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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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A R T I C L E   I N F O

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A B S T R A C T

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

Table 1
Seminal Plasma Malondialdehyde levels and Chromatin Condensation Levels.

	OAT*	CAPE 10**	CAPE 50**	CAPE 100**	NS*	NS CAPE 100**	*P* value
Malondialdehyde(Mmol/L)	17,65 ± 6,57	4,32 ± 3,92	4,09 ± 2,26	3,35 ± 2,40	12,10 ± 7,16	4,98 ± 3,97	0.0001
Choromatin Condensation (%)	0,57 ± 0,15	0,50 ± 0,11	0,52 ± 0,15	0,43 ± 0,14	0,42 ± 0,12	0,14 ± 0,02	0.0033

Note: Values are means ± SD.% Chromatin Condensation: percent DNA fragmentation by the aniline blue stain. CAPE 10: 10 μmol/L CAPE (caffeic acid phenethyl ester), CAPE 50: 50 μmol/L CAPE 100: 100 μmol/L. Interpretation of the table can be followed in the text. OAT: Oligoasthenoteratozoospermia, NS: Normozoospermia.
*Before incubation with CAPE, ** After incubation with CAPE.

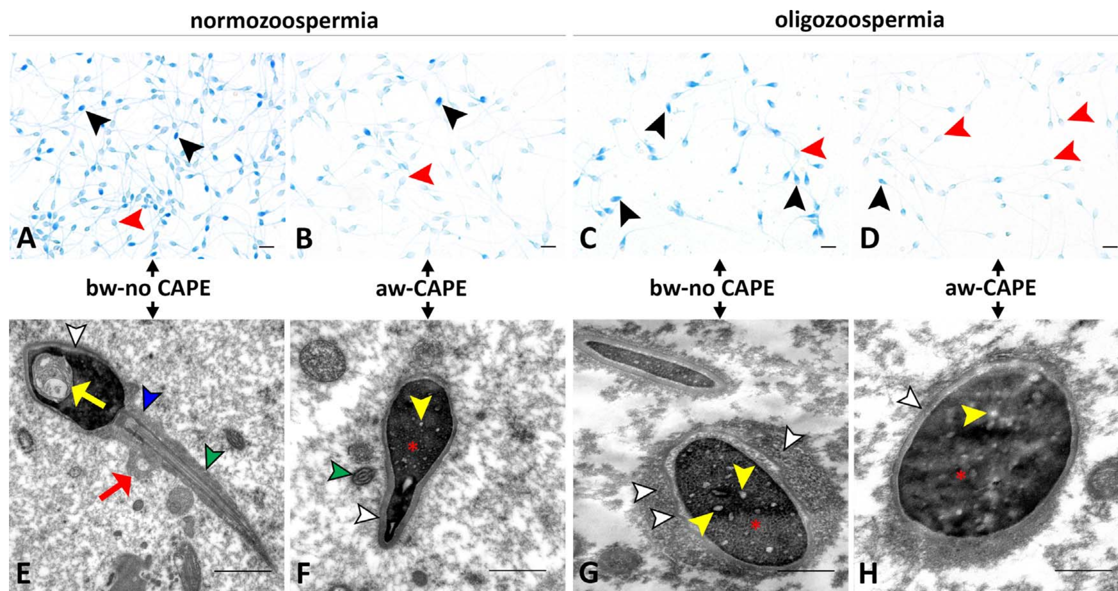

Fig. 1. Effects of CAPE on sperm chromatin condensation and ultrastructural morphology.
Aniline blue stained the spermatozoa with defective chromatin condensation (black arrowheads, A–D). In both normozoospermic (A and B) and oligozoospermic (C and D) samples, CAPE decreased the amount of aniline blue stained spermatozoa (red arrowheads). The decrease was most significant in oligozoospermic samples treated with CAPE (D). Spermatozoa of normozoospermic group usually showed normal acrosomal membrane (white arrowhead, E) with normal neck (blue arrowhead, E) and tail (green arrowhead, E) orientation. Occasional myelin figures (yellow arrow, E) in head or irregularities in plasma membrane (red arrow, E) were not seen after 100 μmol/L of CAPE treatment, depicted by normal acrosomal membrane (white arrowhead, F), normal head with minimum sized vacuoles (yellow arrowhead, F), uniform chromatin condensation (red asterisk, F) and normal tail sections (green arrowhead, F). The nuclei of sperms in oligozoospermic samples were usually observed with insufficiently condensed chromatin (red asterisk, G), prominent vacuoles in head (yellow arrowheads, G) and irregular plasma membrane (white arrowheads, G). 100 μmol/L of CAPE treatment improved the morphology of sperm head with decreased irregularity of plasma membrane (white arrowhead, H), increased smooth and condensed regions (red asterisk) and fewer vacuoles in head (yellow arrowhead, H). CAPE: caffeic acid phenethyl ester, bw: before sperm wash, aw: after sperm wash with density gradient. Bars: A–D = 10 μm, E–G = 3 μm, H = 1 μm

density gradient centrifugation resulted in a significantly lower chromatin condensation percentage when compared with samples that were incubated without CAPE (0.43 ± 0.14) ($p < 0.0033$) (Table 1). Incubations with 10 μmol/L and 50 μmol/L of CAPE showed no significant decrease of DNA fragmentation. Normozoospermic samples were incubated with only 100 μmol/L CAPE following density gradient centrifugation.

The uptake of aniline blue by the spermatozoa, which stands for positive staining for DNA damage, was significantly lower in oligoasthenoteratozoospermic semen samples after incubation with CAPE 100 μmol/L (black arrowhead; Fig. 1D) in comparison to the group without a prior incubation with CAPE (black arrowheads; Fig. 1C). Sperm heads without DNA damage were indicated by red arrowheads in all groups (Fig. 1A–D).

3.2. Seminal malondialdehyde (MDA) content

Without a prior wash, MDA levels in seminal plasma were observed to be high in normozoospermic (12,10 ± 7,16) and oligoasthenoteratozoospermic (17,65 ± 6,57) patients. On the other hand incubation with 10 μmol/L (4,32 ± 3,92), 50 μmol/L (4,09 ± 2,26), 100 μmol/L (3,35 ± 2,40) CAPE after wash revealed a significant decrease in MDA levels ($p < 0,0001$) in oligozoospermic patients (Table 1). Since incubations with 10 μmol/L and 50 μmol/L of CAPE showed no significant decrease of DNA fragmentation as stated above, MDA levels in normozoospermic samples were evaluated only for 100 μmol/L CAPE incubation, which also showed a significant improvement (Table 1).

3.3. TEM analysis

Electron microscopic investigations regarding the ultrastructural morphology of unwashed and washed spermatozoa obtained from normozoospermic group usually revealed relatively normal morphology with common forms of head shapes, undisturbed acrosome and cell membrane (white arrowhead; Fig. 1E), normal neck (blue arrowhead; Fig. 1E), and tail profiles (green arrowhead; Fig. 1E). Occasional myelin figures in head (yellow arrow; Fig. 1E) or irregularities in plasma membrane (red arrow; Fig. 1E) were not seen after 100 μmol/L of CAPE treatment, depicted by normal acrosomal plasma membrane (white arrowhead; Fig. 1F), normal head with minimum sized vacuoles (yellow arrowhead; Fig. 1F), uniform chromatin condensation (red asterisk; Fig. 1F) and normal tail sections (green arrowhead; Fig. 1F). Unwashed and washed samples obtained from oligoasthenoteratozoospermic group usually showed few spermatozoa with normal morphology; as acrosomal disturbances, prominent vacuole formations (yellow arrowheads; Fig. 1G), irregularities in plasma membrane (white arrowheads; Fig. 1G) and chromatin condensation defects (red asterisk; Fig. 1G) were common. Incubation with 100 μmol/L CAPE after density

gradient centrifugation depicted fewer vesicular formations (yellow arrowhead; Fig. 1H) with a well preserved plasma membrane (white arrowheads; Fig. 1H).

4. Discussion

We observed that oligoasthenoteratozoospermic group with increased susceptibility to lipid peroxidation showed higher activity of MDA. We have also showed that preincubation of spermatozoa with CAPE (100 μmol/L) effectively protected spermatozoa from DNA damage caused by ROS.

Directly or indirectly, OS (oxidative stress) has been reported as the main reason of male infertility (Agarwal et al., 2003; Aitken and Baker, 2002). A balanced antioxidant environment is required for fertility, as ROS are necessary for fertilization, acrosome reaction, sperm hyperactivation, sperm motility and sperm capacitation (Aitken and Baker, 2002).

As OS is the result of an imbalance between ROS and existing antioxidants (Halliwell, 1999), this imbalance is consistently associated with sperm abnormalities, resulting in infertility (Agarwal et al., 2014). Increased levels of ROS have been correlated with decreased sperm motility and DNA damage (Gomez et al., 1998). Morphological changes and sperm damage were observed in conditions of OS (*e.g.* oligoteratospermia, varicocele and heat stress), as the incidence of sperm abnormalities and damages to the membrane, acrosome and DNA can be directly associated with the overproduction of ROS in semen (Blumer et al., 2008; Fleming et al., 2004). In this study, we observed an increase in sperm structure defects like injuries of sperm acrosome and plasma membrane, in group highly susceptible to the lipid peroxidation. These results suggest that OS is directly or indirectly related to sperm abnormalities, membrane and acrosome damages, DNA fragmentation, probably due the increased susceptibility of sperm to occasional disruptions of such homeostasis. OS is the main cause of damage to the sperm chromatin (Sakkas and Alvarez, 2010). Sperm DNA entirety is necessary for embryonic development (Fatehi et al., 2006) and for this reason, chromatin damage has been associated with infertility, especially in idiopathic cases (Sharma and Agarwal, 1996). In this study, it can be seen that oligoasthenoteratozoospermic male group with a high susceptibility to lipid peroxidation showed high susceptibility to chromatin damage, as well.

Increased ROS activity and/or production in sperms and reduction in its scavengering capability leads to oxidative stress and can cause infertility in men (Kessopoulou et al., 1992). Indeed, sperm plasma membrane is highly susceptible to oxidative stress because of the high involve of polyunsaturated fatty acids (the ensuing lipid peroxidation is associated with reduced motility and viability), whereas the sperm DNA/chromatin is relatively resistant to this same stress in view of its highly compact configuration (Sawyer et al., 2003). An alternative explanation for these studies is that, in the presence of a lipophilic antioxidant (*e.g.* CAPE), membrane lipid peroxides are formed but are stabilized or sequestered such that they cannot be translocated to the nucleus and therefore cannot cause oxidative DNA injury, since it has been postulated that the release of membrane lipid peroxides by phospholipase A2 may be a cause of DNA damage in sperm (Twigg et al., 1998), (Alvarez and Storey, 1995), (Blair, 2001), (Yang and Schaich, 1996).

Various researches reported a direct correlation between the amount of lipid peroxidation in sperm and sperm dysfunction (Ben Abdallah et al., 2009). Poor penetration of the sperm into the oocyte in oligozoospermic men with high levels of ROS has been identified (Agarwal et al., 2014). Many studies have demonstrated that low levels of the antioxidant capacity of seminal plasma are associated with male infertility (Zini et al., 2001). Total antioxidant capacity of seminal plasma in infertile men is lower than that in fertile men (Carrell et al., 2003).

Sperm processing has to be effective in removal of contaminating leukocytes, abnormal spermatozoa and seminal plasma, and it should allow the recovery of a highly motile sperm fraction. However, the process has to be gentle enough to minimize iatrogenic sperm damage. In particular, minimizing sperm DNA damage is critical because this damage may have an adverse impact on reproductive outcomes of assisted reproduction (Ahmadi and Ng, 1999), (Bungum et al., 2007), (Collins et al., 2008), (Zini et al., 2008). This issue is particularly important since recent experimental evidence shows that sperm DNA fragmentation reduces embryo implantation rates and, in the offspring, it causes reduction of longevity along with elevated the risk of cancer development (Fernandez-Gonzalez et al., 2008), (Perez-Crespo et al., 2008).

The results of this study emphasize the susceptibility of human spermatozoa to oxidative injury *in vitro*. The data suggests that preincubation of spermatozoa with the antioxidant CAPE offers protection against oxidative DNA damage *in vitro*. The data presented by this study is also important to highlight the differential effects of CAPE on sperm DNA integrity.

Conflict of interest

The authors state that they do not have any potential conflict of interest that would compromise the validity of the research conducted.

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