm cells were washed with 1xPBS and permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate for 15 min on ice. After washing with PBS, sperm DNA was labelled by incubating spermatozoa with 50 μl of the TUNEL reaction mixture (Tdt enzyme and FITC-labelled nucleotides) in a humidified atmosphere for 60 min at 37 °C in the dark, with mixing each 15 min. Washed and labelled sperm cells were then resuspended in 1xPBS for flow cytometry analysis. EPICS XL flow cytometer (Beckman Coulter) was used with an argon ion laser at 488 nm. Analysis of the scatter properties (forwarded-angle light scatter (FSC) versus side-angle light scatter (SSC)) was performed to gate spermatozoa and exclude debris and other cell types. 10,000 events were examined for each sample and the percentage of TUNEL-positive cells were estimated from the histograms using the System II software. A negative control (sample without the addition of Tdt enzyme) and a positive control (sample treated with DNase I (3 U/ml, Invitrogen) for 10 min at room temperature to generate DNA strand breaks) were also assessed by TUNEL assay. For each semen sample, sperm DNA fragmentation was evaluated before and after cryostorage.

Measurement of 8-oxoguanine levels by flow cytometry

We used the oxyDNA kit (Biotrin International, Ireland) which is specific for the detection of 8-oxoguanine as one of the major studied oxidised nucleotides. The test is based on the direct binding of a fluorescent probe (FITC conjugate) to DNA adduct 8-oxoguanine. In short, as previously described [22], one aliquot of each semen sample containing 3.10^6 spermatozoa was washed with 1xPBS, fixed, permeabilised with ice-cold 70% ethanol and kept for 1 h at −20 °C. Fixed cells were centrifuged at 1600 rpm for 5 min, washed with PBS, then resuspended in 1 ml wash solution (Tris-buffered saline/Tween 20 containing thrimerosal) and pelleted at 1600 rpm/min for 5 min. Fifty microlitre FITC conjugate were incubated for 1 h with pelleted sperm cells in the dark at room temperature, with mixing each 15 min. Finally, cells were washed, resuspended in 500 μl 1xPBS and analysed in a flow cytometer