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Pretreatment with montelukast and curcumin protect against etoposide-induced testicular toxicity in rats: role of oxidation, inflammation and apoptosis

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Abstract

The success of etoposide for the treatment of testicular cancer is limited by its undesirable side effects on reproductive system, which is generally ascribed to inflammation and oxidative stress. In the current study, the protective effects of montelukast and curcumin on etoposide-induced reproductive toxicity were investigated. Rats were divided into six groups; group 1 was kept as control. In groups 2, 3; montelukast and curcumin were administered at doses of (10 mg/kg/day) and (200 mg/Kg/day) respectively for 10 days. In group 4, etoposide was intraperitoneally administered at a single dose of 10 mg/kg, while in groups 5 and 6; etoposide and montelukast or curcumin, respectively, were given together at the same doses. Etoposide induced oxidative stress via significant increase in MDA level and significant decrease in GSH level as well as SOD and CATA activities. Montelukast and curcumin prevented these effects through antioxidant properties. In addition, the deleterious effects of etoposide on spermatogenesis, serum testosterone level, oxidative stress, ATP, mtDNA and nDNA damage as well as histopathological changes are eliminated by montelukast or curcumin treatment, notably curcumin could normalized some of these parameters. The present study showed that montelukast and curcumin can reverse toxic effects of etoposide on the reproductive system that can be contributed due to anti-oxidant, anti-inflammatory and antiapoptotic potential.

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1. Introduction

Etoposide is a semisynthetic epipodophyllotoxin agent usually used for treatment of testicular cancer (Codelia et al., 2010). The success of etoposide for the treatment of testicular cancer is limited by its undesirable side effects on reproductive system, which are generally ascribed to inflammation and oxidative stress (Ortiz et al., 2009; Kanchana Ganga et al., 2013).

It was shown that etoposide treatment induces germ cell apoptotic death and depletes seminiferous epithelium resulting in adult infertility (Stumpp et al., 2004; Kim et al., 2006). In addition, it has been demonstrated that etoposide treatment leads to a significant decrease in reproductive organ weights (Francisca et al., 2002), sperm quality (Haraguchi, 1992) and inhibition of testosterone secretion from Leydig cells as well as testicular tissue damage (Marcon et al., 2008). However, clinical and experimental data related to the adverse effects of the etoposide treatment on testicular morphology are scanty and fragmentary (Francisca et al., 2002). Hence, there has been a continuous search for biological and pharmacological strategies to control etoposide-induced testicular toxicity and thus permit the administration of high quantities of the drug.

Montelukast is a selective reversible leukotriene (LT) D4 receptor antagonist (Muthuraman and Sood, 2010). Leukotrienes increase in many inflammatory conditions such as peptic ulcer and ischemia/reperfusion (Dengiz et al., 2007; Ozkan et al., 2010). On the other hand, because of its anti-inflammatory property, montelukast provides a significant beneficial effect for preventing oxidative damage in some tissues such as testes, kidney and liver (Ozturk et al., 2010; Kose et al., 2012).

Curcumin exhibits antioxidant property. It can significantly inhibit the generation of reactive oxygen species (ROS) both in vitro and in vivo and act as a scavenger of free radicals (Joe B and Lokesh, 1994; Joe et al., 2004). Curcumin is considered to be a potent cancer chemopreventive agent and a powerful inhibitor of the proliferation of several tumor cells and it exhibits anti-carcinogenic, antiviral and anti-infective properties (Tapia et al., 2012).

Experimental studies showed that administration of antioxidant compound such as melatonin and curcumin may protect reproductive system against chemotherapeutic cytotoxicity to normal tissues which is a critical factor that undermines the curative potential of chemotherapy (Ilbey et al., 2009, Papież, 2013).

Until less toxic agents come into clinical practice, we must find new strategies to minimize the toxic effects of existing drugs. The current study was conducted to gain insights into the possibility of mechanism-based protection by montelukast and curcumin, against the reproductive toxic effects of etoposide.

2. Materials and methods

2.1. Chemicals and drugs

Etoposide (vial, 1 mg/ml) was purchased from (MERCK, France). Montelukast and curcumin were purchased from Sigma- Chemicals Comp., St Louis, MO, USA.

2.2. Animals and experimental design

Seventy two male Albino rats, weighing 180-200 g, were purchased from The Egyptian Organization for Biological Products and Vaccines. Rats were kept in

a well-ventilated room in normal light-dark cycle and temperature (25 ± 2°C). Food and water were provided *ad* libitum. Animals were treated in accordance with the Guidelines for Animal Experimentation (of the Ethics Review Committee) of Faculty of Pharmacy, Suez Canal University. After a 1-week, rats were weighted and randomly assigned into six groups; 12 animals each.

Etoposide was injected once intraperitoneally (i.p.); (10 mg/kg). Montelukast (10 mg/Kg/day) (Muthuraman and Sood, 2010; Ozkan et al., 2010) and curcumin (200 mg/kg/day) (Tirkey et al., 2005) were suspended in distilled water and given orally for 10 consecutive days. Group1; control received distilled water (2ml/Kg); during that a single i.p. injection of 2ml/Kg distilled water was given at day 6. Groups 2 and 3; normal rats received either montelukast or curcumin. Group 4; rats were given distilled water for 10 consecutive days, during that a single i.p. injection of etoposide was given at day 6. Groups 5 and 6; rats received either montelukast or curcumin, during that a single dose i.p. injection of etoposide was given at day 6.

At the end of the experiment, body weights were re-assessed and the percentage change of body weight (BW) was calculated using the formula (final BW-initial BW/final BW) X 100. Survival rate was observed daily.

2.3. Sample collection

Rats were then anaesthetized and sacrificed by decapitation (Vogler, 2006). Five ml blood samples were collected, via cardiac puncture, for serum separation, and stored at -80°C. Abdominal midline incision and orchiectomy was performed. After testes weighing, the right testis was stored at -80°C for biochemical evaluation and the left testis was fixed in Bouin's solution for histopathologic examination. Testicular index was calculated using the formula (testicular weight/final BW) X 100.

2.4. Assessment of spermatogenesis

2.4.1. Serum testosterone level

Serum testosterone was determined by enzyme-linked immunosorbent assay (ELISA) kits (Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.4.2. Semen analysis

2.4.2.1. Sample collection

Orchidectomy was performed. In brief; a midline incision was made and the testicles were milked out of the incision site. The testicles were exposed by incising the tunica vaginalis. The spermatic cord was exposed, ligated and incised. Three ml semen samples were thereafter collected and analyzed immediately (Oyeyemi and Ubiogoro, 2005).

2.4.2.2. Sperm count and motility

Neubauer chamber was used for sperm count with the help of a light microscope at 200×magnifications. In the counting chamber, five small squares for each specimen were used, the resulting number was multiplied by million to obtain the final count. One ml of diluted spermatozoa was also examined to calculate the number and percentage of motile sperms in 3 fields and the mean value was calculated (Pant and Srivastava, 2003).

2.4.2.3. Percentage viability and morphological abnormalities

They were determined from a total count of 300

spermatozoa in each slide. Percent viability (Live/dead ratio) was determined using 1% Eosin and 5% Nigrosin, dead sperms appeared red in color (Wells and Awa, 1970). The number and percentage of abnormal forms from three different fields on each sample were evaluated using a light microscope at 400× magnification (Ciftci et al., 2012).

2.5. Oxidative stress and antioxidant markers

A part of the testis (0.1 g) was ice-cooled, homogenized in 1 ml phosphate buffer (pH 7.4) and centrifuged at 3000×*g* for 15 min. The supernatant was collected and kept at -80°C until the analysis of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CATA) (Ellman, 1970; Aebi, 1974; Marklund, 1992; Preuss et al., 1998) using a UV-visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.6. Inflammatory markers

Serum C-reactive protein (CRP) was measured by ELISA kits DIA MED (Belgium) (Ben Assayag et al., 2009). Further, one hundred mg testicular tissue was homogenized in 0.1 M phosphate buffer (pH 7.2), the homogenate was centrifuged at 2000×*g* for 10 min and the supernatant was used for determination of TNF-α using ELISA kits (Biosource, USA) (Mizutani et al., 2003).

2.7. Adenosine triphosphate (ATP)

ATP was extracted from the tissues with 10 ml of 0.6 M perchloric acid. The extraction mixture was centrifuged for 10 min at 6000×*g*, and 6 ml of the supernatant was taken and quickly neutralized to pH 6.5 with 1 M KOH solution. The supernatant was allowed to stand for 30 min in ice bath to precipitate most of the potassium perchlorate, which was removed by paper filtration. The filtrate solution was stored at -30°C until analysis as described previously (Liu et al., 2006).

2.8. Assessment of mitochondrial DNA integrity

2.8.1. Isolation of mitochondria

One hundred mg of testicular tissue was homogenized in 0.25 M sucrose in 0.7 M Tris-HCl Buffer (pH 7.4) and EDTA. Tissue homogenate was spun at 2500×*g* for 10 min. Supernatant fluid was decanted into centrifuge tubes and span at 10.000×*g* to form primary mitochondrial pellet that is gently re-suspended in 10 ml Tris–sucrose for washing. The pellet was re-centrifuged for several cycles and supernatant fluid was decanted. The final mitochondrial pellet was re-suspended (1ml Tris–sucrose/1 g of original sample) (Gray and Yardley, 1975).

2.8.2. Isolation of mitochondrial DNA (mtDNA)

mtDNA was isolated by using isolation kit (Bio Vision, USA) (Chang et al., 2002). The samples were subjected to 1% agarose gel electrophoresis at 4 V/cm using TAE solution as a running buffer. Furthermore, mtDNA quantity and purity were determined using Nano-Drop™ 1000-Spectrophotometer V3.7 (Thermo-Fisher Scientific-Inc, Wilmington, USA).

2.9. DNA laddering assay

Genomic DNA was extracted from the testes using Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, USA). DNA was then loaded onto agarose gel (15 µg/lane). DNA laddering was determined by constant voltage mode electrophoresis on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. A 1 kb ladder (Sigma, St. Louis, USA) served as DNA base pair marker (Sambrook et al., 2001).

2.10. Histopathologic examination

Thin paraffin wax testicular tissue sections (4 mm thick) fixed in Bouin's solutions were stained with hematoxylin and eosin (H&E) and examined under a light microscope X400 magnification (Olympus, BX 51, Tokyo, Japan). The number of germ cells; namely spermatogonia, pre-leptotene and pachytene spermatocytes as well as elongated spermatids was counted in at least 20 seminiferous tubules selected randomly from at least 3 cross sections/ rat. Mean tubular diameter (μm) (15-20 tubules/ rat) and Sertoli cells number were performed at X400 using Biovis-Image Plus Software for image analysis (Abercrombie, 1946). This analysis was performed with the observer masked to the treatment groups.

2.11. Statistical analysis

Data were expressed as mean ± SEM and analyzed using the Statistical Package of Social Sciences (SPSS program, version 20, SPSS Inc., Chicago, IL, USA). The difference of mean values among groups was assessed by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. All *P* values reported are two-tailed and $P < 0.05$ was considered significant. All possible comparisons were made among groups; amounting to six multiple comparisons for each variable.

3. Results

3.1. Percentage change in BW, testicular index and survival rate

Etoposide resulted in a significant decrease in percentage change of BW (-6.91 ± 1.66%) (**Table 1**). Administration of montelukast (10 mg/Kg) or curcumin (200 mg/kg) resulted in a significant increase in percentage change of BW versus etoposide.

Table 1: Effect of montelukast and curcumin on percentage of change of body weight, testicular index and survival rate in control and etoposide-treated rats

Groups	Percentage change of body weight (%)	Testicular index	Survival rate (%)
Control	14.60 ± 1.58	0.68 ± 0.05	100
Etoposide	-6.91 ± 1.66[a]	0.39 ± 0.01[a]	66.66[a]
Etoposide + Montelukast	5.7 1± 0.96 [a,b]	0.51 ± 0.02 [a,b]	75[b]
Etoposide + Curcumin	9.12 ± 1.07 [a,b,c]	0.48 ± 0.01[a,b]	83.33[a,b]

Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test. $n = 8–12$.
[a]$P < 0.05$ versus control group.
[b]$P < 0.05$ versus etoposide group.
[c]$P < 0.05$ versus montelukast group.

Testicular index in etoposide-treated rats was significantly decreased (**Table 1**). Insignificant change in body and testis weights was recorded in normal animals given montelukast or curcumin in comparison with controls (Data was not shown). Testicular index in montelukast and curcumin-treated groups, were preserved versus etoposide group (**Table 1**).

Control groups showed 100 % survival rate, whereas, etoposide group showed a significant lower survival rate (66.66%). Oral administration of montelukast and curcumin significantly increased the survival rate to (75 and 83.33% respectively) (**Table 1**). Notably, curcumin induced a significant increase in body weight compared to montelukast.

3.2. Testosterone level and semen analysis

Insignificant change in serum testosterone as well as semen analysis was recorded in normal animals given

Table 2: Effect of montelukast and curcumin on serum testosterone and semen analysis [sperm count, motility, viability and abnormal forms] in control and etoposide-treated rats

Groups	Serum testosterone (ng/ml)	Semen analysis			
		Sperm count (X 10^6/ml)	Motility (%)	Viability (%)	Abnormal forms (%)
Control	3.60 ± 0.38	2.60 ± 0.25	89.78 ± 5.66	86.21 ± 6.50	16.11 ± 1.33
Etoposide	0.96 ± 0.07[a]	1.21 ± 0.14 [a]	56.66 ± 4.80[a]	61.54 ± 4.71[a]	42.15 ± 3.25[a]
Etoposide + Montelukast	2.11 ± 0.11 [a,b]	2.11 ± 0.13[a,b]	73.33 ± 6.12[a,b]	71.22 ± 5.32[a,b]	35.50 ± 3.72[a]
Etoposide + Curcumin	2.32 ± 1.07 [a,b]	1.98 ± 0.01[a,b]	72.44 ± 7.11[a,b]	73.68 ± 6.61[a,b]	25.33 ± 2.24[b,c]

Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test. n = 8–12.
[a]$P < 0.05$ versus control group.
[b]$P < 0.05$ versus etoposide group.
[c]$P < 0.05$ versus montelukast group.

montelukast or curcumin in comparison with controls (Data was not shown). Serum testosterone was significantly deceased after etoposide treatment compared to control group (**Table 2**). Oral administration of either montelukast or curcumin elevated this level significantly compared to etoposide group. On the other hand, the results of the current study indicated significant impairment of spermatogenesis upon treatment with etoposide as manifested by significant decrease in sperm count, percent sperm motility, viability and abnormal forms as compared to control group (**Table 2**). While there was insignificant change in abnormal sperm forms upon administration of montelukast, it could significantly increase sperm count, motility and viability compared to etoposide. On the other hand, sperm count, motility and viability were significantly increased after treatment with curcumin compared to etoposide group. Notably curcumin could normalize abnormal sperm forms.

3.3. Biochemical analysis

3.3.1. Lipid Peroxidation and antioxidant effects

The results of the current study indicated that treatment of normal animals with motelukast or curcumin significantly decreased spontaneous lipid

Table 3: Effect of montelukast and curcumin on testicular MDA and antioxidant markers in control and etoposide-treated rats

Groups	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD activity (U/ mg protein)	CATA activity (U/ mg protein)
Control	26.00 ± 2.21	24.00 ± 1.32	86.00 ± 5.81	2.36 ± 0.17
Etoposide	177.00 ± 6.16[a]	4.80 ± 0.42[a]	44.00 ± 3.40[a]	0.47 ± 0.06 [a]
Etoposide + Montelukast	50.00 ± 2.60 [a,b]	14.60 ± 1.27[a,b]	70.00 ± 2.11[a,b]	0.92 ± 0.15[a]
Etoposide + Curcumin	53.00 ± 5.16 [a,b]	22.30 ± 1.17[b,c]	76.00 ± 3.06[b]	2.06 ± 0.15[b,c]

Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. MDA: Malondialdhyde, GSH: reduced glutathione, SOD: superoxide dismutase, CATA: Catalase. Rats were treated for ten days.
[a]$P < 0.05$ compared to control group.
[b]$P < 0.05$ compared to etoposide group.
[c]$P < 0.05$ compared to montelukast group. n = 8-12.

peroxidation levels in the testicular tissues (data not shown). The level of MDA was significantly increased (about 6.7-fold) in rat treated with etoposide. However, this elevation was significantly suppressed (by about 3-fold) when montelukast and curcumin were used with etoposide (**Table 3**).

The current study showed also that treatment of normal rats with curcumin markedly enhanced GSH as well as SOD and CATA activities (data not shown). In contrast, testicular tissue of rats showed significant (**Table 3**) reduction of GSH content as well as SOD and CATA activity after etoposide treatment as compared to control group. Oral administration of montelukast and curcumin increased GSH content and SOD activity significantly compared to etoposide group. While montelukast could not increase CATA activity significantly, curcumin significantly increased CATA activity compared to etoposide group (**Table 3**). Interestingly, curcumin could normalize antioxidant parameters. This suggests that etoposide reduces the antioxidant activity in the testes but on administration of a substance with antioxidant activity, animals try to cope with the condition of

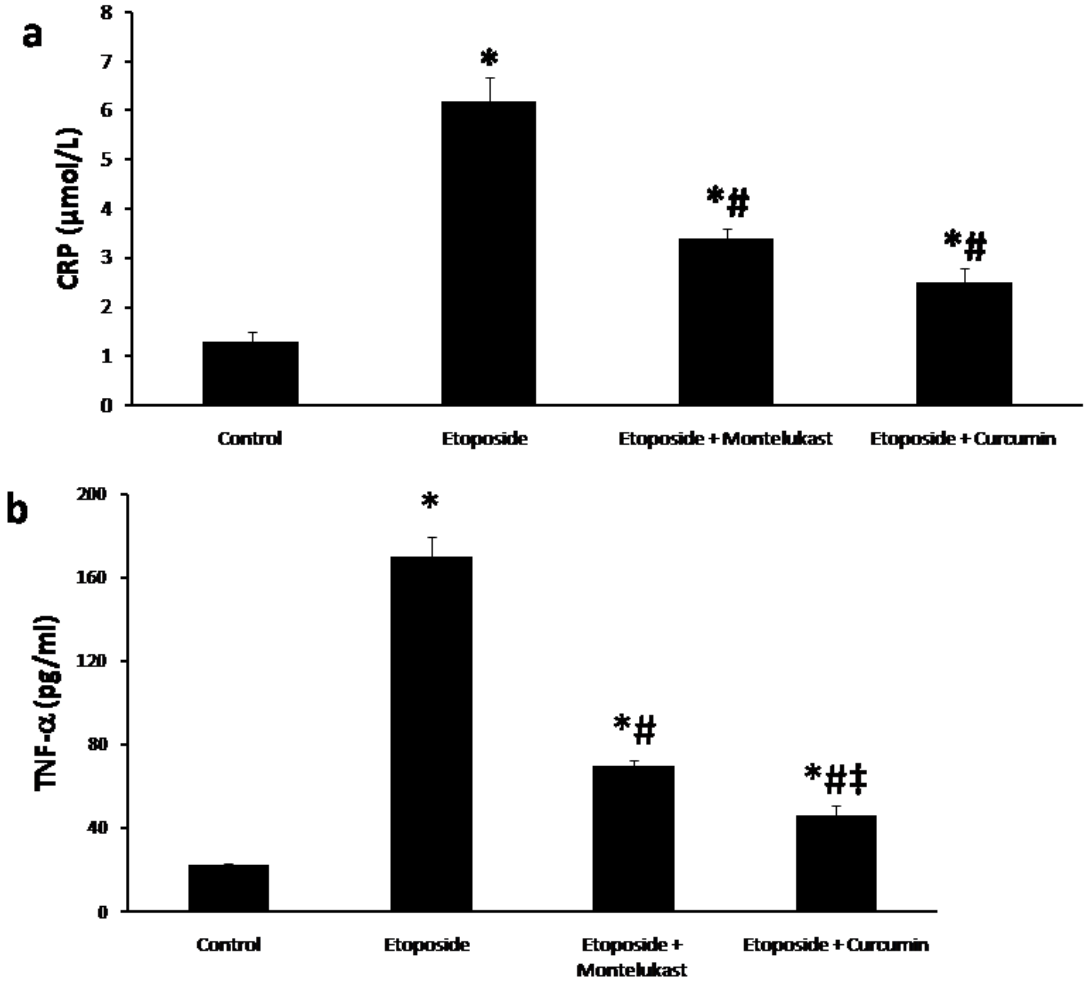

Figure 1: (a) Serum CRP and (b) testicular TNF-α in experimental groups. Either montelukast or curcumin ameliorated the increased levels of CRP and TNF-α in etoposide-treated rats at the end of 10 days' treatment. CRP: C-reactive protein, TNF-α: tumor necrosis factor-α. Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to etoposide group, $^{\ddagger}P < 0.05$ compared to montelukast group. n = 8-12.

oxidative stress and protect the testes from the toxic action of etoposide.

3.3.2. Anti-inflammatory effects

The current data revealed that oral administration of either montelukast or curcumin to normal rats induced insignificant change in serum CRP or testicular TNF-α as compared to control group (data was not shown), while serum level of CRP and testicular level of TNF-α were significantly increased by 7.5 and 4.7 folds respectively in etoposide-treated rats. Both montelukast and curcumin showed anti-inflammatory activity, as manifested by a significant decrease in serum CRP and testicular TNF-α levels compared with etoposide-treated rats (**Figure 1a-b**). Notably, the beneficial effect of curcumin on TNF-α level was significantly better compared to montelukast (**Figure 1b**).

3.3.3. ATP concentration

ATP concentration in different study groups is shown in (**Figure 2a-b**). Etoposide-treated rats showed a significant decrease in ATP level (4.55 ± 0.38) compared to control group (15.60 ± 1.05). Treatment with montelukast or curcumin increased this parameter significantly compared to etoposide group (9.95 ± 1.55 and 13.11 ± 1.67 respectively). ATP level is less reduced by etoposide after curcumin

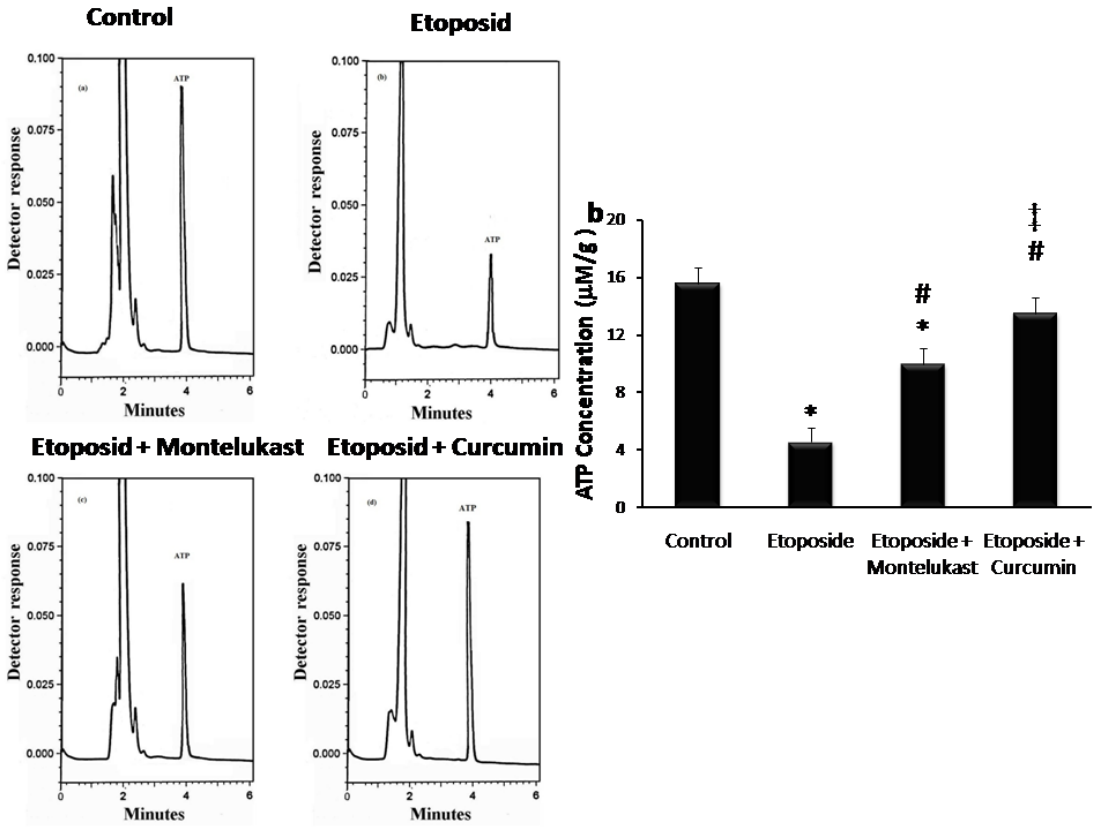

Figure 2: (a) HPLC chromatograms represent ATP level in the testis of normal rats; etoposide-treated rats; Etoposide + montelukast treated rats; and Etoposide + curcumin treated rats. (b) ATP concentration in testicular tissues of experimental groups. Etoposide induced a significant decrease in ATP concentration compared to control group. The etoposide-induced ATP change was significantly decreased upon administration of montelukast or curcumin. Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}P< 0.05$ compared to control group, $^{\#}P< 0.05$ compared to etoposide group, $^{‡}P < 0.05$ compared to montelukast group. *n* = 8-12.

administration (**Figure 2a-b**).

3.3.4. Mitochondrial DNA (mtDNA) integrity and quantity

In normal control animals that received distilled water, montelukast and curcumin, intact form of mitochondrial DNA was electrophoresed as a major band of approximately 16.5 kb (lanes 1-4, respectively, **Figure 3a**). On the other hand, etoposide markedly decreased the amounts of intact mtDNA in the testicular tissue (lane 5). However, mtDNA obtained from animals treated with etoposoide and montelukast (lane 6), or curcumin (lane 7) was electrophoresed in its intact form. This suggested that all the former treatments markedly alleviated etoposide-induced injury of mtDNA.

3.3.4. Fragmentation of nDNA

The electrogram generated from gel electrophoresis shows that nDNA isolated from normal rats (lanes 1, 2, **Figure 3b**), or from normal rats received either

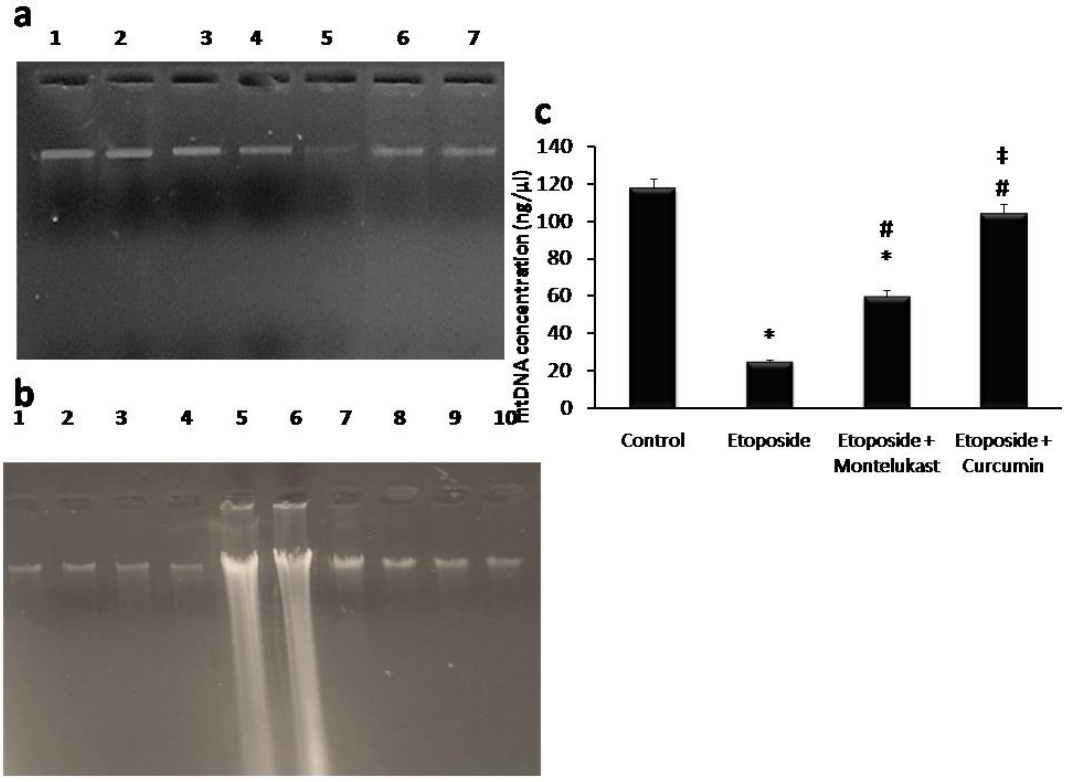

Figure 3: mitochondrial and nuclear DNA in testicular tissue of experimental groups. (a) Mitochondrial DNA (mtDNA) damage. Lane 1 and lane 2 intact mtDNA isolated from normal control animal; lane 3 and lane 4, intact mtDNA samples isolated from normal animals received montelukast and curcumin, respectively. Lane 5, mtDNA sample from the etoposoide treated animals; lane 6 and lane 7 show mtDNA samples isolated from animals treated with montelukast and curcumin, respectively.

(b) Nuclear DNA (nDNA) integrity. Lane M, 100 kb DNA ladder; lane 1 and lane 2 intact nDNA of normal control animals; lane 3 and lane 4, nDNA samples isolated from normal rats received montelukast and curcumin respectively, lanes 5 and 6, nDNA samples of etoposoide-treated animals, lanes 7 and 8 nDNA samples of etoposoide + montelukast-treated animals, lanes 9 and 10, nDNA isolated from rats received curcumin + etoposoide.

(c) Mitochondrial DNA (mtDNA) concentration in testicular tissues of experimental groups. Etoposide induced a significant mtDNA damage compared to control group. The etoposide-induced mtDNA damage was significantly decreased upon administration of montelukast or curcumin. Data were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. $^{*}P< 0.05$ compared to control group, $^{\#}P< 0.05$ compared to etoposide group, $^{‡}P < 0.05$ compared to montelukast group. n = 8-12.

montelukast (lane 3) or curcumin (lane 4) showed total ladder and smear negativity, whereas, etoposide exposure resulted in a marked decrease in the amount of intact nDNA in testicular tissues. Moreover, a dramatic oligonucleosome-length degradation of DNA was observed, characterized by mixed smearing and laddering (lane 5 and lane 6 **Figure 3b**). In contrast, **Figure 3b** showed that no appreciable fragmentation of nDNA was found to occur in animals protected with montelukast (lanes 7, 8) and curcumin (lanes 9, 10). These results demonstrated that the current treatments abolished the ladder pattern of nDNA cleavage in testicular tissue; therefore, they apparently provided protection against etoposide-induced testicular damage.

Figure 3c depicted the intergroup alterations in mtDNA concentration in the testicular tissues of etoposide-treated rats. No significant change was observed in normal rats received montelukast or curcumin (data not shown). In etoposide-treated group, a significant decline was observed in the testicular content of mtDNA, as compared to that of control group (25.10 ± 0.74 vs. 117.30 ± 3.58, $P < 0.05$, **Figure 3c**). However, normalization of the mtDNA content was observed upon supplementation of curcumin (103.80 ± 5.23). Hence, the current data emphasized that etoposide-induced mtDNA damage was significantly decreased upon administration of the current treatments, suggesting a possible protective potential.

3.3.5. Histopathological results

Control animals exhibited normal testicular morphology in the form of tightly coiled seminiferous tubules which constitute most of the testes proper. The tubular structure, diameter and germinal cells number were normal. The center of the tubules shows multiple spermatozoa. Sertoli and Leydig cells also showed normal morphology (**Figure 4a-c**).

In testes of etoposide-treated rats, degenerative changes in the seminiferous epithelium were characterized by the giant cell formation, cytoplasmic vacuolization, Pyknotic nuclei of some germinal cells, depletion and desquamation of germ cells in tubular lumen as well as absence of spermatids and spermatozoa. In addition, a various degree edema was seen in the interstitial spaces (**Figure 4d**). Testicular sections observed in etoposide-treated groups showed normal interstitial tissue, little exfoliation with increased number of germinal epithelial cells within the lumen (**Figure 4e, f**).

Insignificant change in Sertoli cell number was detected compared to controls. The Testicular tubular diameter was significantly decreased compared to controls (**Table 4**). Etoposide also decreased germ cells number compared to controls; spermatogonia; non-pachytene spermatocytes, pachytene spermatocytes and spermatids (**Table 4**).

Compared with etoposide group, the diameters of tubules in montelukast or curcumin groups were significantly increased. Number of Sertoli cells however, didn't change significantly among treatment groups.

Spermatogenesis was significantly preserved in the rats treated with montelukast or curcumin. Montelukast and curcumin increased number of spermatogonia, pachytene spermatocytes and elongated spermatids significantly (**Table 4**) versus etoposide-treated rats. Notably, curcumin normalized the number of pachytene spermatocytes.

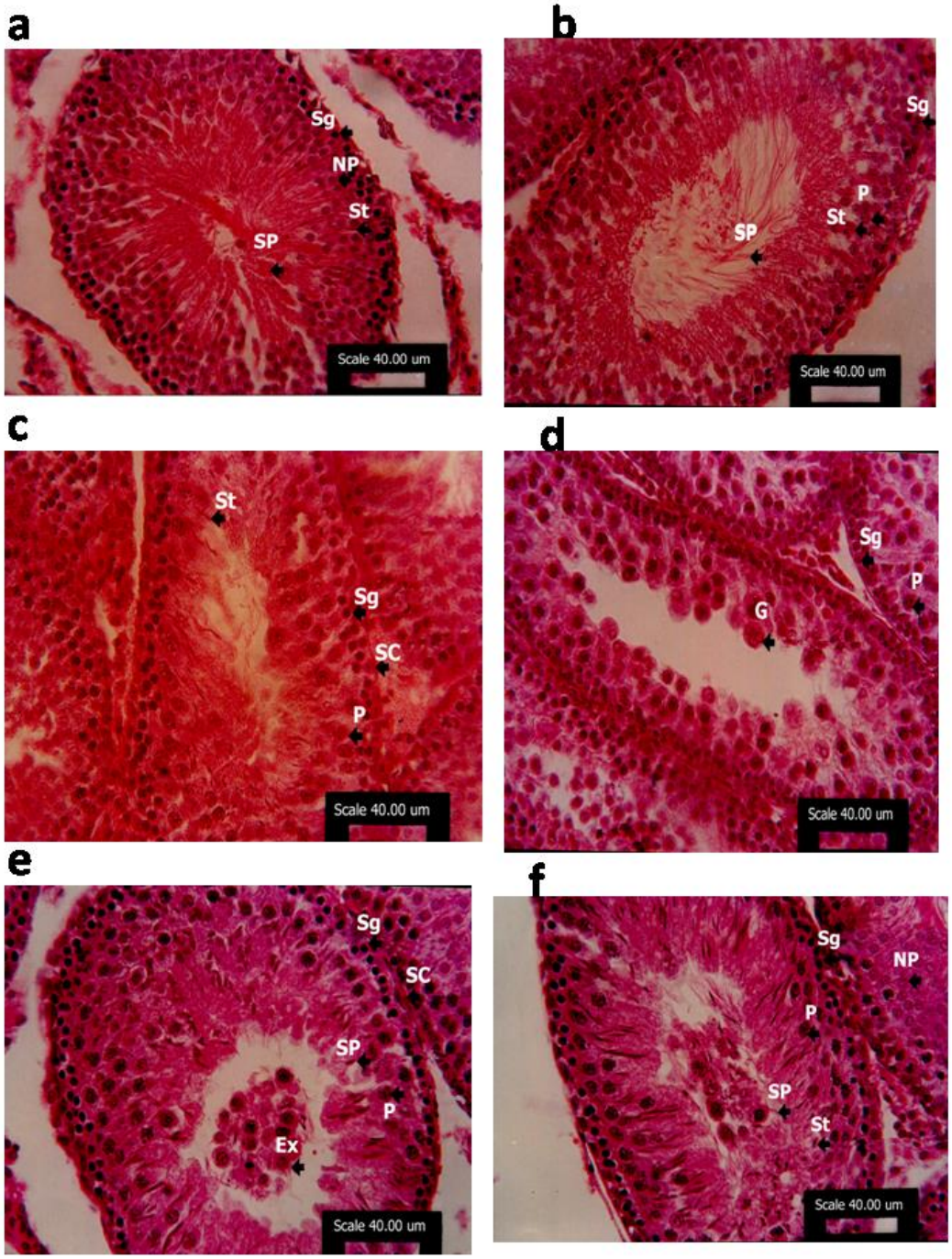

Figure 4 a. Testicular section of control rat showing a seminiferous tubule lined by spermatogenic cells. The center of the tubule shows multiple spermatids and spermatozoa. b. testicular section of normal rat received montelukast showing parts of two seminiferous tubules separated by normal interstitial tissue with Leydig cells. c. testicular section of normal rat received curcumin showing multiple normal sertoli cells and normal interstitial tissue. Many pachytene spermatocytes, spermatids and spermatozoa are also detected. d. Etoposide resulted in decrease in the number of primary spermatocytes and absence of spermatids and spermatozoa in seminiferous tubules. Multiple giant cells and exfoliation of germinal epithelium is also detected. e. Administration of montelukast to etoposide-treated rats induced regeneration of seminiferous tubules and increased number of germinal epithelial cells with many spermatozoa in the lumen. Some exfoliated germinal epithelial cells are still seen in the lumen. f. testicular section of rat received curcumin + etoposide showing normal interstitial tissue between seminiferous tubules. Little exfoliation is seen in the lumen. Many spermatids and spermatozoa are detected in the lumen. Ex: Exfoliated germinal epithelium, G: Giant cells; L: Leydig cells; NP: non- pachytene spermatocytes; P: pachytene spermatocytes; SC: Sertoli cells; Sg: Spermatogonia, St: Spermatids, S: Spermatozoa; Hematoxylin and eosin X 400, Scale bar 40.00 um.

4. Discussion

Etoposide has anticancer properties through generation of breaks in cellular DNA but its therapeutic use is limited due to toxic effects (Bromberg et al., 2003; Kanchana Ganga et al., 2013; Papież, 2013). In this context, the primary aim of this study was to gain insight into the possible mechanism(s) of the toxic effects of etoposide on testicular tissue and how these effects can be prevented.

In the current study, administration of etoposide induced lipid peroxidation, decreased the GSH content and the activities of SOD and CATA in rat testis. Malondialdehyde causes peroxidative damage of cellular lipid content (Berryman et al., 2004). Testis is a viable organ for oxidative stress because of its high content of polyunsaturated membrane lipids (Oguzturk et al., 2012). Reduced glutathione plays an important role in the maintenance of protein and lipid integrity, and provides major protection in oxidative injury (Ognjanovic et al., 2012).

Superoxide dismutase catalyses the dismutation of superoxide anion radicals to hydrogen peroxide and in turn CATA degrades hydrogen peroxide into a molecule of oxygen and water (McCord, 2000).

Lowered activities of SOD and CATA, as noted in the current study, resulted in accumulation of these highly ROS that lead to detrimental effects in different tissues due to imbalance between ROS generation and antioxidant system. The present results are in agreement with previous studies, which reported the induction of oxidative stress and lipid peroxidation in anticancer drugs-treated animals (Reddy et al., 2010; Noori and Mahboob , 2010).

The current study showed that serum testosterone level has significantly decreased with etoposide treatment. This could be attributed to the impairment of Leydig cells or decreased numbers of luteinizing hormone (LH) receptors on Leydig cells (Silici et al., 2009). On the other hand, the weight of the testes is largely dependent on the mass of the differentiated spermatogenic cells, and the reduction in the weight of the testes may be due to the reduced tubule size, spermatogenic arrest and inhibition of steroid

Table 4: Effect of montelukast and curcumin on seminiferous tubular diameter, Sertoli cells number and testicular germ cells population in control and etoposide-treated rats

Groups	Tubular diameter (µm)	Sertoli cells/ tubule	Germ cells number / seminiferous tubule			
			Spermatogonia	Non-pacytene spermatocytes	Pachytene spermatocytes	Elongated spermatids
Control	500.50 ± 9.81	44.11 ± 0.01	2.42 ± 0.19	9.34 ± 0.67	8.11± 0.54	30.28 ± 2.55
Etoposide	371.96 ± 12.17[a]	3.77 ± 0.02	1.05 ± 0.11[a]	4.72 ± 0.31[a]	3.51 ± 0.23[a]	10.87 ± 0.75[a]
Etoposide + Montelukast	425.12 ± 11.11[a,b]	3.55 ±0.01	1.99 ± 0.14[a,b]	5.66 ± 0.48[a]	5.89 ± 0.38[a,b]	18.17 ± 0.34[a,b]
Etoposide + Curcumin	420.30 ± 13.07 [a,b]	3.71 ±0.01	1.88 ± 0.08[a,b]	6.98 ± 0.16[a,b]	7.68 ± 0.61[b]	22.33 ± 1.24[a,b]

Data was expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test. n = 8–12.
[a]$P < 0.05$ versus control group.
[b]$P < 0.05$ versus etoposide group.
[c]$P < 0.05$ versus montelukast group.

biosynthesis of Leydig cell (Chapin et al., 1997). In this context, the spermatogenic inhibition in etoposide-treated rats cannot only be the result of the reduced serum testosterone level. Besides hormonal alteration, the increased lipid peroxidation, decreased antioxidant enzymes and ATP depletion, that is mainly necessary for the flagellum movement of the sperm, may thus represent a key event linked to decreased spermatogenes (Beytur et al., 2012). Spermatozoa are liable to lipid peroxidation as they are rich in polyunsaturated fatty acids that are liable to lipid peroxidation by ROS (El-Sweedy et al., 2007).

Etoposide increased pro-inflammatory cytokines (CRP and TNF-α) in the current study. This may be attributed to activation of the p38 mitogen-activated protein kinase in macrophages which plays a central role in the production of the pro-inflammatory cytokines interleukins 1β and 6 as well as TNF-α (Wood et al., 2006).

In alignment with the oxidative properties of etoposide, it resulted in significant decrease in ATP concentration and increase in mtDNA and nDNA damage in testicular tissue. In agreement, oxidative stress was shown to induce down-regulation of mitochondrial complex I and IV activity and decreased ATP concentrations in diabetic rats (Rastogi et al., 2008). The probability of loss of the reproductive capacity because of high sensitivity of gonads to cytostatic treatment explains the interest of clinicians in studies of the reproductive status of patients treated with etoposide (Pectasides et al., 2004; Ishikawa et al., 2004). In the testis, high concentration of topoisomerase II is present in spermatogonia (Hakovirta et al., 1999), and so, these cells are very sensitive to etoposide. In previous studies, it was shown that etoposide administration to pre-pubertal rats can cause germ cell apoptosis and testicular morphometric alterations, leading to Sertoli cell numerical alternation and serious seminiferous epithelium germ cell harm leading to significant reduction in serum testosterone (Stumpp et al., 2004; Ortiz et al., 2009). In the light of these data, it is reasonable to consider that apoptosis was among causes of reduced spermatozoa production in the current study; however, further investigations are necessary to confirm this hypothesis.

Montelukast, a selective reversible LTD4 receptor antagonist, directly interferes with leukotriene production and their receptors (Muthuraman and Sood, 2010). Treatment with montelukast could prevent lipid peroxidation in testis tissue. Only few studies clarified the effects of montelukast on oxidant/antioxidant status in testicular tissue. However, it was determined that montelukast reduced MDA level in rat testis after ischemic/reperfusion injury (Ozturk et al., 2010).

In harmony with our results, montelukast prevented lipid peroxidation in liver and blood caused by carbon tetrachloride (Cuciureanu et al., 2009). In agreement, it was proved that montelukast can ameliorate lipid peroxidation and increase antioxidant properties in renal tissues after aminoglycosides administration (Kose et al., 2012). However, in contrast to our results, testicular CATA activity and GSH level did not significantly changed upon administration of montelukast to cisplatin intoxicated rats, this may be explained with sensitivity of the rats and duration of

drug treatment (Beytur et al., 2012).

Montelukast treatment reversed the decreased effect of etoposide on testosterone levels. To the best of our knowledge, there was no study about the effects of montelukast treatment on male fertility with etoposide treatment, however, it is thought that this effect of etoposide may occur through interference with LH receptor expression, impairment of the cholesterol mobilization to mitochondrial cytochrome P450, or reduction of the activity of this enzyme, thus interfering with the first steps in testosterone production (Silici et al., 2009).

Additionally, in this study, we showed that montelukast significantly increased sperm count, motility and viability in etoposide-treated rats. Previously, spermatological effects of montelukast with etoposide were not examined. Montelukast treatment can be beneficial due to its antioxidant, anti-inflammatory and antipoptotic properties and may positively affect etoposide-induced infertility.

In histological evaluation, histological damage was decreased when montelukast was combined with etoposide. It was considered that the histopathological effects may attribute to the imbalance between oxidant and antioxidant statuses and/or increased inflammation and DNA damage in testis tissue induced by etoposide that may contribute also to male infertility by reducing sperm count. For this reason, a decrease in elevated oxidative stress in testis tissue with montelukast is very important in terms of infertility (Lirdi et al., 2008).

In this study, curcumin attenuated the etoposide-induced MDA formation and the decreased testicular GSH as well as SOD and CATA activities, possibly due to its intrinsic antioxidant properties (Palipoch et al., 2013). Similarly, curcumin protected against testicular oxidative damage induced by di-n-butylphthalate due to its intrinsic antioxidant properties (Farombi et al., 2007). The antioxidant mechanism of curcumin is due to its specific conjugated structure of two methoxylated phenols and an enol form of β-diketone, the structure showed a typical radical trapping ability as a chain breaking antioxidant (Masuda et al., 2001).

Accordingly, the protective effects of curcumin on spermatogenesis can be explained by the fact that it prevents cellular damage occurring as a result of oxidative stress in spermatogenic cells of seminiferous tubules and Leydig cells and thus relieve the reduction of testosterone level (Aly et al., 2009).

These effects, taken together, improved fertility and testicular performance, through controlling lipoperoxidation which simultaneously affect sperm motility.

Germ cell apoptosis has been reported to play an important role in etoposide-induced testicular damage (Lizama et al., 2011). Curcumin may ameliorate testicular damage due to its anti-apoptotic potential manifested by decrease in mtDNA and nDNA damage. The free radical scavenger activity and improving the antioxidant status of curcumin might justify this protective effect. Mitochondrial dysfunction is characterized by a decrease in energy production in the form of ATP. Mitochondrial dysfunction is caused by oxidative stress and represents an early event in aging and in the pathogenesis of age-related neuronal cell death and degenerative diseases (Eckert et al., 2013).

Curcumin has shown its anti-apoptotic property by significantly reducing DNA fragmentation when compared with etoposide-treated rats. Curcumin has been recently shown to act as an important anti-apoptotic agent (Shehzad et al., 2013).

5. Conclusion

In conclusion, the present study showed that montelukast and curcumin can reverse toxic effects of etoposide on the reproductive system that can be contributed due to anti-oxidant, anti-inflammatory and antiapoptotic potential.

As protective agent, curcumin was found to be more effective in etoposide-induced testicular toxicity. Further clinical and experimental studies are needed to determine the potential beneficial effects of these agents either with etoposide alone or with etoposide-based chemotherapy regimens before their clinical applications against testicular injury.

6. Conflict of interest

The authors report no declaration of conflict of interest.

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