

·Original Article·

Oxidative stress in testicular tissues of rats exposed to cigarette smoke and protective effects of caffeic acid phenethyl ester

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Abstract

Aim: To show the oxidative stress after cigarette smoke exposure in rat testis and to evaluate the effects of caffeic acid phenethyl ester (CAPE). **Methods:** Twenty-one rats were divided into three groups of seven. Animals in Group I were used as control. Rats in Group II were exposed to cigarette smoke only (4 × 30 min/d) and rats in Group III were exposed to cigarette smoke and received daily intraperitoneal injections of CAPE (10 µmol/kg·d). After 60 days all the rats were killed and the levels of nitric oxide (NO) and anti-oxidant enzymes such as superoxide-dismutase, catalase and glutathione peroxidase (GSH-Px) and the level of malondialdehyde were studied in the testicular tissues of rats with spectrophotometric analysis. **Results:** There was a significant increase in catalase and superoxide-dismutase activities in Group II when compared to the controls, but the levels of both decreased after CAPE administration in Group III. GSH-Px activity was decreased in Group II but CAPE caused an elevation in GSH-Px activity in Group III. The difference between the levels of GSH-Px in Group I and Group II was significant, but the difference between groups II and III was not significant. Elevation of malondialdehyde after smoke exposure was significant and CAPE caused a decrease to a level which was not statistically different to the control group. A significantly increased level of NO after exposure to smoke was reversed by CAPE administration and the difference between NO levels in groups I and III was statistically insignificant. **Conclusion:** Exposure to cigarette smoke causes changes in the oxidative enzyme levels in rat testis, but CAPE can reverse these harmful effects. (*Asian J Androl* 2006 Mar; 8: 189–193)

Keywords: testis; cigarette; caffeic acid phenethyl ester; anti-oxidants; nitric oxide

1 Introduction

Cigarette smoke could cause negative effects on every system in an organism due to the various toxic substances and pro-oxidizing and oxidizing materials found

in its structure. Free radicals (FRs) continuously arise in the body as a result of ongoing chemical events, such as oxidative phosphorylation, uric acid metabolism and prostaglandin synthesis [1]. The cellular anti-oxidant defense system consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and melatonin. Vitamin E (α-tocopherol), β-carotene, vitamin C, flavonoids, and coenzymes Q and A are also anti-oxidant factors. The shift of the delicate balance between FRs and the cellular anti-oxidant defense system in favor of FRs might lead to development of oxidative

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stress [1]. Increased activity of free radical scavenger enzymes, such as SOD, CAT and GSH-Px, to prevent the detrimental effects of oxidative stress in different organs has been shown in published reports [2].

Testicular tissue is a highly vascular tissue, and because of the rich blood supply, cigarette smoke may deteriorate the balance between oxidant and anti-oxidant enzyme systems. As a result, FRs are generated, which cause testicular tissue damage. In a recent study it was shown that oxidative stress causes the production of abnormal spermatozoa and affects sperm functions [3].

Caffeic acid phenethyl ester (CAPE) is an active component of honeybee propolis and it is known to have powerful antimicrobial, anti-inflammatory, antineoplastic and anti-oxidizing effects [4]. CAPE exerts its beneficial effects by decreasing free oxygen radicals, and prevents consumption of FR scavenging enzymes acting in parallel to these anti-oxidant enzymes [5, 6]. According to previous studies, CAPE exerts its beneficial effects by decreasing free oxygen radicals and prevents consumption of free radical scavenging enzymes acting in parallel to these antioxidant enzymes [4].

The male factors in fertility assessment are usually based on the evaluation of several semen parameters [7]. It is well known that these parameters are affected by factors such as smoking [7]. Histologic changes in the seminiferous tubular structure, sperm counts and morphology have been mentioned in related articles [8]. But the oxidative stress due to cigarette smoke and the effects of CAPE on this condition have not been studied.

In this experimental study, we aimed to show the oxidative stress exerted on rat testicular tissue due to cigarette smoke and to show the effects of intraperitoneal injection of the anti-oxidizing agent CAPE against this condition.

2 Materials and methods

2.1 Animals

Twenty-one male Wistar-Albino rats (weighing 200–250 g) were used in the study. There were three experimental groups, each consisting of seven rats. The rats in the control group (Group I) breathed normal clean air. In Group II, rats breathed cigarette smoke only (4×30 min/d). The rats in Group III received daily intraperitoneal injections of CAPE ($10 \mu\text{mol/kg}\cdot\text{d}$) with cigarette smoke exposure. International standards for the care of laboratory animals were followed and the

protocol of the study was approved by the responsible local ethical committee.

2.2 Cigarette smoking and isolation of organ

The second-hand subjection of each rat to cigarette smoke inhalation was achieved in a glass cabin ($100 \times 50 \times 20$ cm) with the help of an aquarium air pump. One end of a plastic tube was implanted into the air pump and placed into the glass container with a cigarette on the other end. Non-filtered commercially available cigarettes (Birinci-Tekel) were used. Two lit cigarettes were placed in the cages four times a day, for 30 min each period, during the study period (groups II and III). CAPE ($10 \mu\text{mol/kg}$) was injected intraperitoneally once per day into each rat in Group III. Control rats in Group I breathed normal air. At the end of the experimental period (60 days) the rats were decapitated. Testes of all rats were removed with standard mid-scrotal incisions and frozen immediately in a deep freezer (-85°C).

All of the testicular tissues were washed with cold saline solution and wet tissue weights were obtained. The tissues were cut into small pieces and placed into glass bottles. They were then homogenized in ice-cold Tris-HCl buffer solution (0.2 mmol/L and $50/39.9$ [v/v]), within a homogenizer (Ultra Turrax Type T25-B; IKA Labortechnik, Staufen, Germany) for 2 min at $11\,200 \times g$. The homogenate was centrifuged at $3\,500 \times g$ for 60 min and a supernatant was obtained. The levels of CAT and GSH-Px were determined in the supernatant, and NO and malondialdehyde (MDA) levels were studied in the homogenate. For a further extraction procedure, the supernatant was extracted in ethanol/chloroform mixture ($5/3$, v/v). After a second centrifugation at $3\,500 \times g$ (20 min), the clear upper layer (the ethanol phase) was taken and used in SOD activity determination. All procedures were performed at 4°C and icepacks were used to maintain the temperature during the homogenization procedure.

2.3 SOD activity determination

The principle of the SOD activity determination method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine–xanthine oxidase system as a superoxide radical generator. One unit of SOD was defined as the enzyme activity causing 50% inhibition in the nitroblue tetrazolium reduction rate. SOD activity was expressed as units per mg tissue protein (U/mg prot).

2.4 CAT activity determination

The essentials of the CAT activity determination method were based on the determination of the rate constant of the H_2O_2 decomposition rate at 240 nm. The essentials CAT activity determination method were based on the determination of the rate constant of the H_2O_2 decomposition rate at 240 nm. Results were expressed as U/mg prot.

2.5 GSH-Px activity determination

GSH-Px was measured by the enzymatic reaction which was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, NADPH and glutathione reductase and the change in the absorbance at 340 nm was monitored by spectrophotometer. Activity was given in U/mg prot.

2.6 MDA level determination

Testicular tissue MDA levels were analyzed by a method based on the reaction with thiobarbituric acid at 90–100°C. In the thiobarbituric acid test reaction, MDA or MDA-like substances and thiobarbituric acid react together to produce a pink pigment with an absorption maximum of 532 nm. The results were expressed as nanomol per gram wet tissue (nmol/g wet tissue) calculated by using a standard graphics, which was prepared with serial dilutions of standard 1,1,3,3-tetramethoxypropane.

2.7 NO determination

NO measurement is very difficult in biological specimens, therefore tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured after conversion of nitrate to nitrite by copperized cadmium granules by a spectrophotometer at 545 nm. A standard curve was established with a set of serial dilutions (10^{-8} – 10^{-3} mol/L) of sodium nitrite. Linear regression was carried out using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as nmol/g wet tissue.

2.8 Protein determination

Protein content in the alkaline mixture formed a complex with copper and caused reduction of phosphomolibdate-phosphotungstate reactives. Color change in the

media was proportional with the protein concentration, and absorbance at 700 nm was monitored by spectrophotometer.

2.9 Statistical analysis

Statistical analysis was carried out using SPSS version 10.0 for Windows software (SPSS, Chicago, IL, USA). Distribution of the groups was analyzed with one sample Kolmogorov–Smirnov test. As all groups showed normal distribution, group differences were analyzed using parametric statistical methods, paired independent sample *t*-tests following one-way ANOVA. Results were presented as mean \pm SD. $P < 0.05$ was considered statistically significant.

3 Results

Oxidative stress caused by cigarette smoking and the effects of CAPE on these changes were determined by measuring the level of SOD, CAT and GSH-Px enzyme activity, and tissue levels of NO and MDA. The results are presented in Table 1. SOD activity in testis tissues of rats exposed to cigarette smoke only (Group II) increased significantly compared to the control group ($P < 0.05$), but SOD in Group III decreased to a level closer to the control group ($P = 0.05$). Compared to the control group, CAT activity in Group II increased significantly ($P < 0.05$), but in Group III, CAT activity decreased significantly ($P < 0.05$). The significant decrease in GSH-Px activity in Group II compared to Group I ($P < 0.05$) was reversed in Group III after administration of CAPE ($P < 0.05$), and the difference between Group I and Group III was not significant ($P = 0.05$). In the evaluation of MDA, a statistically significant increase in Group II was observed ($P < 0.05$), but in Group III, a reduction in MDA levels was observed and the difference with the control group was not significant ($P = 0.05$). The elevation of NO was significant in Group II ($P < 0.05$) but, with the administration of CAPE, the testicular tissue NO level in Group III was brought to a level very close to that of the control group ($P = 0.05$; Table 1).

4 Discussion

Smoking is a noxious process that triggers oxidative stress, not only in first-hand smokers but also in those exposed to second-hand smoke. By-products of chemical events, namely FRs and reactive oxygen species,

Table 1. Enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and tissue levels of malondialdehyde (MDA) and nitric oxide (NO) in all experimental groups. Group I, control (normal clean air); Group II, cigarette smoke only (4×30 min/d); Group III, cigarette smoke (4×30 min/d) and daily intraperitoneal injections of caffeic acid phenethyl ester ($10 \mu\text{mol/kg}\cdot\text{d}$). prot, protein; *n*, number. $P < 0.05$ was considered statistically significant.

Experimental group	SOD (U/mg prot)	CAT (U/mg prot)	GSH-Px (U/mg prot)	MDA (nmol/g wet tissue)	NO (nmol/g wet tissue)
I (<i>n</i> = 7)	0.1968 ± 0.0045	0.0092 ± 0.0011	0.0200 ± 0.0011	49.94 ± 3.38	66.48 ± 2.69
II (<i>n</i> = 7)	0.2584 ± 0.0042	0.0202 ± 0.0017	0.0110 ± 0.0013	74.70 ± 2.59	98.67 ± 3.31
III (<i>n</i> = 7)	0.2054 ± 0.0042	0.0124 ± 0.0011	0.0150 ± 0.0011	60.78 ± 2.32	74.85 ± 1.05
<i>P</i> values					
I vs. II	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
I vs. III	= 0.05	> 0.05	< 0.05	= 0.05	= 0.05
II vs. III	< 0.05	< 0.05	= 0.05	< 0.05	< 0.05

cause this oxidative stress. Cigarette smoking is also known to be associated with various cardiovascular diseases and chronic pulmonary diseases, ranging from infection to lung cancer [7–9].

FRs or reactive oxygen species have potentially harmful effects and cause oxidative stress on the metabolic events occurring in molecules. The cellular anti-oxidant defense system controls the effects of these species and this duty is carried by FR scavenger enzymes, such as SOD, CAT and GSH-Px [10]. When there is an impairment in the cellular anti-oxidant defense system and/or FR production exceeds the ability of this defense system to scavenge these species, oxidative stress occurs and FRs attack polyunsaturated fatty acids found widely in cell membranes. Lipid peroxidation develops in cell membranes, which causes production of membrane destruction products, such as MDA [10].

Baskaran *et al.* [11] studied the effects of cigarette smoke on lipid peroxidation and enzyme activities of SOD, CAT, GSH-Px. They observed increased lipid peroxidation in liver, lung and kidneys, and anti-oxidant enzyme activities were also elevated in these organs. Nielsen *et al.* [12] found slightly increased MDA levels in the plasma of smokers when compared to non-smokers. They supported that the plasma MDA level may be a potential biomarker for oxidative stress which occurred due to lipid peroxidation. In another experimental study, elevated MDA levels and decreased GSH and GSH-Px activity were found in rat tissues after the 45-day exposure to cigarette smoke [13].

NO is a water- and lipid-soluble FR synthesized in the vascular endothelium from *L*-arginine by the action of NO synthase enzymes. It plays an important role in the regulation of blood flow in normal and pathologic

situations. There could be two reasons for the elevation of tissue NO levels after exposure to cigarette smoke: increased synthesis because of the injury of the vascular endothelium, or the activation of neutrophils in damaged testicular tissue, leading to synthesis of NO [4]. Determination of the changes in NO levels and the effect of CAPE on NO levels after testicular torsion and detorsion in rats was reported in an experimental study by Koltuksuz *et al.* [4]. Their results showed that NO levels increased with torsion, and administration of CAPE prevented the decrease in NO levels during reperfusion. They concluded that this effect of CAPE on NO levels could be important for protecting testis from such an ischemia/reperfusion injury.

In previous studies, cigarette smoking has been shown to be associated with an overall decreased fertilizing capacity of sperm [14]. The mechanisms of the decrease in male reproductive capacity due to exposure to cigarette smoke ranges from causing erectile dysfunction by inducing some vasculogenic risk factors, to decreasing sperm counts, affecting sperm motility and deteriorating sperm membrane characteristics [15]. Cigarette smoke also leads to secretory dysfunction of both Sertoli and Leydig cells and causes harmful effects on the epididymal sperm maturation process and sperm capacity to penetrate oocytes [14].

Lower seminal plasma anti-oxidant enzyme levels and increased oxidative damage to sperm DNA due to cigarette smoke has been shown by Fraga *et al.* [16]. In addition, mitochondrial and nuclear DNA damage as a result of accumulated oxidative stress and endogenous DNA strand breaks in human spermatozoa after exposure to smoke has also been mentioned in other studies [17, 18].

It has also been shown that the serum levels of cotinine correlate with the number of cigarettes smoked, and passage of the noxious metabolites of cigarette smoke through the blood–testis barrier has been suggested as the basis of the oxidative stress and eventual oxidative damage of testicular tissues [19, 20].

The current study demonstrated that cigarette smoking had harmful effects on testicular tissue by generation of free oxygen radicals. In the CAPE-administered group, the elevation in GSH-Px enzyme activity might be an explanation for the protective effect of CAPE. The rising CAT and SOD activity and MDA levels in the rat testicular tissue due to cigarette smoke exposure were also reversed with administration of CAPE. These results propose the anti-oxidant effect of CAPE by direct or indirect effects on promoting the activity of anti-oxidant enzyme activities, or directly by free oxygen radical scavenging activity. It is also apparent that this oxidative stress will have a negative impact on male fertility potential. Further studies to show the histologic changes in sperm functions, seminiferous tubules, and Sertoli and Leydig cell functions, which associates histopathologic effects with biochemical results, should be performed.

In conclusion, exposure to cigarette smoke results in the overproduction of reactive oxygen species in rat testis. The administration of CAPE significantly decreases the oxidative stress on testicular tissue caused by cigarette smoke. Thus, we can say that CAPE administration in the appropriate dosage and duration prevents the adverse oxidative effects of cigarette smoking on testicular tissue.

References

- 1 Elsayed NM, Bendich A. Dietary antioxidants: potential effects on oxidative products in cigarette smoke. *Nutr Res* 2001; 21: 551–67.
- 2 Karla J, Chaudhary AK, Prasad K. Increased production of oxygen free radicals in cigarette smokers. *Int J Exp Pathol* 1991; 72: 1–7.
- 3 Zalata AA, Ahmed AH, Allamaneni SSR, Comhaire FH, Agarwal A. Relationship between acrosin activity of human spermatozoa and oxidative stress. *Asian J Androl* 2004; 6: 313–8.
- 4 Koltuksuz U, Irmak MK, Karaman A, Uz E, Var A, Ozyurt H, *et al.* Testicular nitric oxide levels after unilateral testicular torsion/detorsion in rats pretreated with caffeic acid phenethyl ester. *Urol Res* 2000; 28: 360–3.
- 5 Ogeturk M, Kus I, Colakoglu N, Zararsiz I, Ilhan N, Sarsilmaz M. Caffeic acid phenethyl ester protects kidneys against carbon tetrachloride toxicity in rats. *J Ethnopharmacol* 2005; 97: 273–80.
- 6 Kus I, Colakoglu N, Pekmez H, Seckin D, Ogeturk M, Sarsilmaz M. Protective effects of caffeic acid phenethyl ester (CAPE) on carbon tetrachloride-induced hepatotoxicity in rats. *Acta Histochem* 2004; 106: 289–97.
- 7 Kunzle R, Mueller MD, Huber AW, Drescher H, Bersinger NA. Seasonality in human semen quality of smokers and non-smokers: effect of temperature. *Asian J Androl* 2004; 6: 243–7.
- 8 Stillman FA, Hartman AM, Graubard BI, Gilpin EA, Murray DM, Gibson JT. Evaluation of the American Stop Smoking Intervention Study (ASSIST): a report of outcomes. *J Natl Cancer Inst* 2003; 95: 1681–91.
- 9 Bai Q, Xu QQ, Jiang H, Zhang WL, Wang XH, Zhu JC. Prevalence and risk factors of erectile dysfunction in three cities of China: a community based study. *Asian J Androl* 2004; 6: 343–8.
- 10 Bozlan AD, Bianchi MS, Bianchi NO. Superoxide dismutase, catalase and glutathione peroxidase activities in human blood: influence of sex, age and cigarette smoking. *Clin Biochem* 1997; 30: 449–54.
- 11 Baskaran S, Lakshmi S, Prasad PR. Effect of cigarette smoke on lipid peroxidation and antioxidant enzymes in albino rat. *Indian J Exp Biol* 1999; 37: 1196–1200.
- 12 Nielsen F, Mikkelsen BB, Nielsen JB. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clinical Chemistry* 1997; 43: 1209–14.
- 13 Rajpurkar A, Dhabuwala CB, Jiang Y, Li H. Chronic cigarette smoking induces an oxidant–antioxidant imbalance in the testis. *J Environ Pathol Toxicol Oncol* 2000; 19: 369–73.
- 14 Kapawa A, Giannakis D, Tsoukanelis K, Kanakas N, Baltogiannis D, Agapitos E, *et al.* Effects of paternal cigarette smoking on testicular function, sperm fertilizing capacity, embryonic development, and blastocyst capacity for implantation in rats. *Andrologia* 2004; 36: 57–68.
- 15 Ryu JK, Song SU, Choi HK, Seong DH, Yoon SM, Kim SJ, *et al.* Plasma transforming growth factor- β^1 levels in patients with erectile dysfunction. *Asian J Androl* 2004; 6: 349–53.
- 16 Fraga CG, Motchnik PA, Wyrobec AJ, Rembel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 1996; 351: 199–203.
- 17 Ballinger SW, Boudier TG, Davis GS, Judice SA, Nicklas JA, Albertini RJ. Mitochondrial genome damage associated with cigarette smoking. *Cancer Res* 1996; 56: 5692–7.
- 18 Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM. Sperm chromatin damage associated with male smoking. *Mutat Res* 1999; 423: 103–11.
- 19 Sofikitis N, Takeana M, Kanakas N, Papadopoulos H, Yamamoto Y, Drakakis P, *et al.* Effects of cotinine on sperm motility, membrane function, and fertilizing capacity *in vitro*. *Urol Res* 2000; 28: 370–5.
- 20 Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. *Hum Reprod Update* 2000; 6: 122–31.