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Research article

Protective effects of caffeic acid phenethyl ester on cadmium-induced testicular injury: A crucial role of antioxidant enzymes in male mice infertility

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ABSTRACT

Infertility in men is referred to inability to achieve pregnancy in fertile females after at least one year of regular intercourse. The lack of oxygen in the environment may lead to an imbalance of testes production. Swiss mice were alienated into four groups 10 mice/each. This included one negative normal control group I. The induction of infertility was achieved with injection of cadmium chloride at dose 3 mg/kg body weight for four consecutive days for the rest groups. Group III received vehicle (saline) from the second day of induction for the similar period during the experiment. Infertile mice determined depending on alterations in morphology, motility, and reduced sperm count. Group IV was treated with 3 mg/kg of caffeic acid phenethyl ester (CAPE) per day, for 6 days from the second day of cadmium intoxication. Data showed effectiveness of CAPE significantly through improving the antioxidant enzymes SOD, GST and GSH in testes homogenate and GSH-Px in mice serum that were treated compared to those in the untreated group II ($P^{<}0.001$). The histopathology, DNA analysis showed marked improvement in spermatogenesis and DNA intact pattern in treated mice testis. Overall, the results demonstrated the ability of CAPE to improve spermatogenic cells. The data analysis indicated the possibility for the future use of CAPE as an inhibitory agent of infertility. Clinical trials and further studies are required to evaluate the definite medical effects of CAPE based on abundant experimental studies, with predictive future applications in human clinical trials.

1. Introduction

Infertility is defined as a condition where there is an inability to establish pregnancy after a year of regular and unprotected sexual intercourse. It affects between 8 and 12% of reproductive-aged couples worldwide [1]. Previously, the occurrence of infertility was attributed to the woman. However, recent scientific developments have provided more clarity regarding sperm production and transmission. Of all infertility cases approximately 30% due to male factor fertility, and up to 90% of these are down to low sperm amount or low sperm value or both [2].

Cadmium (Cd) is a highly toxic heavy metal and considered as an environmental pollutant. Various organs and biochemical systems can be damaged by this metal. It usually resulted in chronic intoxications and can also cause severe, acute toxicity, induce liver, kidney and lungs toxicity. It is also a highly carcinogenic element, causing preferentially prostate, lung and gastro-intestinal cancers [3]. However, Cd used in manufacture of television screens, lasers beams, batteries, pigments used in paint, multiple cosmetics, and galvanization of steel. It can also be used in nuclear fission as a barrier and mixed with zinc to weld seals in water pipes made of lead.

Cigarette smoking is considered as one of the most significant exposures to this metal, which increases the Cd level in the blood compared to non-smokers. Cd is absorbed on the basis of size, through inhalation or ingestion. It has been found 10–50% of inhaled cadmium dust is absorbed, 5–10 % of ingested Cd is absorbed. Iron, calcium, or zinc deficiency increases the intestinal absorption of Cd [4].

There are different mechanisms controlling cadmium reproductive toxicity, including damage of testicular vasculature structure and blood-testes barrier, inflammation, apoptosis, Sertoli and Leydig cell cytotoxicity, oxidative stress, mainly through interference with essential ions,

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interference with selected signalling pathways, and epigenetic regulation of the gene involved in the regulation of the reproductive function [5].

The mechanism of Cd-induced apoptosis is faithfully related to the VDR/CREB1 pathway. As a nuclear hormone receptor involved in metabolism, confirmed that it can participate in the production of ROS and mitochondria reactive oxygen species (mtROS). Mitochondria proved that VDR can influence the oxidative stress response of mitochondria through PI3K/AKT and 17β-estradiol secretion. Moreover, MicroRNAs (miRNAs) play a key role in programmed necrosis and apoptosis of immune organs. Cd exposure caused the apoptosis (BAX, Bcl-2, Caspase 3, Caspase 9) and programmed necrosis (RIP, RIP3, MLKL), increased the expression of CYP enzymes, glycometabolism-related enzymes and production of ROS, while reduced the activities of MDA, SOD, CAT and GSH-PX significantly. Overall, Cd activates oxidative stress and miR-216a-PI3K/AKT axis disorder, thus promoting apoptosis and necrosis [6, 7, 8].

Fenton reaction (creation of hydroxyl radicals from hydrogen peroxide) is responsible for the oxidative stress induced by Cd toxicity, which leads to the alteration of the activities of certain antioxidant enzymes such as Cu- and Zn-superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), and glutathione-S-transferase (GST). Cd toxicity is responsible for reactive oxygen species (ROS) production and lipid peroxidation [9].

Oxidative stress is a condition in which the oxidants are increased to more than the handling capacity of the antioxidant. As a result of this imbalance there is a disturbance in redox signalling and control and/or molecular damage. Oxidative stress plays a great role in increasing the number of cases of male infertility. Undeniably, systemic oxidative stress, which is enhanced as a result of smoking, alcohol abuse, severe respiratory dysfunctions and exposure to toxins, inflammatory processes or other several chronic diseases, reduces male fertility [10].

The sperm membrane which is rich with poly-unsaturated fatty acids is very susceptible to peroxidation by ROS, leading to morphologic sperm changes [11]. High ROS levels can increase the possibility of infertility either directly or indirectly, by inducing oxidative stress (OS) or by acting through the hypothalamic axes of hormone release ROS to diminish male sex hormone levels and interrupt the hormonal balance, respectively. These “endocrine disruptors” not only interfere in the communication between testes and the hypothalamic-pituitary unit but also disrupt the cross-talk between the hypothalamic-pituitary-gonadal (HPG) axis with other hypothalamic hormonal axes [12, 13].

In the plant kingdom there are broadly distributed biologically active compounds named polyphenolic compounds [14]. Some of these compounds have anti-inflammatory effect, carcinostatic, antioxidant, antiviral, and anti-carcinogenic action. One of the most important compounds is propolis, which is a complex resinous mixture used by honeybees to build their hives [15].

Caffeic acid phenethyl ester (CAPE) [2-propenoic acid, 3-(3,4-dihydroxy phenyl)-,2-phenethyl ester] is the main pharmacologically-active component of some propolis types, rich in polyphenols, such as poplar propolis types [16]. CAPE plays a great role in intestinal, colon, hepatic and skin cancer through its chemo-preventive function. CAPE, in a concentration dependent fashion, was shown to inhibit MCF-7 (hormone receptor positive, HR+) and MDA-MB-231 (a model of triple negative BC, TNBC) tumour growth, without affecting normal mammary cells [17].

Furthermore, CAPE suppresses NF-κB activation by decreasing the binding of the p50–p65 complex directly to DNA. The translocation of the p65 subunit of NF-κB to the nucleus is inhibited without affecting the TNF-induced IκBα degradation. There are no inhibitory effects on other transcription factors such as AP-1, TFIID and oct-1. CAPE is found to be a powerful and definite inhibitor of NF-κB activation, and this may provide the molecular basis for its multiple immunomodulatory and anti-inflammatory activities [18].

The current study is aimed at studying whether the administration of the CAPE compound, which displays anti-inflammatory and antioxidant activity, would be beneficial for treating a life crisis disease such as infertility. The antioxidant parameters of SOD, GST, and total GSH in tissue as well as serum GSH-Px parameters will be examines, which are known to be associated with infertility. At the same time, their levels will be correlated with improvement outcomes. More details and investigations of histopathology of the testis and the molecular level of DNA integrity will be assessed, which is necessary to figure out CAPE's mode of action.

2. Materials and methods

2.1. Animals

Adult male Swiss mice weighing 22–25 g were obtained from the animal house of the King Fahad Center for Medical Research, King Abdulaziz University, Jeddah, Saudi Arabia. The mice were accommodated in a pathogen-free situation in the animal house of Albaha University, Saudi Arabia, in a 12/12-h light/dark cycle with food and water available *ad libitum*. Current research project (221/1435) was approved by the Research Ethics Committee, Faculty of Medicine, Albaha University and the animal experimentation was performed strictly following the recommended ethical standards.

2.2. Chemicals and drugs

2.2.1. Cadmium chloride

Cadmium chloride (CdCl2 2.5 H2O) was obtained from Sigma, St. Louis, MO, and dissolved in water. The mice inoculation was performed daily for four repeated days consecutively with different doses of CdCl2 (1, 3, 5 and 7 mg/kg body weight). As the dose of 3 mg/kg induced significant changes of seminiferous tubules in mice, this dose was approved in the following experiments.

2.2.2. Caffeic acid phenethyl ester (CAPE)

CAPE was attained from Sigma Chemical Company. The compound was dissolved in DMSO droplet (Sigma, St. Louis, MO) at 20 mg/ml concentration, standard solution and stowed at −20 °C. Consecutive concentrations in PBS solution were achieved for the compound on the basis of its usage throughout the course of mice handling.

2.2.3. Lethality study in Swiss albino mice

The male Swiss albino mice were randomly divided into different groups of 10 mice each. They were treated with different dose concentrations of CAPE. The number of living mice was recorded daily and the procedure was carried out for 30 days. The mice were subjected to experiments according to ethical standards [19].

2.3. Experimental design

Swiss mice were divided into four groups each comprising 10 mice/group. The induction of infertility was achieved according to the method of Qadori and Al-shaikh [20], with some modifications. On day 1, group I was kept as normal control. Groups II, III, and IV received an intraperitoneal inoculation (ip) of cadmium chloride at the dose 3 mg/kg body weight (b.wt) for four consecutive days. Group II did not receive any treatment, while the group III received vehicle (saline) for a similar period of experimentation. Group IV was treated with 3 mg/kg/day of CAPE for 6 days after cadmium inoculation (treated group). The confirmation of success for all infertile mice was realized according to sperm evaluation, data gained and histopathological examination at the beginning of the experiments. At the end of the experiments, the mice were weighted and scarified (a process of euthanasia used on mice under anaesthesia) 24 h after the last treatment. Blood was withdrawn from all groups, and serum was reserved for analysing GSH-Px parameter. Both left and right testes were collected and weighed. The left testes were

Table 1. Cadmium intoxication effects on sperm count, sperm motility and abnormal sperm levels.

	Normal Control gp I	Cadmium Intoxication gp II	Cadmium Saline Treated gp III	Cadmium CAPE Treated gp IV
Sperm count $\times 10^6$	234.1 ± 0.7	133.8 ± 0.8 ***	129.5 ± 0.8 ***#	203.2 ± 0.8 ***###¶¶¶
Motile sperm (%)	89.0 ± 3.7	62.2 ± 5.4 ***	61.7 ± 4.2 ***	78.3 ± 4.1 ***###¶¶¶
Abnormal sperm (%)	5.2 ± 0.9	13.2 ± 1.9 ***	13.6 ± 2.3 ***	6.8 ± 1.3 ###¶¶¶

Data expressed as mean ± SD SD: standard deviation P: Probability.
Test used: One way ANOVA followed by post-hoc tukey.
*P < 0.05; **P < 0.01; ***P < 0.001 vs. Normal Control gp I.
#P < 0.05; ##P < 0.01; ###P < 0.001 vs. Cadmium Intoxication gp II.
¶P < 0.05; ¶¶P < 0.01; ¶¶¶P < 0.001 vs. Cadmium Saline Treated gp III.

stored at –20 °C until analysis of antioxidant parameters (SOD, GST, and total GSH in tissue). Additionally, the mice right testes were sliced for DNA extraction (Sigma, St. Louis, MO), and apoptosis check. The rest testes were kept in 10% formalin for histopathological examination.

2.4. Sperm count

The sperm cell concentration was estimated. Undiluted semen was withdrawn up to the mark 0.1 and pipette was then filled up to the mark 101 with normal saline that was stained with eosin. Then, the content of the pipette was shaken vigorously by holding the ends of the pipette between the thumb and the index finger. A cover slide was placed over the counting chamber and a drop of diluted semen was spread between the haemocytometer chambers (Muhwa Commerce Co., Ltd), and its cover. Then sperms in 5 large squares (80 small squares) were counted using a high power microscope (40x). The sperm cell concentration was estimated by multiplying the counted number of sperms by 100 (depth) and 1000 (dilution) [21].

2.5. Epididymal sperm abnormalities

A drop of epididymal content for each mouse was mixed with an equal drop of eosin-nigrosin stain (BioGnost's). The semen was carefully mixed with the stain. These films were spread on clean and greasy free slides. Two hundred sperms were randomly observed per mice under the high-power lens of a light microscope (Optika microscope). The description of the abnormal forms of the sperm observed in this study was done according to Mori's classification [22].

2.6. Estimation of ascorbic acid

Ascorbic acid in seminal plasma is oxidized by cupric (Cu+2) to form dehydro ascorbic acid which reacts with acidic 2,4-dinitrophenyl-hydrazine to form a red bis-hydrazone that was measured at 520 nm spectrophotometrically [23].

2.7. Determination of SOD, GST and GSH activity in testis homogenate

The sliced testes were homogenized in phosphate buffered saline (10%) before centrifugation at 4 °C for 20 min at 4000 rpm using cooling centrifuge (DASH Flex12). The resultant supernatant was aspirated using plastic pipette for superoxide dismutase (SOD) (ab65354), glutathione S-transferase (GST) (ab65326) and reduced glutathione (GSH) (ab138881) detection, using spectrophotometer (Spectrophotometer UV7). Briefly, 0.1 M of sodium pyrophosphate buffer, 0.93 μM of phenazine methosulfate (prepared fresh), 0.3 mM nitro blue tetrazolium and 0.47 mM NADH was added to the sample following Nishikimi et al [24]. The absorbance of SOD was measured at 560 nm wavelength and expressed as U/g wet tissue. For determination of GSH level, homogenate was added to tungstate solution in equal volume after centrifugation for 5 min at 2000 rpm. 200 μl of aspirated supernatant was transferred to cuvette containing Tris buffer and 0.2 ml of 5, 5-dithio-bis-2-nitrobenzoic acid reagent. The optical density was measured at 412 nm wavelength after an incubation of 30 min [25]. GST was measured with the spectrophotometric assay of Alin et al [26]. It uses 1-chloro-2,4-dinitrobenzene as the electrophilic substrate that binds to GSH with the participation of the enzyme to form a colored GSH-substrate complex, detected at 340 nm.

2.8. Determination of serum GSH-Px

The blood samples were drawn from all mice model groups. Briefly, about 0.5 ml of the whole blood was collected from each group of mice after the last administration for all groups. The blood was preserved in a 4 °C refrigerator for 30 min, followed by centrifugation at 3000 rpm to obtain about 0.2 ml serum. All the serum was kept at −20 °C before the test. The determination of serum GSH-Px was performed using GSH-Px kits (Sigma) according to the kit's procedures [27].

2.9. DNA electrophoresis

A distinguishing feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi-quantitative way of

Table 2. Cadmium intoxication effects on testicular weight and ascorbic acid level.

	Normal Control gp I	Cadmium Intoxication gp II	Cadmium Saline Treated gp III	Cadmium CAPE Treated gp IV
Testicular Weight (g)	0.803 ± 0.06	0.403 ± 0.03 ***	0.415 ± 0.03 ***	0.682 ± 0.06 ***###¶¶¶
Testicular ascorbic acid level (mg/g tissue)	1.93 ± 0.3	0.08 ± 0.02 ***	0.07 ± 0.02 ***	1.42 ± 0.3 ***###¶¶¶

Data expressed as mean ± SD SD: standard deviation P: Probability.
Test used: One way ANOVA followed by post-hoc tukey.
*P < 0.05; **P < 0.01; ***P < 0.001 vs. Normal Control gp I.
#P < 0.05; ##P < 0.01; ###P < 0.001 vs. Cadmium Intoxication gp II.
¶P < 0.05; ¶¶P < 0.01; ¶¶¶P < 0.001 vs. Cadmium Saline Treated gp III.

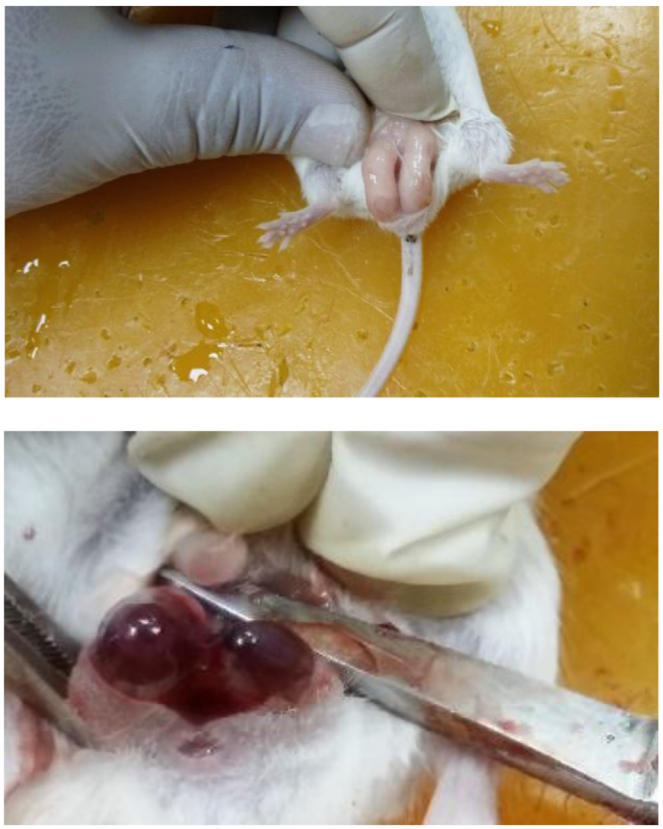

Figure 1. Photograph (Top) of testes in normal mice. (Bottom) testes after cadmium intoxication.

determining apoptosis. Apoptotic changes in testes were evaluated by DNA fragmentation through agarose gel electrophoresis according to the procedure. Testes from all groups were collected after being weighted into Eppendorf tubes and washed twice with cold PBS and homogenized and filtrated. The samples supernatant was re-suspended in 400 μl DNA extraction buffer (10mMTris–HCl PH 8.0, 150mMNacl, 10mM ethylenediaminetetraacetic acid (EDTA) and 2%SDS) (Sigma). Proteinase K (500 ug/ml, Boheringer Mannheim Gmbh, Germany) and RNAse (20 ug/ml; Sigma) were added and these suspensions were incubated for 18 h at 37 C. DNA was extracted twice by means of phenol–chloroform, then precipitated with 0.1 volume of 3M sodium acetate (Nippon Gene Co, Tokyo) and 2.5 volume of 100 cold ethanol, then stored for 1 h at –70 °C. The precipitates were rinsed with 70% ethanol and dried. DNA pellets were resuspended in TE buffer. The quality and integrity of the purified DNA were assessed by gel electrophoresis in 0.8% agarose with DNA size marker 500 bp (Sigma) [28].

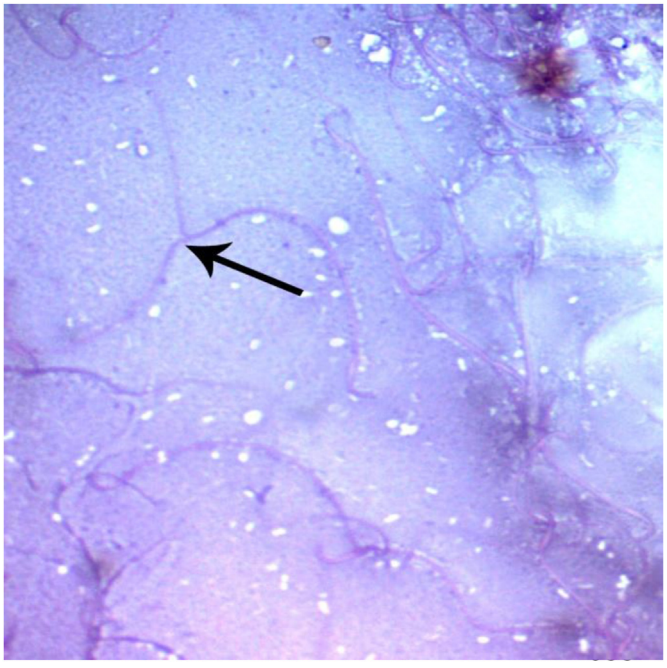

Figure 2. Photomicrograph showing double tail sperm from mice of group II (Nigrosin and Eosin x400).

2.10. Histopathological examination of the testes

The right testes were fixed in 10% paraformaldehyde, dehydrated and embedded in paraffin, sectioned at 5 μm. The sections were stained with haematoxylin and eosin. The morphological changes were examined under a microscope (Eclipse 80i, Nikon, Japan), and the pictures were captured with a video camera (DS-Fi1 digital microscope camera, Nikon, Japan).

2.11. Statistical analysis

Data were analysed using the Statistical Package for Social Science software computer program version 26 (SPSS, Inc., Chicago, IL, USA). Data were parametric and presented in mean and standard deviation. One way analysis of variance (ANOVA) and tukey were used for comparing parametric data. P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cadmium intoxication

In the present study the testes of Cd intoxication were assessed with the following considerations: alterations in morphology, testicular pathology, and reduction in both sperm count and ascorbic acid level, with significant decrease in testicular weight, motile sperms and increase in the incidence of abnormal sperms. The significant decrease observed in parameters was dose dependent in the cadmium intoxication group II. Sperm count ($133.8 \pm 0.8*10^6$) highly significantly decrease ($P < 0.001$) compared to control group ($234.1 \pm 0.7*10^6$). Motile sperm % (62.2 ± 5.4) highly significantly decrease ($P < 0.001$) compared to control group (89.0 ± 3.7). Abnormal sperm % (13.2 ± 1.9) highly significantly increase ($P < 0.001$) compared to control group (5.2 ± 0.9) (Table 1). Testicular weight (0.403 ± 0.03g) was highly significantly decrease in group II ($P < 0.001$) compared to control group (0.803 ± 0.06g) and ascorbic acid level was (1.93 ± 0.3 mg/g) in control group and highly significantly low in the Cd intoxicated group II (0.08 ± 0.02 mg/g) ($P < 0.001$) (Table 2). Furthermore, treated group IV showed a high significant effect and improvement in all parameters verified compared to group II ($P < 0.001$). Moreover, Cd-induced testis injury, testicular edema and haemorrhage were noted (Figure 1). A sperm shape abnormalities in the form of double and coiled tail sperm were recorded in mice exposed cadmium chloride 3 mg/kg.b.wt (Figures 2 and 3).

3.2. Toxicity of CAPE

The mice treated with CAPE (1, 3, 6, 9 and 12 mg/kg) (I.P) did not shown any sign of toxicity in their body weight, overall appearance and organ pathology. The LD_{50} dose of CAPE was 9 mg/kg (I.P). In the second day after cadmium injection, all mice groups were suffering infertility. The CAPE treatment with 3 mg/kg b.wt. for six consecutive days had a median survival time of 26 days. The mice improved after treatment and were surviving without any indications of adverse effects. Moreover, two mice were found to be completely cured. It was observed that the ongoing treatment with 3 mg/kg caused no sores or irritability in the mice.

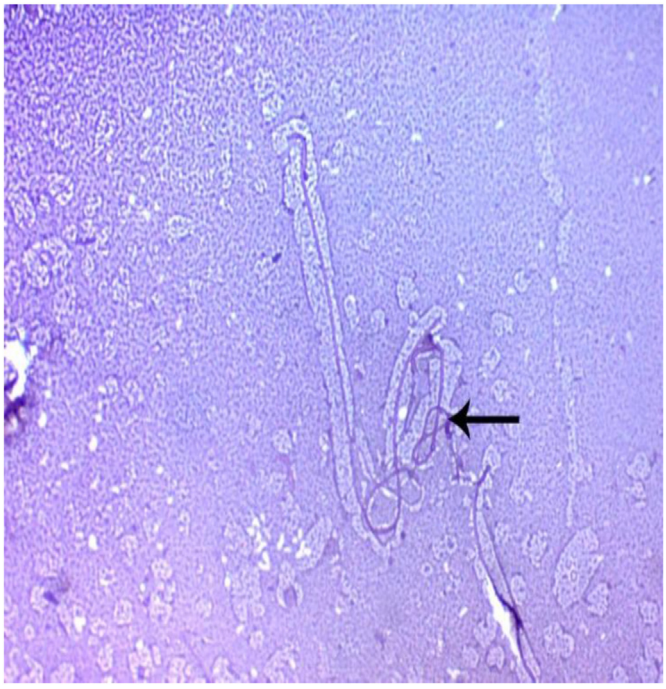

Figure 3. Photomicrograph showing coiled tail sperm group II mice (Nigrosin and Eosin x400).

3.3. Effect of CAPE on the tissue SOD, GST and GSH activities

The mean activities of antioxidant enzymes including SOD, GST and GSH were studied (Table 3) in the testes homogenates of the Cd intoxication group. The SOD, GST and GSH were significantly lower in group II compared with the control group. The results showed that the CAPE at dose 3 mg/kg in group IV with IP treatment provoked high significant increase in the level of SOD, GST and reduced GSH enzymes activities compared with the infertile group II ($P < 0.001$). These results suggested that treatment with CAPE at the selected dose (3 mg/kg b.wt) resulted in increased ROS removal in mice testis.

3.4. Effect of CAPE on the serum GSH-Px activity

GSH-Px contents in serum were found to be highly significantly lowered ($P < 0.001$) in the intoxicated mice group II when compared to the normal control group I ($P < 0.001$). However, the serum level of GSH-Px in the group of mice undergoing intoxication followed by CAPE treatment group IV was significantly higher ($P < 0.001$) in comparison with the intoxicated mice of group II (Table 4).

3.5. DNA fragmentation

Cd induced DNA damage in testes was evaluated by detecting DNA ladders in agarose gel electrophoresis. The results showed that Cd caused marked DNA fragmentation in testis lane 2 compared to control lane 1 which showed complete intact form. Simultaneous treatment with CAPE significantly decreased Cd-induced DNA fragmentation in testis lane 4. Moreover, DNA fragmentation in response to Cd treated saline represented a series of fragments and did not show any improvement in DNA form lane 3 (Figure 4). Treatment with CAPE led to significant protection against Cd-induced DNA fragmentation. The results showed that CAPE could counteract Cd-induced apoptosis in mice testes.

3.6. Histopathological results

The histopathological examination of H&E stained sections for the testis of the control mice showed thick fibrous tissue capsule underneath blood vessels. Seminiferous tubules indicated normal structure. They were lined by spermatogonia, followed by successive layers of germinal epithelium at various stages of spermatogenesis with normal supporting cells of Sertoli (Figures 5 and 6).

The current study revealed severe histological changes in 78 % of the examined mice in the form of extensive widening of interstitial spaces (Figure 7) due to diffuse oesinophilic, edematous vacuolated fluids infiltration, necrosis (Figure 8), congestion and haemorrhage. However, in 22 % of the mice, there was complete necrosis and sloughing of all layers of seminiferous tubules (Figures 9 and 10). Furthermore, in 38% of the mice there was marked cystic dilatation, atrophy with fibrosis and complete absence of the sperms.

Table 3. The SOD, GST and GSH levels on mice different groups.

	Normal Control gp I	Cadmium Intoxication gp II	Cadmium Saline Treated gp III	Cadmium CAPE Treated gp IV
SOD U/g wet tissue.	49.8 ± 0.45	30.03 ± 0.49 ***	31.01 ± 0.52 ***#	44.46 ± 1.25 ***###¶¶¶
GST U/g wet tissue.	55.36 ± 0.77	33.86 ± 0.65 ***	32.72 ± 0.70 ***#	50.02 ± 1.01 ***###¶¶¶
GSH U/g wet tissue.	70.16 ± 0.45	50.65 ± 0.58 ***	50.25 ± 0.43 ***	62.38 ± 0.66 ***###¶¶¶

Data expressed as mean ± SD SD: standard deviation P:Probability.
Test used: One way ANOVA followed by post-hoc tukey.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. Normal Control gp I.
#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ vs. Cadmium Intoxication gp II.
¶$P < 0.05$; ¶¶$P < 0.01$; ¶¶¶$P < 0.001$ vs. Cadmium Saline Treated gp III.

Table 4. Serum level GSH-Px on mice different groups.

Group	Normal Control gp I	Cadmium Intoxication gp II	Cadmium Saline Treated gp III	Cadmium CAPE Treated gp IV
GSH -Px mg/dl	4.82 ± 0.04	3.92 ± 0.08 ***	3.83 ± 0.09 ***	4.7 ± 0.18 ###¶¶¶

Data expressed as mean ± SD SD: standard deviation P: Probability.
Test used: One way ANOVA followed by post-hoc tukey.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. Normal Control gp I.
#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ vs. Cadmium Intoxication gp II.
¶$P < 0.05$; ¶¶$P < 0.01$; ¶¶¶$P < 0.001$ vs. Cadmium Saline Treated gp III.

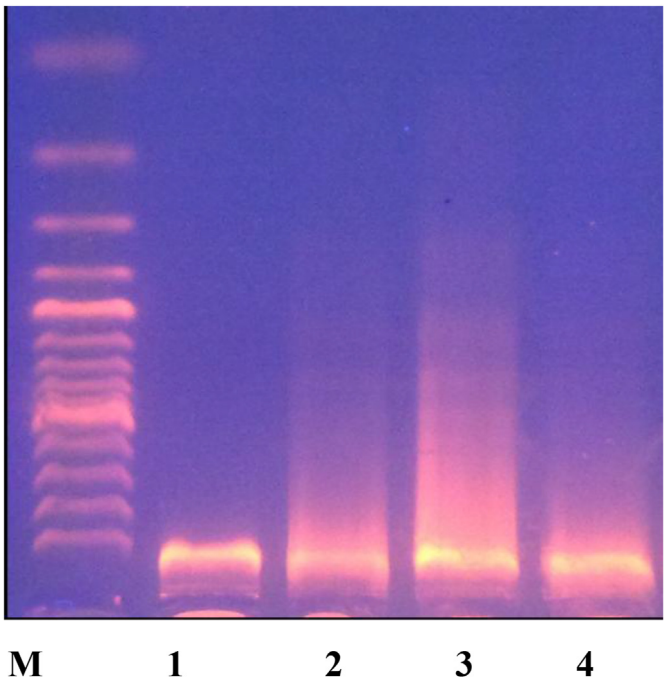

Figure 4. Agarose gel electrophoreses showing DNA fragmentation in mouse testis induced by cadmium: Lane M DNA molecular weight marker. Lane 1 represents control testis, lane 2 cadmium-intoxicated group II, Lanes 3 represents DNA fragmentation in group III, and lane 4 treated mice with CAPE 3 mg/kg b.wt.

Mice of Cd exposure group IV treated with CAPE showed regenerative changes with complete destruction of spermatogonia, as these changes were irreversible. The regenerative changes were in the form of improvment congestion and edema, disappearance of mononuclear infiltrates and reconstruction of layer of spermatogenesis and appearance of spermatids and mature sperm (Figures 11 and 12).

4. Discussion

Male infertility is one of the most common problems in many societies and there are traditional reasons that may lead to its occurrence such as,

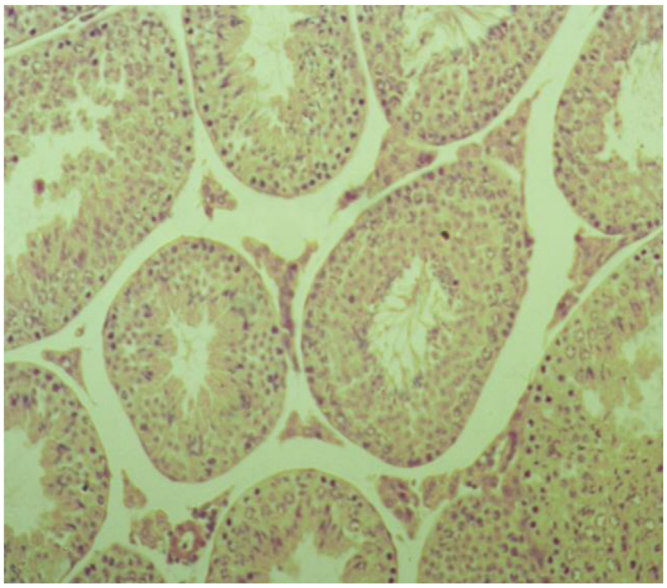

Figure 5. Photomicrograph, showings normal structures of seminiferous tubules and spermatocytes of normal control group I (H&Ex400).

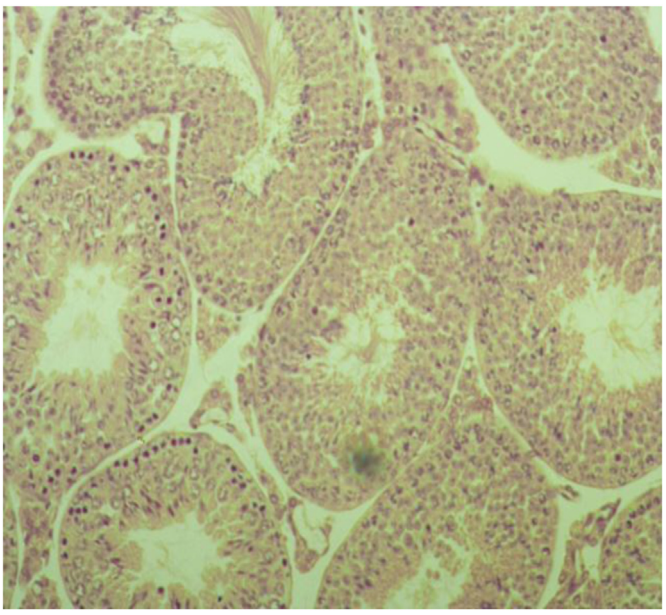

Figure 6. Photomicrograph, showings normal structures of seminiferous tubules and spermatocytes of normal control group I (H&Ex400).

infections and wounds fibrosis, trauma, tumours, disappearance of one testicle, and varicocele. In recent years, free radicals are considered a major cause for infertility. In other words, the spermatogenic cells, like any other cells, rely entirely on the central respiratory system necessary for its physiological process as normal cells. In line with this, any affect in oxygen rate or deficiency may cause change in cells' life span, function, and morphology and consequently lead to infertility [29, 30, 31, 32].

Consequently, it will sheds light on the importance of using extracts of natural sources such as, caffeic acid phenethyl ester and evaluate its effectiveness as an antioxidant and anti-inflammatory compound. This study examines the impact of CAPE on experimental animal induced

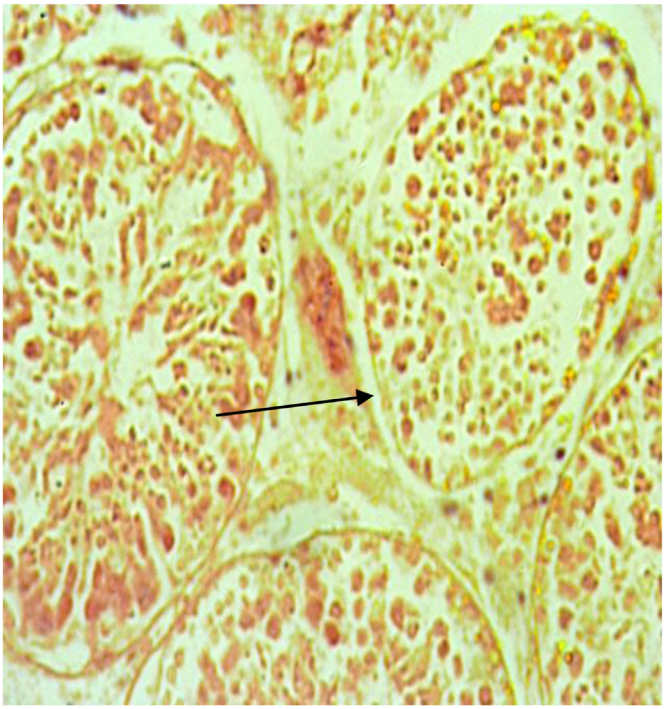

Figure 7. Seminiferous tubule-extensive widening of interstitial spaces (black arrow) in cadmium exposure toxicity (x400).

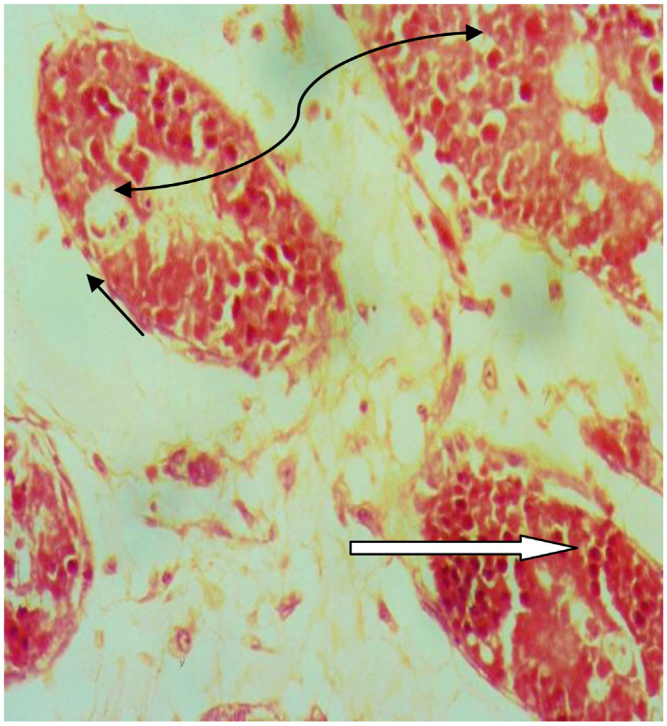

Figure 8. Seminiferous tubule-extensive widening (black) vacuolations (curved), necrosis (white) in cadmium toxicity (x400).

infertility as well as the extent of its ability to reduce the level of free radicals and protect the spermatogenic cells from destruction. In addition, it examines the high rate of infertility due to free radicals increases.

The infertility model mice used in this study offered two advantages. First, the seminiferous tubules vacuolations, disruption of spermatocyte cells and edema in this model closely resemble those of human disease. Secondly, the mice involved are normal and do not have any underlying

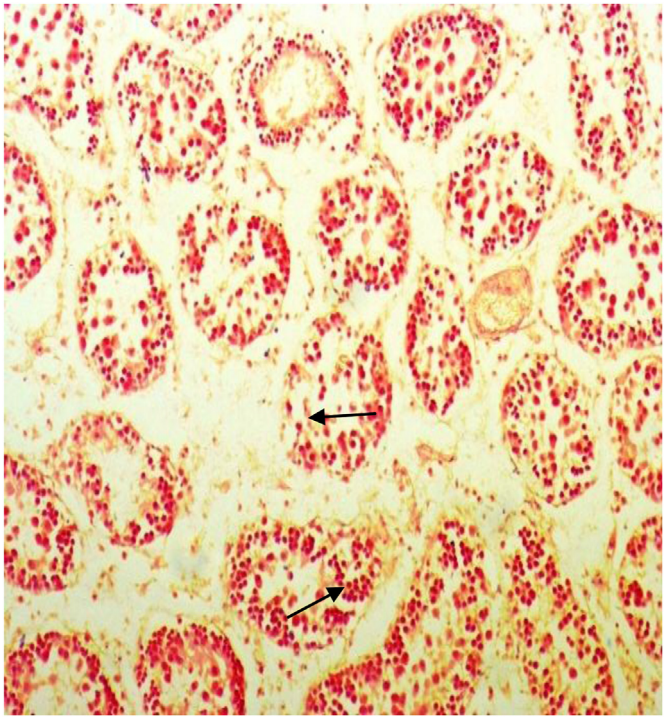

Figure 9. Seminiferous tubules showings vacuolations, disruption of spermatocyte, edema in cadmium exposure toxicity (x200).

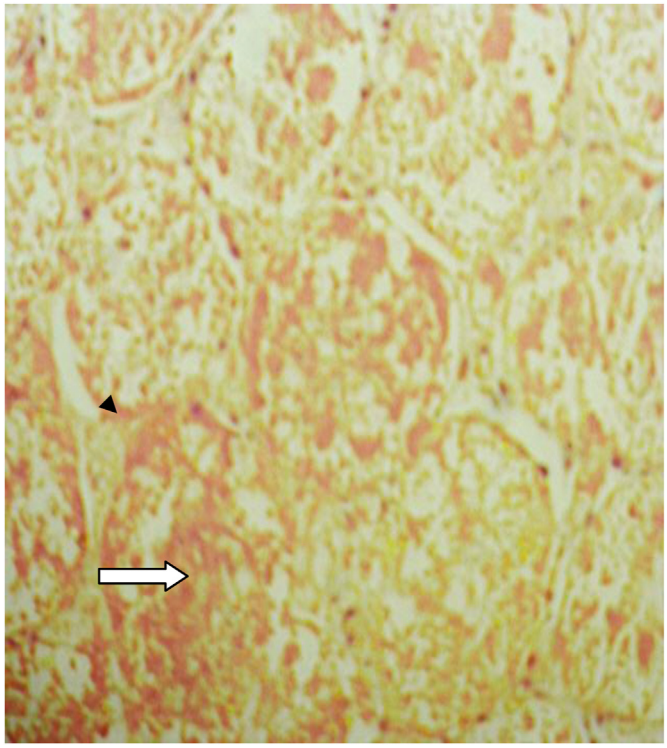

Figure 10. Ghosts of Seminiferous tubule showings extensive necrosis (white), vacuolations (head), in cadmium exposure (x200).

abnormalities that may complicate the study. Concerning Cd intoxication, our results showed a marked effect and induction of infertility (Tables 1 and 2; Figures 1, 2, and 3) similar to the results gained by Adaramoye and Akanni who documented that Cd affects both testes and sperms. Moreover, it was observed that there was decrease in experimental animals' testes weight during the administration of Cd with different duration time and doses [33]. Cupertino et al. also reported that animals' body weight was not affected by Cd exposure, while their testicular and parenchyma weights decreased [34]. CAPE has the ability

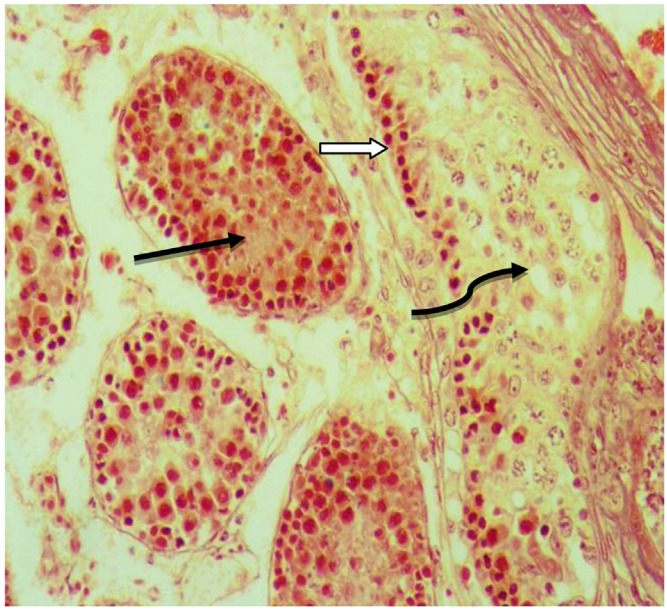

Figure 11. Seminiferous tubules necrosis (black), vacuolations (curved) peritubular edema, regenerative changes (white) in CAPE treated group IV (x200).

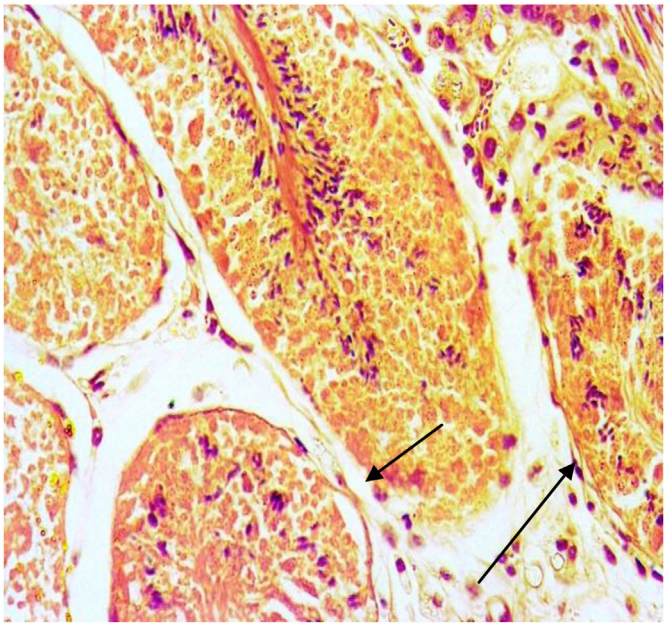

Figure 12. Seminiferous tubules with regenerative changes (black), edema and interstitial infiltrates in treated CAPE group IV (x400).

to prevent the live weight loss caused by Cd [35]. Moreover, Akyol et al. stated that testes weight loss in rats were prevented by the use of CAPE administered together with Cd [36]. Zakaria and Al Busadah specified that the weights of both testes reduced significantly with cadmium as well as a significant decrease in a live sperm and motility (%). Moreover, stated that cadmium also induced significant reduction in sperm count ($30 * 10^7$/ml) when compared to control group ($100 * 10^7$/ml) also they noticed alterations in morphology and reduced ascorbic acid level [37].

In the last decade, an increase in oxidative stress and oxidant–antioxidant imbalance has emerged as major contributors to the pathogenesis and damage. The damage can be controlled by persistent production of free radical scavengers at significantly high concentration for a longer duration. Present data suggest that a localized rise in enzymatic and non-enzymatic antioxidant levels in testes tissue may be beneficial in the treatment of infertility. In this study, Cd injection to the mice model deteriorated the activity of the tissue SOD, GST and GSH as well GSH-Px serum level (Tables 3 and 4, respectively). In accordance with these findings, Bashandy et al. illustrated also the toxic effect of cadmium on testicular damage. Thus, Cd administration at lower concentration by oral route reduces SOD activity to (25U/mg protein) [38]. Furthermore, in accordance with the results derived in this study, Kini et al. demonstrated in their study the effect of cadmium on testes, noticing a significant decrease in GSH (8.34 mg g^{-1}) and SOD (0.76 mg g^{-1}) in the Cd injected group [39].

Likewise, the results clarified that the administration of CAPE at 3 mg/kg/day for 6 days after Cd injection proved beneficial for the host in two ways. First, CAPE induced a marked antioxidant effect activity on antioxidant enzymes, as demonstrated by the high significant increase in the level of SOD and GST and reduced GSH enzymes activities compared with the infertile group II ($P < 0.001$). Furthermore, this data revealed nearly a normal value at the end of the treatment period in the treated group IV of infertility compared to the untreated group II. Second, the improvement of tissue antioxidant levels appeared parallel to a significant increase in the circulating serum level of GSH-Px after treatment. This finding seems to be consistent with other similar findings reported by Zakaria and Al Busadah who studied the effect of pentoxifylline and reported its protective antioxidant role after Cd injection to their model [37]. In connection to this, Ige et al. mentioned that the Cd significantly reduced testicular SOD (1.2 mg/U), while Allium cepa rats treated showed a significant rise in testicular SOD (1.8 mg/U) and catalase activities when compared to the normal control group I [40]. On the other hand, the results of this study revealed that serum GSH-Px has correlations with GSH and SOD levels in tissue homogenates (Figure 13 A, B). Furthermore, a correlation was observed in both GSH and SOD levels (Figure 13C) in tissue homogenate. All correlations were highly significant.

The results of the present study proved that Cd affects the primary spermatocytes by direct toxicity, causing DNA damage. The most important step in reproduction is the maintenance of DNA integrity in the paternal genome. Fertility can be impaired when lesions occur in DNA germ cells, consequently affecting reproduction outcomes. Furthermore, the data showed fragmentation of DNA in the untreated infertile mice group II lane 2, compared to the treated group IV lane 4. This fact helped us confirm the results of CAPE's inhibitory and counteract effects *in vivo* against Cd intoxication. The precise mechanism of CAPE in the enzyme activities is not fully clear. However, two mechanisms can be speculated. First, CAPE may affect the transcriptional and/or translational pathways of these antioxidant enzymes. Second, it prevents the induction of the enzymes by the inhibition of toxic oxidative products. In this study we

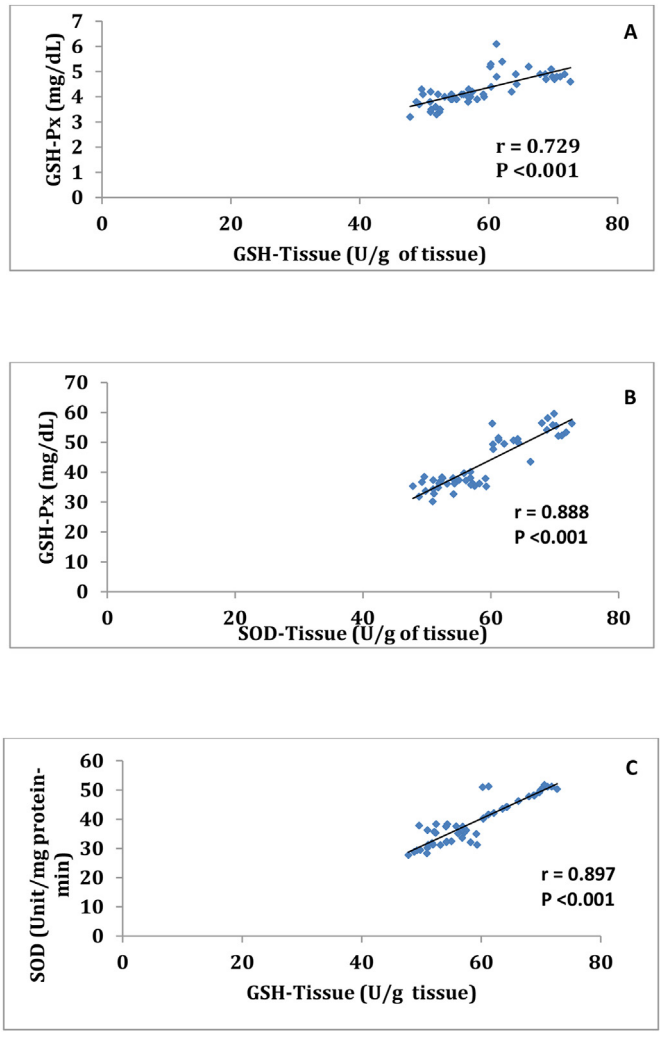

Figure 13. The solid lines represent, the linear regression and correlation coefficient (r), P is the significance. (A) There is a moderate positive highly significant correlations between GSH-Px and GSH in tissue ($r = 0.729$, $p < 0.001$). (B) There is a strong positive highly significant correlations between GSH-Px and SOD in tissue ($r = 0.888$, $p < 0.001$). (C) There is a strong positive highly significant correlations between SOD and GSH in tissue ($r = 0.897$, $p < 0.001$).

illustrated that simultaneous treatment with CAPE significantly decreased Cd-induced DNA fragmentation in testes lane 4. Moreover, DNA fragmentation in response to saline treatment group III represented a series of fragments with multiples base pairs in lane 3 and did not show any changes or improvement (Figure 4). Treatment with CAPE led to significant protection against Cd-induced DNA fragmentation. These results agree with the results of Erboga et al. who observed multiple potential sites of testes where Cd affects the cells. However, treatment with CAPE markedly reduced Cd-induced germ cell and Leydig cell apoptosis in testis [41].

The testes are the major target organ for Cd, with high sensitivity to the metal. Spermatogenesis is affected by Cd toxicity through two ways; the first is degeneration in spermatogenic cells, through damage to the blood-testis barrier. The second is the disruption of the connection complex between Sertoli cells [42]. Concerning histopathological examination of testes sections, the results showed extensive widening of interstitial spaces (Figure 7) due to diffuse oesinophilic, edematous vacuolated fluids infiltration, necrosis (Figure 8), congestion and hemorrhage. Cd causes necrosis in seminiferous tubules, casting of seminiferous tubule epithelial cells into the lumen and multiple histopathological changes. Moreover, in some cases there is an occurrence of a complete necrosis and sloughing of all layers of seminiferous tubules (Figures 9 and 10), with complete absence of the sperms. These results are almost in harmony with those studied by Mouro et al. who presented some changes in testes architecture found the animals exposed to toxicity by Cd show seminiferous tubules degeneration with absence of germ cells and generalized vacuolization of the seminiferous epithelium [43]. In addition, Obianime and Roberts, who demonstrated an effect of Cd on kidney and testis, confirmed the findings of the present study; they noticed damage to the histology of the testis at a dose of 0–40 mg/kg of Cd. Furthermore, germ cells and semniferous tubules destruction, vascular congestion, and focal necrosis of tissue were found. Additionally, they found a decrease in spermatocytes, pyknosis, nucleus destruction, and oedema in the interstitial tissue [44].

Caffeic acid phenethyl ester has the ability to be distributed very well through systemic circulation. It passes easily from the serous membranes and also has *in vivo* effects, besides its proven *in vitro* effect against Cd-induced toxicity [45]. The results of the current study indicated that CAPE-treated group IV showed regenerative changes compared to cadmium exposure group II. The regenerative changes were in the form of improvement of congestion and edema, disappearance of mononuclear infiltrates and reconstruction of layer of spermatogenesis and appearance of spermatids and mature sperm (Figures 11 and 12). Our findings are in line with the results of Balaha et al. who clarified in their study the potentiality of CAPE against Cd-induced toxicity. This is demonstrated in certain antioxidant effect of CAPE on tissue damage [46]. This is also in agreement with the results of Abdallah et al. who affirmed that a section in testes of a rat treated with Cd and CAPE showed an increase of spermatogenic cells. Finally, they concluded in their study that CAPE protects the testes from injury [47].

Overall, it is obvious that a significant oxidative stress has been noted in all the pathological models of the reproductive organs, such as testicular injury, which was used as an example. Treatment with CAPE has protecting effects on the oxidative stress in testes. These findings clearly show that CAPE has an inhibitory effect on testicular damage, induced by Cd and this effect is shared by the most possible pathway, scavenging ROS extensively by CAPE. Clinical studies are needed to validate the correct usage of CAPE either alone or in combination therapy.

5. Conclusion

Based on the findings of this study, we can conclude the following: It can be hypothesized that the CAPE has an inhibitory effect against Cd induced testicular damage, oxidative stress, apoptosis and exhibit regenerative improvement as well inhibit congestion and edema of testes. Moreover, our results indicated disappearance of mononuclear infiltrates and reconstruction of layer of spermatogenesis in mice model used throughout this study. Consequently, it is beneficial to administer CAPE to counter the adverse effects of these toxic metals. Finally, CAPE confirmed many biological and pharmacological properties. Clinical trials and further studies are needed to evaluate the definite medical effects of CAPE based on abundant experimental studies, with predictive future applications in human clinical trials.

Declarations

Author contribution statement

El-Refaei MF: Conceived and designed the experiments; Performed the experiments; Analyzedand interpreted the data.

Eman A. A. Abdallah: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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