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*Hum Exp Toxicol* 2008 27: 901
DOI: 10.1177/0960327108102046

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What is This?
Protection of cyclophosphamide-induced toxicity in reproductive tract histology, sperm characteristics, and DNA damage by an herbal source; evidence for role of free-radical toxic stress

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Cyclophosphamide (CP) as an anticancer alkylating agent has been known as a male reproductive tract toxicant. The aim of this study was to examine whether Satureja khuzestanica essential oil (SKEO) as an established herbal antioxidant, might protect tract rat reproductive system from toxicity of CP. To reach this aim, total antioxidant power (TAP) and lipid peroxidation (LPO) in testis and plasma, blood levels of sex hormones, sperm characteristics, DNA integrity and chromatin quality, and fertility in male rats were tested. Histopathological analysis of testes and epididymides and staining of mast cells were performed for assessment of spermatogenic disorders. CP (6 mg/kg/day) and SKEO (225 mg/kg/day) were administered alone or in combination by gavage for 28 days. In the CP-exposed rats, testicular and plasma LPO increased, TAP decreased, plasma testosterone diminished, and both spermatogenesis and fertility were impaired. In CP-treated rats, a decrease in sperm quality was associated with increased DNA damage and decreased chromatin quality. Coadministration of SKEO significantly improved CP-induced changes in plasma testosterone, sperm quality, spermatogenesis and fertility, toxic stress, and DNA damage. It is concluded that CP-induced toxic effects on androgenesis and spermatogenesis is mediated by free radicals. SKEO protects reproductive system from toxicity of CP through its antioxidant potential and androgenic activity.

Key words: antioxidants; cyclophosphamide; DNA damage; fertility; lipid peroxidation; oxidative stress; Satureja khuzestanica essential oil; sperm quality; spermatogenesis; testicular toxicity; testosterone; toxic stress

Introduction

Exposure to toxins during spermatogenesis may target male germ cells that results in abnormal functioning and adverse pregnancy outcomes.1 Cyclophosphamide (CP) as an alkylating agent is the most commonly used anticancer and immunosuppressant drug. It is used for the treatment of chronic and acute leukemia, multiple myeloma, lymphomas, rheumatic arthritis, bone marrow transplantation, and some immune-related diseases.2

Phosphoramide mustard and acrolein are two active metabolites of CP.3 The antineoplastic effect of CP is associated with phosphoramide mustard, whereas acrolein is linked to toxic effects like cell death, apoptosis, oncosis, and necrosis.4 Despite its wide spectrum of clinical uses, reproductive toxicity of CP is known as the main adverse effect seen in humans and animals.5,6 A number of reports have indicated that CP alters human fertility.6 Adult male patients treated with CP (1–2 mg/kg/day) for more than 4 months showed oligozoospermia or azoospermia.7 Studies on rats or mice have confirmed the potential of CP to cause testicular weight loss, transitory oligospermia, reduced DNA and protein synthesis in spermatogonia and spermatids, and histological alterations in the testes and epididymides.5,8,9

Although, the precise mechanism by which CP causes testicular toxicity is undefined, it is known

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that CP disrupts the redox balance of tissues resulting in oxidative stress.\textsuperscript{10–12} It has been reported\textsuperscript{13} that oxidative DNA damage is caused by hydroperoxide derivative of CP through generation of \( \text{H}_2\text{O}_2 \). Also, acrolein has been found to interfere with the tissue antioxidant defense system\textsuperscript{14} and produces highly reactive oxygen free-radicals\textsuperscript{15} that are mutagenic to mammalian cells.\textsuperscript{16} Consequently, from these aforementioned studies, we hypothesized that the administration of a potent and safe antioxidant might reduce CP-induced reproductive toxicity. *Satureja khuzestanica* Jamzad (Marzeh Khuzestani in Persian, family of *Lamiaceae*) is an endemic plant that is widely distributed in the Southern part of Iran. It is famous for its analgesic and antiseptic properties in folk medicine.\textsuperscript{17} In the recent years, compositions of the essential oils of wild and cultivated *S. khuzestanica* have been well established.\textsuperscript{18,19} As reported recently, oral administration of *S. khuzestanica* essential oil (SKEO) to rats induced a marked antioxidant, antidiabetic, antihyperlipidemic, and reproduction stimulatory effects without occurrence of any toxic or adverse effects. Furthermore, in female rats that received SKEO, a marked increase in the number of implantation and live fetuses was observed.\textsuperscript{20} SKEO improved recovery from inflammatory bowel disease in animals by reduction of oxidative stress biomarkers.\textsuperscript{21} SKEO was also shown to act against malathion-induced toxicity by improving oxidative stress, acetylcholinesterase activity, and mitochondrial glycolysis and gluconeogenesis in rats.\textsuperscript{22,23} Furthermore, results of a new study showed that administration of SKEO (225 mg/kg) to male rats increases follicle stimulating hormone (FSH) and testosterone, and improves weights of testes, epididymides, and accessory sex glands. Histopathological analysis also showed that in male rats treated with SKEO (150, 225 mg/kg), the number of spermatogonium, spermatid cords, Leydig cells, and spermatozooids increased and the Sertoli cells were found hypertrophic.\textsuperscript{24} On the basis of these findings, the present study was intended to examine whether coadministration of SKEO can prevent CP-induced reproductive toxicity in rats and how free radicals play a role.

**Methods**

**Chemicals**

CP (Endoxan Baxter\textsuperscript{®}), 2-thiobarbituric acid (TBA), Ham's F10, NaHCO\textsubscript{3}, trypan blue, eosin-Y, Nigrosin, ethanol, hematoxylin, paraffin, toluidine blue, Car-}

**Preparation of essential oil**

The aerial parts of the plant were collected during the flowering stage of the plant from Khoaramabad of Lorestan province in Iran. The plant was identified by botanist. The collected parts of the plant were air-dried at ambient temperature in the shade and hydrodistilled using a Clevenger type apparatus for 5 h, giving yellow oil in 0.9% yield. The oil was dried over anhydrous sodium sulfate and stored at 4 °C. The density of the essence was 0.98.

**Animals**

Adult sexually mature male (4 months of age weighing 200–230 g), and female (3 months of age weighing 180–200 g) albino rats of Wistar strain were obtained from animal house of Veterinary School of Urmia University. They were housed in a specific pathogen-free environment under standard conditions of temperature (23 ± 1 °C), relative humidity (55 ± 10%), and 12/12 h light/dark cycle, and fed with a standard pellet diet and water *ad libitum*. Animals were checked weekly for body weight and occurrence of any toxic signs. All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by Institute Review Board.

**Treatment**

In this study, four groups each containing eight male rats were considered. Treatment groups were as follows: group 1 received olive oil by oral gavage daily, group 2 received CP (6 mg/kg/day) by gavage, group 3 received an effective dose of SKEO at 225 mg/kg/day, and group 4 received SKEO (225 mg/kg/day) and CP (6 mg/kg/day) by gavage. All groups were treated for 4 weeks. The protocol for this study, including doses and duration of treatment for CP and SKEO, were all designed according to previous studies.\textsuperscript{24–26}

**Examination of male fertility**

Three male rats from each group were mated on day 28 with sexually mature normal females presenting...
at least three regular estrus cycles confirmed by analysis of daily vaginal smears. Females in the proestrus stage were mated with males for 1 week (2 females per male); the presence of sperm in the vaginal smear was taken as the proof of mating and was considered as day zero of pregnancy. At the end of pregnancy, the number of pregnant female, litter size, and fecundity and fertility indices were recorded as follows:

Fertility index = (number of males impregnating females/number of males used in the test) × 100. Fecundity index of male = (number of pregnancy/number of copulation) × 100.

Sampling
At the end of the specified treatment, the animals were euthanized by CO2 exposure and were killed by decapitation. Blood samples were collected in vials containing heparin. The plasma was separated and kept at −80 °C until analysis of luteinizing hormone (LH), FSH, testosterone, and toxic stress markers including cellular lipid peroxidation (LPO) and total antioxidative power (TAP). Testes and accessory sex glands were removed, cleared of adhering connective tissue, weighed and perfused with cold (0.9% NaCl), and used for the histopathological study. One testis was kept frozen at −80 °C until homogenized for further analyses of toxic stress markers and a histochemical analysis. Radioimmunoassay kits determined the concentrations of sex hormones LH, FSH, and testosterone.

LPO and TAP
Concentration of LPO in plasma and testis was determined by measurement of malondialdehyde and other lipid peroxide aldehydes that react with TBA known as TBA-reactive substances (TBARS). The absorption of the TBARS was determined spectrophotometrically at 532 nm by use of 1,1,3,3-tetraethoxypropan as the standard. The absorption of the TBARS was determined spectrophotometrically at 532 nm by use of 1,1,3,3-tetraethoxypropan as the standard.

TAP of plasma and testis was determined by measuring their ability to reduce Fe3+ to Fe2+. The complex between Fe2+ and TPTZ gives a blue color with absorbancy at 593 nm. Both methods have been described in details in our previous report.27

Sperm characteristics
Epididymal sperms were collected by slicing the epididymides in 5 mL of Ham’s F10 and incubating for 5 min at 37 °C in an atmosphere of 5% CO2 to allow sperm to swim out of the epididymal tubules. One drop of sperm suspension was placed on a microscope slide, and a cover slip was placed over the droplet. At least 10 microscopic fields were observed at 400× magnification using a phase contrast microscope, and the percentage of motile sperm28 was evaluated microscopically within 2–4 min of their isolation from the epididymides and was expressed as a percentage of motile sperm of the total sperm counted.

Epididymal sperm counts were obtained by the method described in the WHO Manual (1999).28 Briefly, a 5 μl aliquot of epididymal sperm was diluted with 95 μl of diluent (0.35% formalin containing 5% NaHCO3 and 0.25% trypan blue) and approximately 10 μl of this diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent drying. The cells sediment during this time and were counted with a light microscope at 400×.

A 20 μl of sperm suspension was mixed with an equal volume of 0.05% eosin-Y. After 2 min incubation at room temperature, slides were viewed by bright-field microscope with magnification of 400×. Dead sperms appear pink and live sperms are not stained. Two hundred sperms were counted for each sample and viability percentages were calculated.

For the analysis of morphological abnormalities, sperm smears were drawn on clean and grease-free slides, and allowed to air dry overnight. The slides were stained with 1% eosin-Y/5% nigrosin and examined at 400× for morphological abnormalities such as amorphous, hook less, bicephalic, coiled, or abnormal tails.29

Assessment of DNA integrity and chromatin quality
Staining of spermatozoa with acridine orange Acridine orange staining was used to monitor the effects of CP on cauda epididymal sperm. To perform this assay with fluorescent microscope, thick smears were fixed in Carnoy’s fixative (methanol: acetic acid 1: 3) for at least 2 h. The slides were stained for 5 min and gently rinsed with deionized water. Two-hundred sperms from each staining protocol were evaluated and graded as normal DNA (green) or damaged DNA (yellow to red).30

Staining of spermatozoa with aniline blue Protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by aniline blue.31 Slides were prepared by smearing 5 μl of either a raw or washed semen sample. The slides are air-dried and fixed for 30 min in
3% glutaraldehyde in phosphate buffered saline. The smear was dried and stained for 5 min in 5% aqueous aniline blue solution (pH 3.5). Sperm heads containing immature nuclear chromatin stain blue and those with mature nuclei do not take up the stain. The percentage of spermatozoa stained with aniline blue was determined by counting 200 spermatozoa per slide under bright field microscope.

Sample preparation for light microscopy and histopathological analysis
After fixation of testis and epididymides in a 10% formalin solution, they were directly dehydrated in a graded series of ethanol and embedded in paraffin. Thin sections (4–5 μm) were cut using a microtome and stained with hematoxylin and eosin and examined using a light microscope. The qualitative changes of testes and epididymides were recorded. In the lumen of the epididymides, sperm density was observed and graded as normal (+++), moderately decreased (++), or as severely decreased (+) depending on the concentration of spermatozoa in the tubular cross-sections. Degenerating Leydig cells and abnormal Sertoli cells were considered. One hundred seminiferous tubules were counted, and tubule differentiation index (TDI), which is the percentage of seminiferous tubules containing at least three differentiated germ cells and spermatid index (SPI), which is the percentage of seminiferous tubules with normal spermatiation were determined for each group.

Toluidine blue staining of mast cells
In order to evaluate mast cells number, tissue slices of all testis and cauda epididymal sections were routinely processed for paraffin embedding. The sections were prepared and stained with toluidine blue.

Statistical analysis
Values are reported as mean ± SEM. Statistical significance between groups was computed by analysis of variance and Tukey multiple comparison posthoc tests. Data relating to fecundity index, fertility index, litter size, and number of pregnant female were subjected to Exact Fischer Test. P values lower than 0.05 were considered significant.

Results
There was no significant change in animal mass gain among treated groups. Administration of CP increased LPO in plasma and testis as compared to control (Table 1). Administration of SKEO reduced plasma and testis LPO in comparison to control. Coadministration of CP and SKEO resulted in restoration of CP-induced LPO change in plasma and testis of CP-treated animals. Administration of SKEO increased TAP in plasma and testis as compared to control. CP decreased TAP in plasma and testis as compared to control. Coadministration of CP and SKEO restored CP-induced reduction of TAP in plasma and testis of CP-treated animals.

The weight of testes, epididymis, seminal vesicle, and ventral prostate decreased by CP treatment, whereas all changes were restored by SKEO cotreatment (Table 2). There was no significant difference in LH and FSH levels between groups. When compared to control, administration of SKEO increased plasma testosterone concentrations (Table 2), whereas CP decreased plasma testosterone concentration. Coadministration of CP and SKEO recovered CP-induced reduction of plasma testosterone. As shown in Table 2, treatment of male rats with CP resulted in reduced sperm count and motility, while the number of dead and abnormal sperms increased. Treatment with SKEO improved semen quality and minimized toxic effects of CP.

The formation of DNA strand breaks in CP-treated group was evident from decreased percentage of double stranded DNA in the epididymal sperm (Table 2). SKEO suppressed CP-induced DNA damage. Percentage of immature sperms in CP-treated rats was higher than those of controls. Cotreatment of animals with SKEO and CP improved the chromatin quality as compared with the CP-treated group.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of CP and SKEO on oxidative stress biomarkers in plasma and testes of male rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Plasma LPO (μmol/mL)</td>
<td>2.07 ± 0.2</td>
</tr>
<tr>
<td>Testis LPO (μmol/mL)</td>
<td>2.04 ± 0.26</td>
</tr>
<tr>
<td>Plasma TAP (mmol/mL)</td>
<td>595.3 ± 24.6</td>
</tr>
<tr>
<td>Testis TAP (mmol/mL)</td>
<td>1508 ± 83.97</td>
</tr>
</tbody>
</table>

SKEO, *Satureja khuzestanica* essential oil; CP, Cyclophosphamide; LPO, lipid peroxidation; TAP, total antioxidant power.
<sup>a</sup>Significantly different between control and other groups at P < 0.05.
<sup>b</sup>Significantly different between cyclophosphamide and other groups at P < 0.05.
The fecundity and fertility indices and litter size of the male rats treated with CP was lower than those of the controls (Table 2). Administration of SKEO improved fecundity and fertility indices and litter size in CP-treated animals. Histopathological studies of the testes revealed that CP-disintegrated seminiferous tubules and impaired spermatogenesis with central debris that was devoid of sperms. CP increased the number of sloughing tubules (Table 3, Figure 1). Most of the tubules showed hypoplasia of the Leydig cells and dispersion with picnotic nuclei (Figure 2). Testicular damage in CP-treated rats was evident as interstitial edema and increased interstitial space (Figure 2). CP fragmented the junction of Sertoli cells and induced amorphous Sertoli cells with flattened nucleus. As seen in Table 3, both TDI% and SPI% reduced in CP-treated animals. SKEO treatment prevented from CP-induced reduction in sperm concentration. The number of mast cells in the testicular and cauda epididymal tissues was higher in CP-treated animals than in controls (Table 3). Coadministration of CP and SKEO resulted in restoration of CP-induced mast cells being surplus.

### Discussion

Results of both biochemical and histological examinations indicate that CP induces a marked reproductive toxicity through induction of free-radical toxic stress that is protectable by SKEO coadministration. The marked reduction in testicular weight by CP is explained by diminished number of germ cells, atrophy of Leydig cells and a significant lower rate of spermatogenesis as confirmed by our histopathological findings. Reduction in the weight of testes and accessory reproductive organs in CP-treated animals was higher in CP-treated animals than in controls (Table 3). Coadministration of CP and SKEO resulted in restoration of CP-induced mast cells being surplus.

### Table 2 Effects of CP and SKEO on the organ weight, sex hormone levels, sperm characteristics, and fertility of male rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SKEO</th>
<th>CP</th>
<th>CP + SKEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis weight (g)</td>
<td>1.52 ± 0.01</td>
<td>1.57 ± 0.03(^{a,b})</td>
<td>1.41 ± 0.02(^a)</td>
<td>1.49 ± 0.03(^{b})</td>
</tr>
<tr>
<td>Epididymal weight (g)</td>
<td>0.476 ± 0.02</td>
<td>0.522 ± 0.01(^{a,b})</td>
<td>0.398 ± 0.02(^a)</td>
<td>0.468 ± 0.00(^b)</td>
</tr>
<tr>
<td>Ventral prostate weight (g)</td>
<td>0.281 ± 0.00</td>
<td>0.297 ± 0.02(^{a,b})</td>
<td>0.217 ± 0.01(^a)</td>
<td>0.260 ± 0.00(^{b})</td>
</tr>
<tr>
<td>Vescicle seminal weight (g)</td>
<td>0.542 ± 0.03</td>
<td>0.576 ± 0.03(^{a,b})</td>
<td>0.437 ± 0.01(^a)</td>
<td>0.513 ± 0.00(^{b})</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>0.25 ± 0.001</td>
<td>0.24 ± 0.004</td>
<td>0.25 ± 0.008</td>
<td>0.25 ± 0.003</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>0.22 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>0.25 ± 0.05</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Testosterone (ng/dl)</td>
<td>46.66 ± 9.39</td>
<td>151.6 ± 7.76(^{a,b})</td>
<td>21.6 ± 3.7(^a)</td>
<td>106 ± 13.7(^{b})</td>
</tr>
<tr>
<td>Sperm count (10^6/mL)</td>
<td>171.86 ± 2.73</td>
<td>172.13 ± 2.19(^{b})</td>
<td>79.66 ± 4.08(^a)</td>
<td>158.66 ± 5.78(^{a,b})</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>79.59 ± 3.63</td>
<td>84.46 ± 8.68(^{a,b})</td>
<td>51.72 ± 1.72(^a)</td>
<td>73.46 ± 1.85(^{a,b})</td>
</tr>
<tr>
<td>Dead sperms (%)</td>
<td>18.3 ± 7.36</td>
<td>11.3 ± 4.34(^{b})</td>
<td>47.9 ± 8.46(^a)</td>
<td>25.2 ± 6.51(^b)</td>
</tr>
<tr>
<td>Abnormal sperms (%)</td>
<td>7.9 ± 1.91</td>
<td>7.1 ± 1.59(^{b})</td>
<td>36.2 ± 6.16(^a)</td>
<td>18.9 ± 1.79(^{a,b})</td>
</tr>
<tr>
<td>Positive acridine orange staining (%)</td>
<td>13.92 ± 3.04</td>
<td>13.92 ± 1.65(^{a,b})</td>
<td>13.92 ± 1.65(^{a,b})</td>
<td>13.92 ± 1.65(^{a,b})</td>
</tr>
<tr>
<td>Positive aniline blue staining (%)</td>
<td>1.35 ± 0.77</td>
<td>1.06 ± 0.43(^{b})</td>
<td>2.33 ± 1.44(^{a})</td>
<td>1.26 ± 0.47(^{b})</td>
</tr>
<tr>
<td>Number of impregnated females</td>
<td>6</td>
<td>6(^b)</td>
<td>2(^a)</td>
<td>5(^b)</td>
</tr>
<tr>
<td>Total live litter</td>
<td>47</td>
<td>63(^{a,b})</td>
<td>3(^a)</td>
<td>27(^{a,b})</td>
</tr>
<tr>
<td>Fecundity (%)</td>
<td>100</td>
<td>100(^{b})</td>
<td>40(^a)</td>
<td>83.3(^{a,b})</td>
</tr>
<tr>
<td>Fertility (%)</td>
<td>100</td>
<td>100(^{b})</td>
<td>33.3(^a)</td>
<td>66.6(^{a,b})</td>
</tr>
</tbody>
</table>

SKEO, *Satureja khuzestanica* essential oil; CP, Cyclophosphamide; FSH, ; LH, .

\(^{a}\)Significantly different from control as $P<0.05$.

\(^{b}\)Significantly different from cyclophosphamide $P<0.05$.

### Table 3 Comparative assessment of histopathological findings in male rats treated by CP and SKEO

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SKEO</th>
<th>CP</th>
<th>CP + SKEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDI (%)</td>
<td>94.58 ± 0.95</td>
<td>94.75 ± 1.34(^{a,b})</td>
<td>59.41 ± 1.25(^{a})</td>
<td>83.46 ± 1.71(^{a,b})</td>
</tr>
<tr>
<td>SPI (%)</td>
<td>96.74 ± 0.70</td>
<td>97.91 ± 0.31(^{b})</td>
<td>42.16 ± 0.88(^{a})</td>
<td>87.08 ± 2.36(^{a,b})</td>
</tr>
<tr>
<td>Sloughing tubules (%)</td>
<td>1.53 ± 0.56</td>
<td>1.39 ± 0.27(^{b})</td>
<td>17.34 ± 0.97(^{a})</td>
<td>5.66 ± 0.47(^{a,b})</td>
</tr>
<tr>
<td>Mast cells number of testis (mm²)</td>
<td>1.59 ± 0.13</td>
<td>1.58 ± 0.15(^{b})</td>
<td>2.83 ± 0.14(^{a})</td>
<td>2 ± 0.37(^{b})</td>
</tr>
<tr>
<td>Mast cells number of cauda epididymides (mm²)</td>
<td>2.98 ± 0.09</td>
<td>2.81 ± 0.1(^{b})</td>
<td>4.22 ± 0.07(^{a})</td>
<td>3.13 ± 0.27(^{b})</td>
</tr>
<tr>
<td>Cauda epididymal sperm concentration</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Degenerating and scattering of Leydig cells</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Flattened nucleus and deformed Sertoli cells</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

SKEO, *Satureja khuzestanica* essential oil; CP, Cyclophosphamide; TDI, tubular differentiation index; SPI, spermiation index.

\(^{a}\)Significantly different from control as $P<0.05$.

\(^{b}\)Significantly different from cyclophosphamide $P<0.05$. 

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Protection of cyclophosphamide-induced free-radical toxic stress

MA Rezvanfar, et al.

905

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reflect the reduced availability of androgens.\textsuperscript{35} In spite of a marked reduction in plasma testosterone by CP, no significant change in LH and FSH levels was observed. Thus, altered plasma testosterone by CP is assumed to be resulting from a direct toxic effect of CP on the structure of Leydig cells that is confirmed by histological findings.

Increased generation of free radicals is one of the possible mechanisms involved in CP-induced Leydig cell dysfunction.\textsuperscript{36} It has been reported\textsuperscript{37} that toxic stress reduces levels of key enzymatic and nonenzymatic antioxidants in Leydig cells resulting in a decline in testosterone secretion. CP-treated rats in our study had a significant increase in plasma and testis LPO and a significant drop in TAP suggesting the presence of free-radical toxic stress within the testis. The potential of CP and its metabolite acrolein for generating free radicals and LPO in the rat has been previously reported.\textsuperscript{38} Various studies\textsuperscript{10,11} suggested that a strong correlation exists between CP-induced toxicity and the induction of LPO. Therefore, toxic stress could play a critical role in the induction of sperm abnormalities, namely a higher susceptibility of sperm DNA to denature and fragment.\textsuperscript{39,40}

In the present study, epididymal sperm count and motility decreased by CP treatment while the number of dead and abnormal sperms increased. Spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity.\textsuperscript{41} Thus, they are very susceptible to free radical attack, which results in disturbed sperm motility,\textsuperscript{40} presumably by a rapid loss of intracellular ATP leading to damage in sperm flagellum, decreased sperm viability, and increased morphology defects (amorphous, hook less, bicephalic, coiled, or abnormal tails) with deleterious effects on sperm capacitation and acrosome reaction.\textsuperscript{42} Activity of Na-K-ATPase is highly sensitive to toxic stress.\textsuperscript{43} Thus, it may be plausible that CP-induced toxic stress lead to depletion of Na-K-ATPase and reduction of sperm motility.

A direct toxic effect of CP on the spermatogenesis in the seminiferous tubules may be considered as one of the mechanisms of action of CP in producing abnormal and dead sperms.\textsuperscript{5} Also, DNA damage may be responsible for the increased percentage of abnormal sperm forms. As demonstrated by acridine orange staining, exposure to CP causes single/double

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**Figure 1** Photomicrograph of testis from treated rats of control (A) CP (B&C) and SKEO + CP (D). *Satureja khuzestanica* Essential Oil (SKEO); Cyclophosphamide (CP). The control group presented normal architecture with active spermatogenesis (A) ×400. The CP group showed disintegrated seminiferous tubules, impaired spermatogenesis, hypoplasia and dispersion of Leydig cells, interstitial edema, and increased interstitial space, failure of spermatiation (B) ×400, increase in the number of sloughing tubules, and deformed spermatid cord (C) ×100. SKEO cotreated animals show nearly normal architecture (D) ×100.
strand breaks in spermatozoa DNA. Moreover, aniline blue staining showed poor chromatin-packed spermatozoa, confirming immature spermatozoa in CP-treated animals. It is thought that damaged spermatozoa are the source of free radicals.44

Free radical damage to mature sperms during their comigration from the seminiferous tubules to the epididymides forms immature sperms as an important cause of male infertility.45 Moreover, toxic stress-induced DNA damage may accelerate the process of germ cell apoptosis leading to a decline in sperm counts.45 In addition, toxic stress has been shown to affect the integrity of the sperm genome by induction of high frequencies of single- and double-strand DNA breaks, which are often detected in the ejaculates of infertile men.46

The adverse progeny outcomes associated with paternal exposure to CP after 3 weeks is due to accumulation of DNA damage in round spermatids as a result of spermiogenic germ cell phase-specific susceptibility to damage.25 Moreover, chronic exposure to CP decreases the expression of all antioxidant genes that are detected within pachytene spermatocytes and round spermatids. This effect contribute to a redox imbalance making these cells susceptible to toxic stress.47 Supporting this idea, a correlation between increased sperm DNA damage and poor blastocyst formation in vitro has been shown.48

In the present study, it has been shown that in rats treated with CP for 28 days, there were significant impairments in fecundity and fertility indices, and litter size and a significant decrease in impregnated female rats. These changes were in association with reduction in sperm quality and increased rate of DNA damage in cauda epididymal spermatozoa. Supporting our results, it has been reported that chronic paternal exposure of low dose CP can impair male fertility49 and alter the growth and development of the next generation50 through alteration of sperm chromatin structure.26

As shown in the present study, CP causes a significant decrease in the TDI and SPI and increases the number of sloughing tubules in testis. Structural development and maturation of germ cells and spermatocytes are important functions of Sertoli cells.51 Therefore, a potential explanation for the failure of...
spermiogenesis in the CP-treated males is disruption of testosterone-dependent attachment of round spermatids to Sertoli cells. In response to SKEO treatment, the SPI and TDI increased, possibly because of steroidogenic properties of SKEO that prevented their degeneration or loss into the lumen.

To explain the positive effect of SKEO on spermatogenesis, sperm quality, and fertility parameters, several ideas can be considered. One explanation is the androgenic property of SKEO. Another explanation is that SKEO protects the DNA structure and integrity of the spermatozoa during spermatogenesis and prevents a dominant lethal effect in chromatin. Our results demonstrated that CP-induced increase of mast cells in the testis and cauda epididymal tissues is recovered by SKEO. Several studies have confirmed that increased amount of testicular mast cells is associated with infertility and spermatogenetic disorders, which is due to the augmentation of free radicals from degranulated mast cells after allergic and immunologic stimulus. The dysfunction of the blood–testis barrier has also been attributed to an excess number of mast cells. Mast cell blockers like ketotifen or tranilast have been used in patients with severe oligospermia resulting in improvement of sperm count and pregnancy rates. Therefore, SKEO seems to have the same mast cell blocking activity remaining to be elucidated in future.

Increasing evidence support the fact that SKEO is beneficial where free radicals are known to play a predominant role in toxicity. Previous studies have shown concomitant antioxidant and reproductive stimulatory effects of SKEO in rats. The present findings supported our previous reports on the positive effects of SKEO in improving fertility and reproduction by its antioxidant potential. The Iranian SKEO has been well analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GS-MS) and found to have mainly carvacrol (93.9%), flavonoids, p-cymene, and thymol. Carvacrol, as the main constituent of SKEO, has marked antioxidant properties. Incubation of HepG2 (human hepatoma cells) and Caco-2 (human colonic cells) in the presence of carvacrol or thymol protect the H2O2-induced DNA strand breaks. Another study has confirmed that both carvacrol and thymol reduce H2O2-induced DNA damage in human cells cultured in vitro. Also antimutagenic property of carvacrol has been reported. In fact, all flavonoids are able to inhibit cellular LPO and attenuate other processes involving reactive oxygen species. Recently, it has been revealed that SKEO protects from malathion-induced oxidative stress in the hemopoietic system and interferes with malathion-induced stimulation of hepatic cells.

glycogenolysis and gluconeogenesis. Supporting this finding, there are several reports on the benefit of antioxidants in protecting male reproductive system from toxic effects of CP. It was found that ascorbic acid reduces reproductive toxicity of chronic low doses of CP. There is also evidence that Yukmijihwang-tang as a multi-herbal medicinal formula can improve reproductive toxicity of CP through reduction of oxidative stress. Two studies from the same researchers indicated that supplementation with lipoic acid as an antioxidant reduces CP-induced reproductive toxicity by the same mechanism.

Collectively, the present results support the idea that reproductive toxicity of CP is mediated through oxidative stress, and SKEO, as a strong and safe antioxidant, can protect the reproductive system from CP-induced damages that usually result in infertility. Fortunately, SKEO in its first clinical trial has been shown to improve blood lipid and oxidant–antioxidant status in diabetic patients with hyperlipidemia. Supplementation with SKEO in middle-aged men, who take CP, will elucidate the benefit of this herbal source antioxidant.

Acknowledgments

This study is the outcome of a joint study of the Veterinary and Pharmacy Schools and was partially supported by a grant from the Excellence Center of Toxicology of TUMS. This work was presented in EUROTOX 2008 Congress in Rhodes, Greece and the abstract was published in Toxicology Letters.

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Protection of cyclophosphamide-induced free-radical toxic stress

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Protection of cyclophosphamide-induced free-radical toxic stress
MA Rezvanfar, et al.


