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Attenuation of cyclosporine A-induced testicular and spermatozoal damages associated with oxidative stress by ellagic acid

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ABSTRACT

This study was conducted to investigate the possible protective effect of ellagic acid (EA) on cyclosporine A (CsA)-induced testicular and spermatozoal damages associated with oxidative stress in male rats. Forty adult male Sprague–Dawley rats were divided into 4 groups of 10 animals each. Control group was used as placebo. Cyclosporine group received CsA at the dose of 15 mg/kg/day. Ellagic acid group was treated with EA (10 mg/kg/day). Cyclosporine plus ellagic acid group received CsA + EA. Reproductive organs were weighed and epididymal sperm characteristics and histopathological structure of testes were examined along with malondialdehyde (MDA) and glutathione (GSH) levels, glutathione-peroxidase (GSH-Px) and catalase (CAT) activities in testicular tissue. CsA significantly decreased the weights of testes and ventral prostate, epididymal sperm concentration, motility, testicular tissue glutathione (GSH), glutathione-peroxidase (GSH-Px) and catalase (CAT), diameters of seminiferous tubules and germinal cell layer thickness, and it significantly increased malondialdehyde (MDA) level and abnormal sperm rates along with degeneration, necrosis, immature germ cells, congestion and atrophy in testicular tissue. However, the CsA plus EA treatment attenuated all the CsA-induced negative changes observed in the testicular tissue, sperm and oxidant/antioxidant parameters. In conclusion, CsA-induced oxidative stress leads to the structural and functional damages in the testicular tissue and sperm quality of rats, and also EA has a protective effect on these damages.

1. Introduction

Mythic literatures richly describe the transplantation as a cure for diseases although the clinical practice of transplantation relatively spans a few decades. Today, organ and bone marrow transplants are successfully performed. But, the rejection reactions occur when a foreign organ is attacked by the body's immune system. Many immunosuppressive drugs are used to prevent the rejection reactions. Although the modern era of pharmacologic immunosuppression has initiated with the introduction of the antiproliferative drug, 6-mercaptopurine, the major advance in immunosuppression in the past three decades has been the development of pharmacologic agents, the prototype of which is cyclosporine A [1].

CsA, a neutral lipophilic cyclic undecapeptide ($C_6H_{11}N_{11}O_{12}$) was isolated from the fungus *Tolypocladium inflatum gams.* Although it was firstly identified in 1976 as a novel antibiotic agent, it was subsequently discovered to be a powerful immunosuppressive agent [2]. Nowadays, CsA is successfully used in transplant medicine and in the therapy of autoimmune diseases such as uveitis [3], rheumatic arthritis [4] and psoriasis [5]. The potent immunosuppressant effect of CsA is attributed to its specific inhibiting feature on the lymphokine generation, differentiation and signal transduction pathways of T cell receptor [6,7]. However, it has been reported that CsA causes renal [8,9], hepatic and cardiac damages [9] and gingival hypertrophy, tremor, increased blood pressure [6] as well as testicular [10–12] and spermatozoal toxicity [12–16] in experimental animals.

Spermatozoa are germ cells that have a pivotal role at fertilisation. Infertility is a problem with a large magnitude. Sperm damages are one of the factors that cause infertility. Reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\cdot}$) or molecules and/or hydroxyl radical ($^{\cdot}OH$) affect both male and female gametes [17]. ROS produced by spermatozoa play an important role in normal physiologic processes such as sperm capacitation, acrosome reaction, oocyte fusion, and stabilization of the mitochondrial capsule in the mid-piece [18–20]. However, uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress, which is harmful to spermatozoa through a variety of mechanisms [20–22]. It has been documented that the biochemical mechanism of CsA-induced toxicity in many organs including kidney [8,9], liver, heart [9] and ovary [23] is attributed to the oxidative

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stress. CsA-induced direct damages in hypo-gonadic axis [13–15] and Sertoli cell phagocytic function [11] have been considered for testicular and spermatozoal toxicity up to our earlier study was published in 2007 [12]. In our study we additionally demonstrated that CsA causes both testicular and spermatozoal toxicity by affecting the oxidant/antioxidant balance of testis [12].

It has been reported that exogenous antioxidants such as lycopene [8], N-acetylcysteine [24], black grape extract [23] and taurine [25] could have a therapeutic role against CsA-induced different organ toxicity in non-transplanted experimental animals. Recently, there is growing interest in understanding the role and mechanism of the phytochemicals: polyphenolics, flavonoids and phenyl propanoids as inhibitors of oxidative stress [26]. Among all phytochemicals, ellagic acid (EA; 2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) has been receiving the most attention because of its wide array of biological properties, such as radical scavenging, chemopreventive [27–29], antiatherogenic, antiapoptotic [30] and estrogen receptor modulator [31] properties. Raspberries, strawberries, walnuts, longan seed, mango kernel [32,33] and pomegranate [34] are rich plants with respect to EA. EA contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage [35]. Our recent report [29] has demonstrated that exogenous EA administration to rats protects testicular tissue and spermatozoa from cisplatin, a chemotherapeutic agent-induced toxicity by decreasing lipid peroxidation and increasing enzymatic antioxidant activities. In the light of above information, the present study was designed to investigate whether EA has possible protective effect against CsA-induced negative changes in epididymal sperm characteristics and testicular tissue associated with oxidative stress in rats.

2. Materials and methods

2.1. Chemicals

CsA (Sandimmun® enj. sol., 50 mg/ml) was purchased from Novartis (Istanbul, Turkey). EA was supplied from Fluka (Steinheim, Germany) and the other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Animals, experimental design and sample collection

Forty healthy adult male Sprague–Dawley rats (8 weeks old, 200–240 g body weight) were used in this study. The animals were obtained from Fırat University, Experimental Research Centre (Elazığ, Turkey) and were housed under standard laboratory conditions (temperature 24±3 °C, humidity 40–60%, a 12-h light:12-h dark cycle). A commercial pellet diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water were given *ad libitum*. The protocol for the animal use was approved by the Institutional Review Board of the National Institute of Health and Local Committee on Animal Research.

CsA was subcutaneously injected to the animals at the dose of 15 mg/kg. EA is hardly dissolved under natural condition. Therefore, it was dissolved in alkaline solution (0.01 N NaOH; approximately pH 12). pH of the final solution after the addition of EA was approximately 8. This final solution (pH≈8) was administered to the animals by gavage at the dose of 10 mg/kg. All treatments were maintained daily for 21 days. The dose and administration period of CsA and EA were selected, according to previous studies [9,12,29]. The animals were randomly divided into four experimental groups of 10 rats in each. These groups were arranged as follows: the control group was used as placebo and, was given subcutaneous injection of 0.5 ml isotonic saline + 0.5 ml slightly alkaline solution. The cyclosporine group received subcutaneous injection of CsA + 0.5 ml slightly alkaline solution. The ellagic acid group was administered subcutaneous injection of 0.5 ml isotonic saline + EA. The cyclosporine plus ellagic acid group received subcutaneous injection of CsA + EA.

The rats were killed under slight ether anaesthesia at the end of a 21 day treatment period. Testes, epididymides, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. One of the testes was fixed in 10% formalin solution for histopathological examinations. The other testes samples were also stored at −20 °C until biochemical analyses. For the enzymatic analyses, testicular tissues were minced in a glass and homogenized by a teflon–glass blender in cold physiological saline on ice. Then, the tissues were diluted with a 9-fold volume of phosphate buffer (pH 7.4).

2.3. Evaluation of sperm parameters

The epididymal sperm concentration was determined with a hemocytometer using a modified method described by Türk et al. [12,29]. The right epididymis was finely minced by anatomical scissors within 1 ml of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2 min, and then allowed to incubate at room temperature for 4 h to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue-fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymal spermatozoa was drawn into the capillary tube up to 0.5 lines of the pipette designed for counting red blood cells. The solution containing 0.595 M sodium bicarbonate, 1% formalin and 0.025% eosin was pulled into the bulb up to 101 lines of the pipette. This gave a dilution rate of 1:200 in this solution. Approximately 10 µl of the diluted sperm suspension was transferred to both counting chamber of Improved Neubauer (Deep 1/10 mm, LABART, Darmstadt, Germany) and allowed to stand for 5 min. The sperm cells in both chambers were counted with the help of light microscope at 200× magnification.

The percentage of sperm motility was evaluated using a light microscope with heated stage as described by Sönmez et al. [36]. For this process, a slide was placed on a light microscope with a heated stage warmed up to 37 °C, and then several droplets of Tris buffer solution [0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid] were dropped on the slide and a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris buffer solution and mixed by a cover-slip. The percentage of sperm motility was evaluated visually at 400× magnification. Motility estimates were performed from 3 different fields in each sample. The mean of the 3 successive estimations was used as the final motility score. To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide (3000 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage [12,29].

2.4. Biochemical analyses

The testicular tissue lipid peroxidation levels were measured according to the concentration of thiobarbituric acid reactive substances (TBARs) [37]. The amount of produced malondialdehyde (MDA) was used as an index of lipid peroxidation. Briefly, one volume of the test sample and two volume of stock reagent (15%, w/v trichloroacetic acid in 0.25 N HCl and 0.375%, w/v thiobarbituric acid in 0.25 N HCl) were mixed in a centrifuge tube. The solution was heated in boiling water for 15 min. After cooling, the precipitate was removed by centrifugation at 1500 *g* for 10 min, and then absorbance of the supernatant was read at 532 nm against a blank containing all

reagents except test sample on a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). The MDA level was expressed as nmol/ml.

The reduced glutathione (GSH) level of testicular tissue was measured at 412 nm using the method of Sedlak and Lindsay [38]. The samples were precipitated with 50% trichloroacetic acid, and then centrifuged at 1000 *g* for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris–EDTA buffer (0.2 mol/l; pH 8.9) and 0.1 ml of 0.01 mol/l 5,5′-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer. The level of GSH was expressed as nmol/ml.

The glutathione-peroxidase (GSH-Px) activity in testicular tissue was determined according to the method of Lawrence and Burk [39]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 IU/ml oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM H_2O_2. Enzyme source (0.1 ml) was added to 0.8 ml of the above mixture and incubated at 25 °C for 5 min before initiation of the reaction with the addition of 0.1 ml of peroxide solution. The absorbance at 340 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (the enzyme was replaced with distilled water) was subtracted from each value. The protein concentration was also measured by the method of Lowry et al. [40]. The GSH-Px activity was expressed as IU/g protein.

The testicular tissue catalase (CAT) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm, according to the method of Aebi [41], and was expressed as kU/g protein, where k is the first-order rate constant.

2.5. Histopathological examination

The testicular tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm and were stained with haematoxylin and eosin [42]. Light microscopy was used to measure diameters of seminiferous tubules (DST) and of germinal cell layer thicknesses (GCLT) and to evaluate the damages in testicular tissue. The degree of damages was graded as follows: mild (+), moderate (++) and severe (+++).

2.6. Statistical analysis

All values were presented as mean ± S.E.M. Differences were considered to be significant at $P<0.05$. One-way analysis of variance (ANOVA) and *post hoc* Tukey-HSD test were used to determine differences between groups. The SPSS/PC program (Version 10.0; SPSS, Chicago, IL) was used for the statistical analysis.

3. Results

3.1. Effects of CsA and EA treatments on reproductive organ weights and epididymal sperm characteristics

Table 1 shows the changes in the reproductive organ weights and epididymal sperm characteristics in response to various treatments for a 21 day treatment period. No statistically significant differences were observed between control and ellagic acid groups in terms of reproductive organ weights. However, alone CsA administration caused a statistically significant reduction ($P<0.01$) in weights of testes and ventral prostate and insignificant reduction ($P>0.05$) in epididymal and seminal vesicle weights compared to the control group. A marked ($P<0.01$) increase in cyclosporine + ellagic acid group was observed in testis weight compared to alone cyclosporine group. Increments observed in epididymal, seminal vesicle and ventral prostate weights in cylosporine + ellagic acid group were statistically insignificant in comparison to the alone cyclosporine group.

Table 1
Mean ± SEM values of reproductive organ weights and sperm characteristics belonging to each group.

Parameters		Groups			
		Control ($n=10$)	Ellagic acid ($n=10$)	Cyclosporine ($n=10$)	Cyclosporine + ellagic acid ($n=10$)
Testes (g)		1.388 ± 0.03	1.372 ± 0.02	1.004 ± 0.07[a]	1.256 ± 0.03[b]
Epididymides (g)		0.361 ± 0.027	0.407 ± 0.004	0.314 ± 0.017[c]	0.376 ± 0.010
Seminal vesicles (g)		0.868 ± 0.03	0.980 ± 0.04	0.506 ± 0.10[c]	0.852 ± 0.13
Ventral prostate (g)		0.442 ± 0.04	0.462 ± 0.02	0.250 ± 0.04[a]	0.336 ± 0.04
Epididymal sperm concentration (million/g tissue)		317.2 ± 17.0	350.4 ± 24.1	203.3 ± 25.2[d]	275.8 ± 41.8
Sperm motility (%)		71.99 ± 5.23	77.99 ± 2.71	49.05 ± 6.33[d]	71.33 ± 5.64[e]
Abnormal sperm rate (%)	Head	2.93 ± 1.02	1.93 ± 0.49	6.48 ± 0.68[d]	3.60 ± 0.53
	Tail	3.53 ± 0.20	2.80 ± 0.64	9.93 ± 1.57[a]	5.80 ± 0.36
	Total	6.46 ± 1.13	4.73 ± 0.52	16.41 ± 1.91[a]	9.40 ± 0.62[b]

[a] Different from control group ($P<0.01$).
[b] Different from cyclosporine group ($P<0.01$).
[c] Different from ellagic acid group ($P<0.01$).
[d] Different from control group ($P<0.05$).
[e] Different from cyclosporine group ($P<0.05$).

Only EA administration for 21 days did not markedly alter all the studied sperm characteristics when compared to the control group. Alone CsA treatment significantly decreased sperm concentration and sperm motility and, it increased the percentage of head ($P<0.05$), tail and total ($P<0.01$) abnormality of sperm in comparison to the control group. The administration of EA to CsA-treated rats significantly protected the CsA-induced negative changes in sperm motility ($P<0.05$) and total abnormality ($P<0.01$) versus alone cyclosporine group. Although the values of sperm concentration were numerically higher and, head and tail abnormality percentages were also numerically lower in cyclosporine + ellagic acid group than values in alone cyclosporine group, the differences were not statistically significant.

3.2. Effects of CsA and EA treatments on biochemical parameters

Markers of testicular tissue lipid peroxidation and antioxidant enzyme activities of all the groups are given in Table 2. Alone EA treatment remained ineffective on MDA and GSH levels, GSH-Px and CAT activities in comparison to the control group. While CsA administration resulted in a significant ($P<0.01$) increase in MDA level when compared to the control group, the CsA + EA treatment provided a marked reduction ($P<0.01$) in the increased MDA levels versus alone cyclosporine group.

Alone CsA treatment decreased the GSH levels, GSH-Px and CAT ($P<0.01$) activities when compared to the control group. However,

Table 2
Mean ± SEM values of testicular tissue malondialdehyde (MDA) and glutathione (GSH) levels and glutathione-peroxidase (GSH-Px) and catalase (CAT) activities belonging to each group.

Parameters	Groups			
	Control ($n=10$)	Ellagic acid ($n=10$)	Cyclosporine ($n=10$)	Cyclosporine + ellagic acid ($n=10$)
MDA (nmol/ml)	14.59 ± 0.24	14.48 ± 0.68	21.63 ± 0.34[a]	16.42 ± 0.72[b]
GSH (nmol/ml)	3.72 ± 0.20	4.16 ± 0.11	2.61 ± 0.08[a]	3.27 ± 0.24[b,c]
GSH-Px (IU/g protein)	47.70 ± 1.96	54.09 ± 2.44	29.48 ± 1.22[a]	43.30 ± 0.35[b,c]
CAT (kU/g protein)	60.24 ± 2.43	63.86 ± 3.02	44.05 ± 1.54[a]	54.59 ± 2.19[b]

[a] Different from control group ($P<0.01$).
[b] Different from cyclosporine group ($P<0.01$).
[c] Different from ellagic acid group ($P<0.01$).

Table 3
Diameters of seminiferous tubules (DST), germinal cell layer thicknesses (GCLT) and the degree of damages in testicular tissue [mild (+), moderate (++), severe (+++)].

Parameters	Groups			
	Control ($n=10$)	Ellagic acid ($n=10$)	Cyclosporine ($n=10$)	Cyclosporine + ellagic acid ($n=10$)
Immature spermatogonia and spermatocytes	ND	ND	1.14 ± 0.26^{a}	0.29 ± 0.18^{b}
Interstitial oedema	ND	ND	0.57 ± 0.20^{a}	ND[b]
Reduction in germinal cell layer thickness	ND	ND	1.29 ± 0.18^{a}	$0.43 \pm 0.20^{a,b}$
Atrophy in seminiferous tubules	ND	ND	1.14 ± 0.26^{a}	0.43 ± 0.20^{a}
Capillary congestion	ND	ND	0.57 ± 0.30	0.29 ± 0.20
Necrosis in seminiferous tubules	ND	ND	0.71 ± 0.29^{a}	0.29 ± 0.18
Degeneration in seminiferous tubules	ND	ND	1.57 ± 0.30^{a}	$1.00 \pm 0.00^{a,b}$
Spermatogenic arrest	ND	ND	1.29 ± 0.18^{a}	$0.71 \pm 0.18^{a,b}$
DST (μm)	223.68 ± 2.08	221.04 ± 2.31	205.44 ± 3.04^{a}	$213.20 \pm 2.73^{a,b}$
GCLT (μm)	55.12 ± 0.84	55.04 ± 0.59	48.56 ± 0.95^{a}	$52.56 \pm 0.64^{a,b}$

ND: not detected.
[a] Different from both control and ellagic acid groups ($P<0.01$).
[b] Different from cyclosporine group ($P<0.01$).

administration of EA to CsA-treated rats prevented the CsA-induced decreases in these endogenous antioxidants.

3.3. Effects of CsA and EA treatments on testicular histopathology

Measured DST and GCLT values and scored histopathological changes are presented in Table 3. While alone EA application did not significantly alter the DST and GCLT compared to the control group, only CsA administration caused a significant ($P<0.001$) decrease in these parameters. CsA plus EA treatment provided a marked ($P<0.001$) amelioration in these measurements. When the structure of testes was histopathologically examined; it was observed that histological appearances of testicular tissues of control (Fig. 1C) and ellagic acid (Fig. 1D) groups were normal. The histopathological changes were observed in alone cyclosporine and cyclosporine + ellagic acid groups. Atrophy in DST, necrosis in germinal cells, degeneration, interstitial oedema, capillary congestion and spermatogenic arrest were marked damages in testicular tissue in alone cyclosporine group. Spilled immature spermatogonia and spermatocytes were encountered in lumens of some seminiferous tubules of alone CsA-treated group (Fig. 1A). It was determined that there are marked decreases in immaturation, necrotic and degenerative changes in germinal cells of rats given CsA along with EA compared to the cyclosporine group (Fig. 1B).

4. Discussion

The use of CsA has improved quality of life and survival of transplant patients by largely contributing to the decrease in

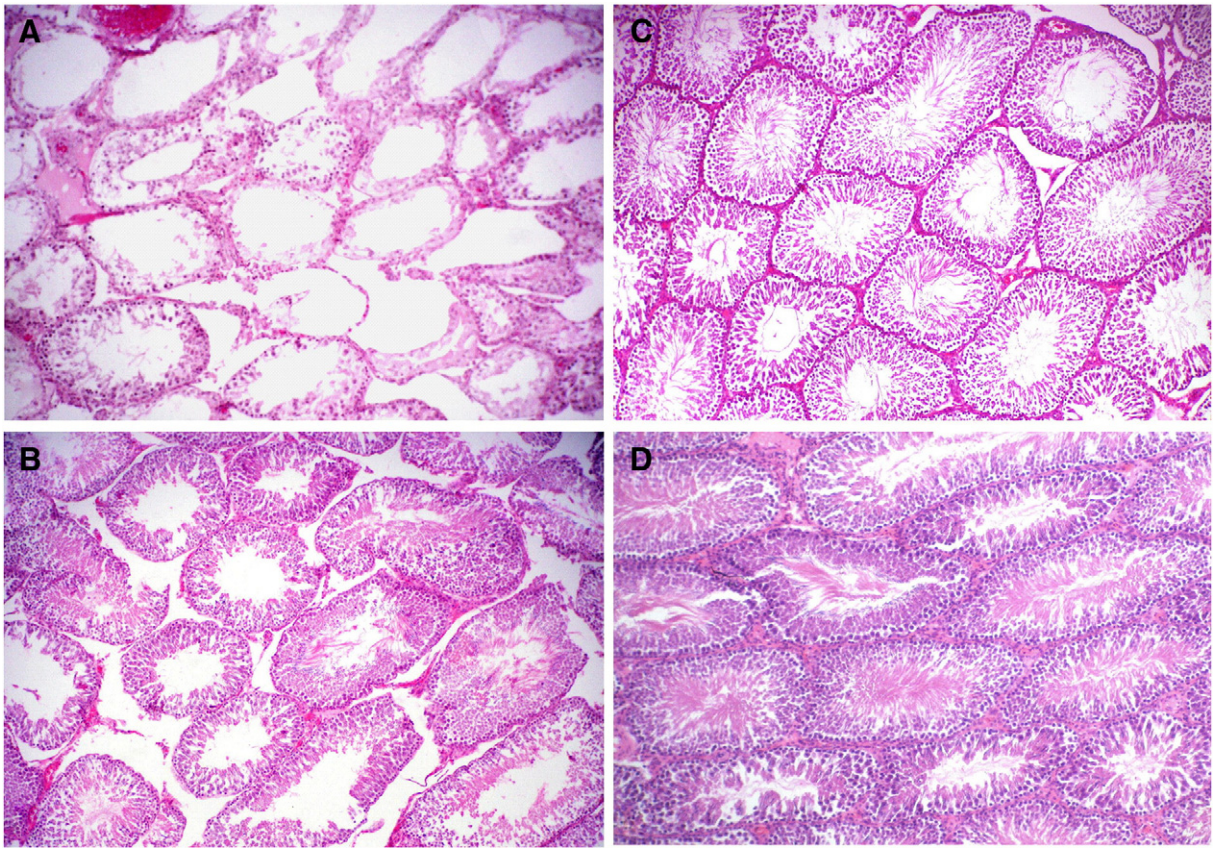

Fig. 1. A. Severe atrophy in DST, necrosis in germinal cells, degeneration, spermatogenic arrest, interstitial oedema and congestion in alone CsA-treated group. B. Mild disorganisation in germinal cells, degeneration and interstitial oedema in CsA + EA group. C. Normal histological view of seminiferous tubules in control group. D. Normal histological view of seminiferous tubules in EA group (H&E × 100).

morbidity, rejection reactions and hospitalization days [6]. However, clinical use of CsA is limited due to its unwanted side-effects in different organs such as kidney, heart, liver in transplanted patients [43–45]. It has been reported that the CsA administration causes a dose-dependent decline (20 mg/kg or higher) in the reproductive organ weights of non-transplanted male rats [13–15]. However the findings of studies of our earlier [12] and Monteiro et al. [46] have demonstrated that CsA has no significant effect on testicular weight. Alone CsA administration caused a significant decrease in testicular weight in the present study. The differences among present, our earlier [12] and Monteiro et al. [46] studies can be attributed to the sensitivity of rats used in different works. The reduction in testicular weight may be explained by CsA-induced direct or indirect (via ROS) atrophy in DST and decreased GCLT. CsA-induced increased ROS levels or its direct effects lead to decrease in testosterone production [47] and volumetric proportion of Leydig sells [46]. The reduction in prostatic weight observed in the study may be explained that the secretion of this organ likely decreased because testosterone production was diminished by CsA.

Spermatozoa are particularly susceptible to the damage induced by excessive ROS, because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes [18,19]. ROS can attack to the unsaturated bonds of the membrane lipids in an autocatalytic process, with the genesis of peroxides, alcohol and lipidic aldehydes as by-product of the reaction. Thus, the increase of free radicals in cells can induce the lipid peroxidation by oxidative breakdown of polyunsaturated fatty acids in membranes of cells. Obviously, peroxidation of sperm lipids destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases [12,29]. Xu et al. [48] have reported that different dosages of CsA affect sperm morphology of human after renal transplantation. Misro et al. [16] reported that in vitro addition of 1 mg/kg CsA reduces human sperm motility. In our earlier study [12], we showed that 15 mg/kg CsA caused reduced epididymal sperm concentration, sperm motility and increased morphologically abnormal sperm in non-transplanted healthy rats. Daily administration of CsA significantly reduced epididymal sperm concentration, sperm motility and increased abnormal sperm rate compared to the control group in this study. Our findings are in agreement with above reports. The negative changes observed in sperm quality in the present study may be attributed to the peroxidation of polyunsaturated fatty acids in plasma membranes of spermatozoa, damaged flagellum which important machinery for the sperm motility, directly impairing of spermatogenic cell development, impaired maturation or spermiation, and altered membrane porosity caused by CsA administration.

CsA leads to seminiferous epithelium degeneration, resulting in Sertoli cell vacuolization, abnormal round and elongated spermatids, large accumulation of residual cytoplasm at the epithelium border next to the lumen of tubules, reduced haploid cell population, pachytene spermatocytes, decreases in DST and GCLT, necrosis, interstitial oedema, desquamative germinal cells and the deceleration of spermatogenesis [10,12,46]. In this study, similar lesions and findings were observed in alone CsA-treated rats. The damages observed in the histological structure of testis in this work may be elucidated with the direct or indirect effect of CsA which latter induces lipid peroxidation that is a chemical mechanism capable of disrupting the structure and function of testis.

The use of oxygen during normal metabolism produces ROS, some of which are highly toxic and deleterious to cells and tissues. The most abundant ROS formed in the course of cellular metabolism is $O_2^{-\cdot}$ [49]. Dismutation of the $O_2^{-\cdot}$ or directly from the action of oxidase enzymes gives rise to H_2O_2. This molecule is not a free radical itself but, in the presence of transition metals (Cu^{+2}, Fe^{+2}) via the Fenton reaction, it is rapidly converted to the $^{\cdot}OH$. The $^{\cdot}OH$ is widely accepted as being the most damaging ROS produced by cells [50]. CsA-induced-free radicals have a great potential to react rapidly with lipids which in turn leads to lipid peroxidation. Alone CsA treatment caused significant increase in MDA level of testicular tissue in the present study.

When ROS begin to accumulate, testes exhibit a defensive mechanism using various antioxidant enzymes. The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of $O_2^{-\cdot}$ when they are converted to H_2O_2 and diatomic oxygen. The enzyme at this step is superoxide dismutase (SOD). Two enzymes participate in the removal of H_2O_2 from the cellular environment, peroxidases and CAT. The most abundant peroxidase is the GSH-Px. This enzyme uses reduced GSH as a substrate to transfer electrons to H_2O_2 (and other peroxides) thereby converting it into two molecules of water [51]. In the present study, it was found that only CsA administration decreased the GSH levels, GSH-Px and CAT activities when compared to the control group. Decreases in enzymatic and non-enzymatic antioxidants observed in this work may be attributed to excessive utilisation of these antioxidants in order to scavenge the free radicals lead to lipid peroxidation.

EA inhibits generation of $O_2^{-\cdot}$ and $^{\cdot}OH$ in both enzymatic and non-enzymatic systems by its metal-chelating property, thus providing protection against lipid peroxidation [9]. In our earlier studies we found that EA protected cisplatin-induced testicular and spermatozoal toxicity by decreasing lipid peroxidation in testes [29]. Additionally, it has been reported that EA decreases MDA level and increases GSH level [35], GSH-Px and CAT activities [9] in various organs of rats treated with CsA. In the present study, administration of EA provided total or partial improvements in sperm parameters, testicular histology, lipid peroxidation and antioxidant enzyme activities in CsA-treated rats. This status may be explained with partial attenuation of CsA-induced degeneration, reduction in GCLT, and possibly enhancement of sperm concentration in the epididymis, and increment in fluids of accessory glands due to the decreased lipid peroxidation and increased antioxidant enzyme activities caused by EA administration. The declining of lipid peroxidation in testicular tissue apparently indicates that EA potently scavenged the free radicals, and suppressed oxidative DNA damage.

In conclusion, this study apparently suggests that EA has a protective effect against testicular and spermatozoal toxicity induced by CsA. This protective effect of EA seems to be closely involved with the suppressing of oxidative stress. Therefore, EA may be used combined with CsA after transplantation and in autoimmune diseases to improve CsA-induced injuries in sperm quality and oxidative stress parameters.

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