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Antioxidant activity of CAPE (caffeic acid phenethyl ester) *in vitro* can protect human sperm deoxyribonucleic acid from oxidative damage

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ABSTRACT

Purpose: Sperm processing (e.g., centrifugation) used in preparation for assisted reproduction can result in excessive generation of reactive oxygen species (ROS) and potential sperm damage. The use of antioxidants during sperm processing has been shown to prevent iatrogenic sperm damage, including DNA damage. In this study, we evaluated the effect of caffeic acid phenethyl ester (CAPE) on oxidative stress mediated sperm dysfunction and DNA damage.

Methods: Semen samples were obtained to liquefy at room temperature. After centrifugation and washing protocols, spermatozoa were incubated in a single step supplemented medium with either of 10, 50 or 100 µmol/L CAPE for 2 hours at 36 °C. After incubation period, MDA levels of seminal plasma were measured. The fragmentation in sperm DNA was detected by light microscopy via use of an aniline blue assay, while ultrastructural morphology was analyzed by transmission electron microscopy.

Results: Significant increase has been observed in percent chromatin condensation (assessed by aniline blue staining) and Malondialdehyde (Mmol/L) in oligoasthenoteratozoospermia group before the centrifugation (0.57 ± 0.15). Incubation of samples with 100 µmol/L CAPE after centrifugation resulted in a significantly lower percent chromatin condensation compared to samples incubated without CAPE (0.42 ± 0.12) ($P < 0.0033$). Incubation of all samples with CAPE (10 µmol/L, 50 µmol/L, 100 µmol/L.) after centrifugation resulted in a significantly lower percentage of Malondialdehyde levels.

Conclusions: The data suggests that preincubation of spermatozoa with the antioxidant CAPE offers protection against oxidative DNA damage in vitro.

1. Introduction

Sperm, like all aerobic cells, lives in a permanent oxygen paradox. Oxygen is necessary for life; however, metabolites of aerobic respiration can be extremely detrimental. During the process of spermatogenesis, reactive oxygen species (ROS) are created by sperm metabolism. Collect of ROS can cause an imbalance in relation to the activity of antioxidant molecules, leading to oxidative stress (OS), which is quite damaging to all sperm components. Maintaining a balance between generation neutralization of ROS occurs due to the antioxidant capacity of sperm and seminal plasma. The enzymatic and non-enzymatic antioxidants in seminal plasma protect plasma membrane from peroxidation (Lenzi et al., 2000).

Sperm preparation techniques used for assisted reproductive technologies may result in release of increased amounts of reactive oxygen species (ROS) (Aitken and Clarkson, 1988). Production of ROS has even more damaging effects on morphologically abnormal spermatozoa obtained from infertile men (Iwasaki and Gagnon, 1992; Zini et al., 1993;

Abbreviations: ROS, reactive oxygen species; TEM, transmission electron microscope; WHO, World Health Organization

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Zini et al., 2000). ROS was shown to cause peroxidation of lipids of sperm membranes, a situation that results in a decrease of sperm motility and vitality (Alvarez et al., 1987), a long with sperm chromatin and DNA damage (Aitken et al., 1998). Use of antioxidants in sperm preparation has been presented to prevent the iatrogenic sperm damage (Hughes et al., 1998; Zini et al., 2010).

Structurally related to flavonoids, caffeic acid phenethyl ester (CAPE) is the biologically active component of honeybee propolis extract and it has been used as a local medicine with no harmful effects on normal cells. The compound is known to have powerful antimicrobial, anti-inflammatory, antineoplastic and antioxidant effects (Ilhan et al., 1999; Koltuksuz et al., 2000). Local medicine takes advantage of CAPE as a dietary supplement for therapeutic effects (Tolba et al., 2013). A study has demonstrated the healing effects of CAPE in the treatment of oxidative stress. (Song et al., 2012). Caffeic Acid Phenethyl Ester (CAPE) is a propolis constituent that has gained attention due to its broad pharmacological activities (Bankova, 2005) antibacterial, antiproliferative, antiparasitic and antioxidant effects, among others (Alday-Provencio et al., 2015; Hernandez et al., 2007). CAPE exerts its beneficial effects by decreasing free oxygen radicals, and prevents consumption of free radical scavenging enzymes by acting in parallel to these antioxidant enzymes (Kus et al., 2004; Ogeturk et al., 2005). However, to the best of our knowledge, there is hardly any report in the literature regarding the effect of CAPE in sperm dysfunction and DNA damage.

Therefore, in this experimental study, we aimed to evaluate the effect of CAPE on oxidative stress mediated sperm dysfunction and DNA damage to examine the antioxidant properties of CAPE and gain insight into the potential role of CAPE in male reproduction.

2. Materials and methods

In this study normozoospermic (n = 30) and oligoasthenoteratozoospermic (n = 30) males who applied to Zeynep Kamil Women's and Children's Disease Training and Research Hospital were included. Patients with a history of varicocele, urogenital system infections, testicular tumors, chemotherapy, radiotherapy, congenital defects, endocrine system diseases and systemic problems were not included. Patients with congenital penile anomalies, sexual disorders, and retrograde ejaculation were also not taken into account.

2.1. Sample preparation

Following 3–5 days of sexual abstinence, samples were collected in a sterile semen container and let to liquefy. After complete liquefaction, concentration and motility of spermatozoa (in 10 µl of semen sample) were evaluated manually according to WHO 2012 criteria, under a Makler's counting chamber. Samples with $< 15 \times 10^6$/ml sperm concentration, < 4% normal morphology and < 32% progressive motility were evaluated as oligoasthenoteratozoospermic, whereas samples with $> 15 \times 10^6$/ml sperm concentration, > 4% normal morphology and > 32% progressive motility were stated as normozoospermic.

Before semen wash, samples were stained with aniline blue for depiction of DNA fragmentation and, from each sample at least 200 spermatozoa were evaluated. In order to accomplish malondialdehyde (MDA) analysis, seminal plasma was obtained after centrifugation at 2500 rpm for 10 min and 1 ml of sample was frozen at −80° C for each case. One microliter of semen sample was spared for the transmission electron microscopic (TEM) examinations.

Semen samples were then prepared by density gradient centrifugation method (v/v = 1:1, 40%–80% gradient solution) and centrifuged at 1200 rpm for 10 min. After centrifugation and wash, spermatozoa were incubated in single step medium supplemented with either 10, 50 or 100 µmol/L of CAPE (Sigma Aldrich C8221), for 2 h at 36° C. The incubation was followed by the same set-up of semen, DNA fragmentation, MDA and TEM analyses experiments.

2.2. DNA fragmentation analysis (Aniline blue staining)

DNA fragmentation was assessed by aniline blue staining (Cat. No: 415049 Sigma, USA). Aniline blue stain was used to show the presence of excessive histones in spermatozoon nuclei. Concentrated sample was spread and air dried as a smear on a glass slide, fixed with alcohol, stained with 20 µl drop of aniline blue for 5 min, washed with PBS and dried in air. In order to accomplish the quantitative analysis, at least 200 spermatozoa were counted on each slide and spermatozoa stained blue with the dye were scored to have damaged DNA (Histone positive).

2.3. Lipid peroxidation

Lipid peroxidation in spermatozoa and seminal plasma was measured by reaction of thiobarbituric acid (TBA) with MDA according to protocol proposed by Yagi et al. (Yagi, 1984). Content of MDA was measured spectrofluorometrically using a Jasco FP-6200 (Japan) spectrofluorometer (excitation at 515 nm, emission at 553 nm). The MDA fluorescence intensity of spermatozoa and seminal plasma was determined using various concentrations of tetraethoxypropane as standards. The results were expressed as nmol MDA/10×10^6 cells and nmol MDA/ml seminal plasma.

2.4. TEM analysis

Following liquefaction semen samples were centrifuged at 2000 rpm for 5 min in washing medium and the supernatant was expelled. The pellet was immersion fixed at 4° C for 4 h in 2,5% glutaraldehyde (Cat. No: 354400, Merck Millipore, USA) solution prepared in 0.1 M PBS (pH = 7,2). Following wash in a buffer solution, the pellet was post-fixed in 0,1% Osmium tetroxide (Cat. No: 19160, EMS Diasum, USA) for one hour. Dehydration, was then maintained by immersion in grading series of alcohol (70%, 90%, 96%, 100%). After application of propylene oxide, the sample was embedded in 1:1, 1:2 and pure epon at 60 °C respectively. Contrasted grids were examined by a Jeol Sx TEM (USA) to reveal the qualitative morphology changes at head, neck and tail regions.

2.5. Ethics

All patients signed an informed consent and the information for this study remained confidential within the institution. This study was approved by the ethical review board of Zeynep Kamil Women's and Children's Disease Training and Research Hospital.

2.6. Statistical analysis

All statistical analyses were performed using SPSS software program (SPSS Inc., Chicago, USA). Results were expressed as mean ± SD. Differences between treatments (normozoospermic, oligozoospermic, CAPE 10: 10 µm/ml CAPE, CAPE 50: 50 µm/ml, CAPE 100: 100 µm/ml) were analysed statistically with one-way ANOVA analysis of variance followed by Dunnett's *t*-test. The paired *t*-test was used to check for differences for "before sperm wash" and "after sperm wash". For all analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of CAPE on sperm DNA damage

The quantitative analysis of DNA fragmentation rate revealed a significant difference between oligoasthenoteratozoospermic and normozoospermic samples ($p < 0.0033$). Significant increase has been observed in per cent chromatin condensation assessed by aniline blue staining in oligoasthenoteratozoospermia group before semen wash (0.57 ± 0.15). Incubation of samples with 100 µmol/L CAPE following