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

MA Rezvanfar, RA Sadrkhanlou, A Ahmadi, H Shojaei-Sadee, MA Rezvanfar, A Mohammadirad, A Salehnia and M Abdollahi

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

Human & Experimental Toxicology (2008) **27:** 901–910
www.het.sagepub.com

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

MA Rezvanfar[1], RA Sadrkhanlou[1], A Ahmadi[1], H Shojaei-Sadee[1], MA Rezvanfar[2], A Mohammadirad[2], A Salehnia[2] and M Abdollahi[2]

[1]Laboratory of Embryology, Department of Embryology and Histology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; and [2]Laboratory of Toxicology, Faculty of Pharmacy, and Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran, Iran

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

Correspondence to: Prof. Mohammad Abdollahi, Laboratory of Toxicology, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran. Email: mohammad.abdollahi@utoronto.ca

10.1177/0960327108102046

that CP disrupts the redox balance of tissues resulting in oxidative stress.[10–12] It has been reported[13] that oxidative DNA damage is caused by hydroperoxide derivative of CP through generation of H_2O_2. Also, acrolein has been found to interfere with the tissue antioxidant defense system[14] and produces highly reactive oxygen free-radicals[15] that are mutagenic to mammalian cells.[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.[17] In the recent years, compositions of the essential oils of wild and cultivated *S. khuzestanica* have been well established.[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 were observed.[20] SKEO improved recovery from inflammatory bowel disease in animals by reduction of oxidative stress biomarkers.[21] SKEO was also shown to act against malathion-induced toxicity by improving oxidative stress, acetylcholinesterase activity, and mitochondrial glycogenolysis and gluconeogenesis in rats.[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 spermatozoids increased and the Sertoli cells were found hypertrophic.[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®), 2-thiobarbituric acid (TBA), Ham'sF10, NaHCO3, trypan blue, eosin-Y, Nigrosin, ethanol, hematoxylin, paraffin, toluidine blue, Carnoy's fixative (methanol/Acetic acid; 1/3), glutaraldehyde, phosphate buffered saline, acridine orange, aniline blue, 1,1,3,3,-tetra ethoxy-propane, trichloroacetic acid, n-butanol, hexadecyl trimethyl ammonium bromide, 2,4,6-tripyridyl-s-triazine (TPTZ), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide, acetic acid, and phosphate buffer from Merck Chemical Co. (Tehran) were used in this study.

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) dissolved in distilled water following pretreatment with olive oil by gavage, group 3 received an effective dose of SKEO at 225 mg/kg/day dissolved in olive oil by gavage, 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.[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 CO_2 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 antioxidant 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 malonedialdehyde 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.

TAP of plasma and testis was determined by measuring their ability to reduce Fe^{3+} to Fe^{2+}. The complex between Fe^{2+} and TPTZ gives a blue color with absorbency 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% CO_2 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 sperm [28] 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% $NaHCO_3$ 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[32] and spermiation index (SPI), which is the percentage of seminiferous tubules with normal spermiation 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.[33]

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 1 Effects of CP and SKEO on oxidative stress biomarkers in plasma and testes of male rats

	Control	*SKEO*	*CP*	*CP + SKEO*
Plasma LPO (μmol/mL)	2.07 ± 0.2	1.59 ± 0.01[a,b]	2.63 ± 0.28[a]	1.93 ± 0.08[b]
Testis LPO (μmol/mL)	2.04 ± 0.26	1.41 ± 0.01[a,b]	3.1 ± 0.22[a]	2.28 ± 0.15[b]
Plasma TAP (mmol/mL)	595.3 ± 24.6	809.9 ± 49.9[a,b]	471 ± 25.6[a]	675 ± 56.7[b]
Testis TAP (mmol/mL)	1508 ± 83.97	1830 ± 52.9[a,b]	1200.8 ± 10.36[a]	1493.6 ± 80[b]

SKEO, *Satureja khuzestanica* essential oil; CP, Cyclophosphamide; LPO, lipid peroxidation; TAP, total antioxidant power.
[a]Significantly different between control and other groups at $P < 0.05$.
[b]Significantly different between cyclophosphamide and other groups at $P < 0.05$.

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

	Control	*SKEO*	*CP*	*CP + SKEO*
Testis weight (g)	1.52 ± 0.01	1.57 ± 0.03[a,b]	1.41 ± 0.02[a]	1.49 ± 0.03[b]
Epididymal weight (g)	0.476 ± 0.02	0.522 ± 0.01[a,b]	0.398 ± 0.02[a]	0.468 ± 0.00[b]
Ventral prostate weight (g)	0.281 ± 0.00	0.297 ± 0.02[a,b]	0.217 ± 0.01[a]	0.269 ± 0.00[b]
Vesicule seminal weight (g)	0.542 ± 0.03	0.576 ± 0.03[a,b]	0.437 ± 0.01[a]	0.513 ± 0.00[b]
FSH (mlU/mL)	0.25 ± 0.001	0.24 ± 0.004	0.25 ± 0.008	0.25 ± 0.003
LH (mlU/mL)	0.22 ± 0.02	0.28 ± 0.04	0.25 ± 0.05	0.30 ± 0.06
Testosterone (ng/dl)	64.66 ± 9.39	151 ± 7.76[a,b]	21.6 ± 3.7[a]	106 ± 13.7[b]
Sperm count (10^6/mL)	171.86 ± 2.73	172.13 ± 2.19[b]	79.66 ± 4.08[a]	158.66 ± 5.78[a,b]
Motility (%)	79.59 ± 3.63	84.46 ± 0.86[a,b]	51.72 ± 1.72[a]	73.46 ± 1.85[a,b]
Dead sperms (%)	18.7 ± 7.13	11.2 ± 4.34[b]	47.9 ± 8.46[a]	25.2 ± 6.51[b]
Abnormal sperms (%)	7.9 ± 1.91	7.1 ± 1.59[b]	36.2 ± 6.16[a]	18.9 ± 1.79[a,b]
Positive acridine orange staining (%)	13.92 ± 3.04	8.07 ± 1.65[a,b]	41.76 ± 4.76[a]	17.61 ± 4.61[b]
Positive aniline blue staining (%)	1.13 ± 0.77	1.06 ± 0.43[b]	23.13 ± 1.44[a]	12.66 ± 0.47[b]
Number of impregnated females	6	6[b]	2[a]	5[b]
Total live litter	47	63[a,b]	3[a]	27[a,b]
Fecundity (%)	100	100[b]	40[a]	83.3[a,b]
Fertility (%)	100	100[b]	33.3[a]	66.6[a,b]

SKEO, *Satureja khuzestanica* essential oil; CP, Cyclophosphamide; FSH, ; LH, .
[a]Significantly different from control as $P < 0.05$.
[b]Significantly different from cyclophosphamide $P < 0.05$.

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-treated animals showed minimal histologic abnormalities and recovered from CP-induced lesions. Histopathological assessment of sections provided from concentrated sperms of cauda epididymides of CP-treated animals showed a severe decline in the lumen mature spermatozoa. 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.[34] Reduction in the weight of testes and accessory reproductive organs in CP-treated animals

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

	Control	*SKEO*	*CP*	*CP + SKEO*
TDI (%)	94.58 ± 0.95	94.75 ± 1.34[b]	59.41 ± 1.25[a]	83.46 ± 1.71[a,b]
SPI (%)	96.74 ± 0.79	97.91 ± 0.31[b]	42.16 ± 0.88[a]	87.08 ± 2.36[a,b]
Sloughing tubules (%)	1.53 ± 0.56	1.39 ± 0.27[b]	17.34 ± 0.97[a]	5.66 ± 0.47[a,b]
Mast cells number of testis (mm^2)	1.59 ± 0.13	1.58 ± 0.15[b]	2.83 ± 0.14[a]	2 ± 0.37[b]
Mast cells number of cauda epididymides (mm^2)	2.98 ± 0.09	2.81 ± 0.1[b]	4.22 ± 0.07[a]	3.13 ± 0.27[b]
Cauda epididymal sperm concentration	++	+++	+	++
Degenerating and scattering of Leydig cells	—	—	+++	+
Flattened nucleus and deformed Sertoli cells	—	—	++	+

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 $P < 0.05$.

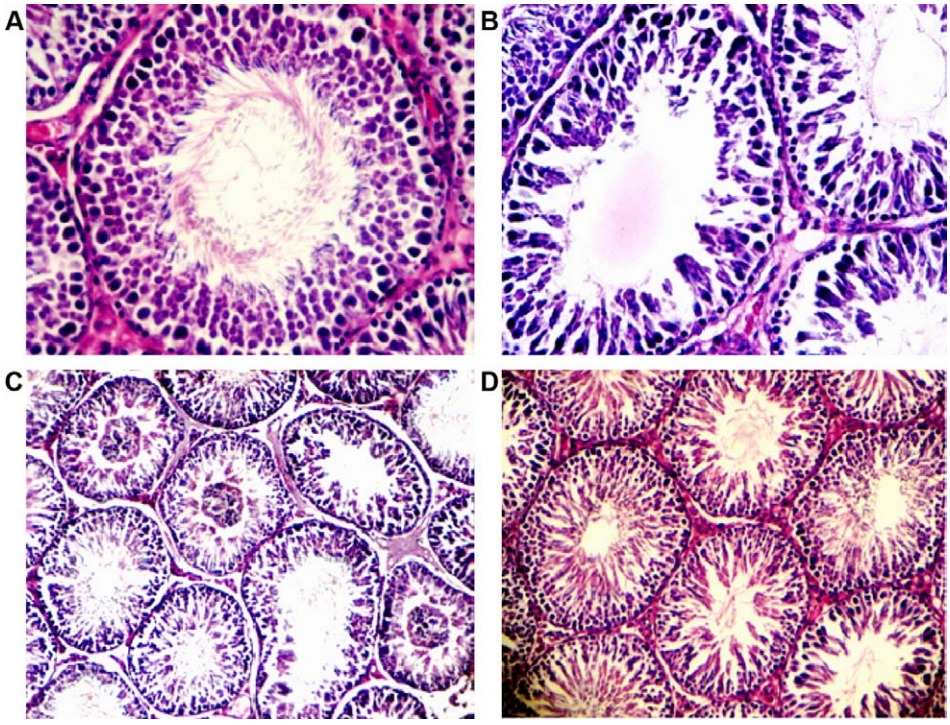

Figure 1 Photomicrograph of testis from treated rats of control (A) CP (B&C) and SKEO + CP (D). *Satureja khuzestanica* Essential Oil (SKEO); Cyclophosphamide (CP). The control group presented normal architecture with active spermatogenesis (A) ×400. The CP group showed disintegrated seminiferous tubules, impaired spermatogenesis, hypoplasia and dispersion of Leydig cells, interstitial edema, and increased interstitial space, failure of spermiation (B) ×400, increase in the number of sloughing tubules, and deformed spermatid cord (C) ×100. SKEO cotreated animals show nearly normal architecture (D) ×100.

reflect the reduced availability of androgens.[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.[36] It has been reported[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.[38] Various studies[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.[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.[41] Thus, they are very susceptible to free radical attack, which results in disturbed sperm motility,[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.[42] Activity of Na-K-ATPase is highly sensitive to toxic stress.[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.[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

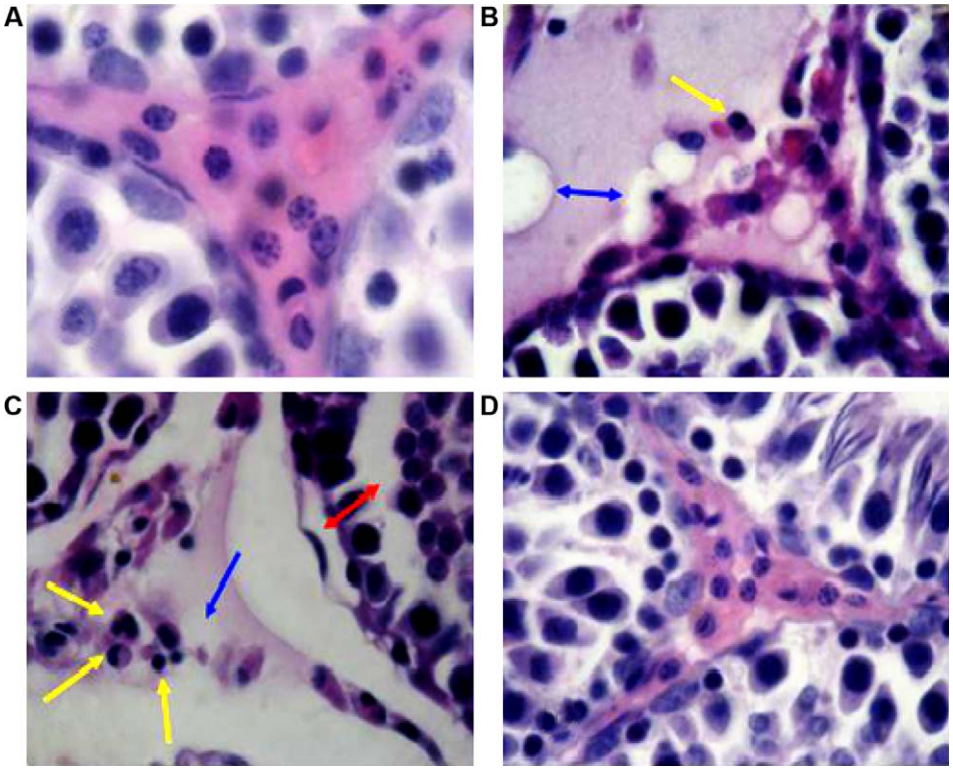

Figure 2 Interstitial histograms of testis from treated rats of control (A), CP (B and C), and SKEO + CP (D). *Satureja khuzestanica* Essential Oil (SKEO); Cyclophosphamide (CP). The control group presented interstitial tissue with normal architecture. Leydig cells are accumulated and have acidophilic cytoplasm with normal nucleus (A) ×1000. Scattered and degenerated Leydig cells in the testis interstitium, hypoplasia, and vacuolization of their cytoplasm along with picnosis of their nuclei and detachment of germ cells are evident in CP-treated sample (B & C) ×1000. SKEO cotreated animals showed minimal histologic abnormalities and the lesions from CP (D) ×1000.

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 fertility[49] and alter the growth and development of the next generation[50] 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 spermiation 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[24,52] 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[53] and spermatogenetic disorders, which is due to the augmentation of free radicals from degranulated mast cells after allergic and immunologic stimulus.[54] The dysfunction of the blood–testis barrier has also been attributed to an excess number of mast cells.[55] Mast cell blockers like ketotifen or tranilast have been used in patients with severe oligospermia resulting in improvement of sperm count and pregnancy rates.[56] 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.[20–22] Previous studies have shown concomitant antioxidant and reproduction stimulatory effects of SKEO in rats.[20] The present findings supported our previous reports on the positive effects of SKEO in improving fertility and reproduction by its antioxidant potential.[20,24] 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.[18,19] Carvacrol, as the main constituent of SKEO, has marked antioxidant properties.[57–59] Incubation of HepG2 (human hepatoma cells) and Caco-2 (human colonic cells) in the presence of carvacrol or thymol protected the H_2O_2-induced DNA strand breaks.[60] Another study has confirmed that both carvacrol and thymol reduce H_2O_2-induced DNA damage in human cells cultured *in vitro*.[61] Also antimutagenic property of carvacrol has been reported.[62] In fact, all flavonoids are able to inhibit cellular LPO and attenuate other processes involving reactive oxygen species.[63] Recently, it has been revealed that SKEO protects from malathion-induced oxidative stress in the hemopoeitic system and interferes with malathion-induced stimulation of hepatic cells glycogenolysis and gluconeogenesis.[22,23] 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[10] 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.[64] Two studies from the same researchers indicated that supplementation with lipoic acid as an antioxidant reduces CP-induced reproductive toxicity by the same mechanism.[65,66]

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.[67] 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.

References

1 Robaire, B, Hales, BF. Paternal exposure to chemicals before conception. *BMJ* 1993; **307**: 341–342.
2 Dollery, C. Cyclophosphamide. In: Dollery, C, Editor, Therapeutic Drugs. Edinburg: Churchill Livingstone; 1999. p. 349–353.
3 Ludeman, SM. The chemistry of the metabolites of cyclophosphamide. *Curr Pharm Des* 1999; **5**: 627–643.
4 Kern, JC, Kehrer, JP. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. *Chem Biol Interact* 2002; **139**: 79–95.
5 Anderson, D, Bishop, JB, Garner, RC, Ostrosky-Wegman, P, Selby, PB. Cyclophosphamide: review of its mutagenicity for an assessment of potential germ cell risks. *Mutat Res* 1995; **330**: 115–181.
6 Howell, S, Shalet, S. Gonadal damage from chemotherapy and radiotherapy. *Endocrinol Metab Clin North Am* 1998; **27**: 927–943.
7 Qureshi, MS, Pennington, JH, Goldsmith, HJ, Cox, PE. Cyclophosphamide therapy and sterility. *Lancet* 1972; **2**: 1290–1291.

8 Meistrich, ML, Parchuri, N, Wilson, G, Kurdoglu, B, Kangasniemi, M. Hormonal protection from cyclophosphamide-induced inactivation of rat stem spermatogonia. *J Androl* 1995; **16**: 334–341.
9 Kaur, F, Sangha, GK, Bilaspuri, GS. Cyclophosphamide-induced structural and biochemical changes in testis and epididymidis of rats. *Indian J Exp Biol* 1997; **35**: 771–775.
10 Das, UB, Mallick, M, Debnath, JM, Ghosh, D. Protective effect of ascorbic acid on cyclophosphamide- induced testicular gametogenic and androgenic disorders in male rats. *Asian J Androl* 2002; **4**: 201–207.
11 Ghosh, D, Das, UB, Ghosh, S, Mallick, M, Debnath, J. Testicular gametogenic and steroidogenic activities in cyclophosphamide treated rat: a correlative study with testicular oxidative stress. *Drug Chem Toxicol* 2002; **25**: 281–292.
12 Manda, K, Bhatia, AL. Prophylactic action of melatonin against cyclophosphamide-induced oxidative stress in mice. *Cell Biol Toxicol* 2003; **19**: 367–372.
13 Murata, M, Suzuki, T, Midorikawa, K, Oikawa, S, Kawanishi, S. Oxidative DNA damage induced by a hydroperoxide derivative of cyclophosphamide. *Free Radic Biol Med* 2004; **37**: 793–802.
14 Arumugam, N, Sivakumar, V, Thanislass, J, Devaraj, H. Effects of acrolein on rat liver antioxidant defense system. *Indian J Exp Biol* 1997; **35**: 1373–1374.
15 Mythili, Y, Sudharsan, PT, Selvakumar, E, Varalakshmi, P. Protective effect of DL-alpha-lipoic acid on cyclophosphamide induced oxidative cardiac injury. *Chem Biol Interact* 2004; **30**: 13–19.
16 Kawanishi, M, Matsuda, T, Nakayama, A, Takebe, H, Matsui, S, Yagi, T. Molecular analysis of mutations induced by acrolein in human fibroblast cells using supF shuttle vector plasmids. *Mutat Res* 1998; **417**: 65–73.
17 Jamzad, Z. A new species of the genus Satureja (Labiatae) from Iran. *Iran J Bot* 1994; **6**: 215–218.
18 Farsam, H, Amanlou, M, Radpour, MR, Salehinia, AN, Shafiee, A. Composition of the essential oils of wild and cultivated Satureja khuzistanica Jamzad from Iran. *Flav Frag J* 2004; **19**: 308–310.
19 Sefidkon, F, Ahmadi, S. Essential oil of Satureja khuzistanica Jamzad. *J Essent Oil Res* 2000; **12**: 427–428.
20 Abdollahi, M, Salehnia, A, Mortazavi, SH, Ebrahimi, M, Shafiee, A, Fouladian, F, *et al.* Antioxidant, antidiabetic, antihyperlipidemic,reproduction stimulatory properties and safety of essential oil of Satureja Khuzestanica in rat in vivo: a toxicopharmacological study. *Med Sci Monit* 2003; **9**: 331–335.
21 Ghazanfari, G, Minaie, B, Yasa, N, Nakhai, L, Mohammadirad, A, Nikfar, S, *et al.* Biochemical and histopathological evidences for beneficial effects of Satureja Khuzestanica Jamzad essential oil on the mouse model of inflammatory bowel diseases. *Toxicol Mech Methods* 2006; **16**: 365–372.
22 Basiri, S, Esmaily, H, Vosough-Ghanbari, S, Mohammadirad, A, Yasa, N, Abdollahi, M. Improvement by Satureja khuzestanica essential oil of malathion-induced red blood cells acetylcholinesterase inhibition and altered hepatic mitochondrial glycogen phosphorylase and phosphoenolpyruvate carboxykinase activities. *Pestic Biochem Physiol* 2007; **89**: 124–129.
23 Abdollahi, M, Ranjbar, A, Shadnia, S, Nikfar, S, Rezaie, A. Pesticides and oxidative stress: a review. *Med Sci Monit* 2004; **10**: 141–147.
24 Haeri, S, Minaie, B, Amin, G, Nikfar, S, Khorasani, R, Esmaily, H, *et al.* Effect of Satureja khuzestanica essential oil on male rat fertility. *Fitoterapia* 2006; **77**: 495–499.
25 Codrington, AM, Hales, BF, Robaire, B. Spermiogenic germ cell phase-specific DNA damage following cyclophosphamide exposure. *J Androl* 2004; **25**: 354–362.
26 Codrington, AM, Hales, BF, Robaire, B. Exposure of male rats to cyclophosphamide alters the chromatin structure and basic proteome in spermatozoa. *Hum Reprod* 2007; **22**: 1431–1442.
27 Afshari, M, Larijani, B, Rezaie, A, Mojtahedi, A, Zamani, MJ, Astanehi-Asghari, F, *et al.* Ineffectiveness of allopurinol in reduction of oxidative stress in diabetic patients; a randomized, double-blind placebo-controlled clinical trial. *Biomed Pharmacother* 2004; **58**: 546–550.
28 World Health Organization (WHO). WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999.
29 Wyrobek, AJ, Gordon, LA, Burkhart, JG, Francis, MW, Kapp Jr, RW, Letz, G, *et al.* An evaluation of the mouse sperm morphology test and other sperm tests in nonhuman mammals. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 1983; **115**: 1–72.
30 Erenpreiss, J, Bars, J, Lipatnikova, V, Erenpreisa, J, Zalkalns, J. Comprative study of cytochemical tests for sperm chromatin integrity. *J Androl* 2001; **22**: 45–53.
31 Hammadeh, M, Zeginiadov, T, Rosenbaum, P. Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. *Arch Androl* 2001; **46**: 99–104.
32 Porter, KL, Shetty, G, Meistrich, ML. Testicular edema is associated with spermatogonial arrest in irradiated rats. *Endocrinology* 2006; **147**: 1297–1305.
33 Gaytan, F, Aceitero, J, Lucena, C, Aguilar, E, Pinilla, L, Garnelo, P, *et al.* Simultaneous proliferation and differentiation of mast cells and Leydig cells in the rat testis. Are common regulatory factors involved. *J Androl* 1992; **13**: 387–397.
34 Katoh, C, Kitajima, S, Saga, Y, Kanno, J, Horii, I, Inoue, T. Assessment of quantitative dual-parameter flow cytometric analysis for the evaluation of testicular toxicity using cyclophosphamide- and ethinylestradiol-treated rats. *J Toxicol Sci* 2002; **27**: 87–96.
35 Patil, S, Patil, S, Londonkar, R, Patil, SB. Effect of pethidine on spermatogenesis in albino rats. *Indian J Pharmacol* 1998; **30**: 249–253.
36 Debnath, D, Mandal, TK. Study of quinalphos (an environmental oestrogenic insecticide) formulation (Ekalux 25 E.C.)-induced damage of the testicular tissues and antioxidant defence systems in Sprague-Dawley albino rats. *J Appl Toxicol* 2000; **20**: 197–204.
37 Cao, L, Leers-Sucheta, S, Azhar, S. Aging alters the functional expression of enzymatic and non-enzymatic anti-oxidant defense systems in testicular rat Leydig cells. *J Steroid Biochem Mol Biol* 2004; **88**: 61–67.

38 Sudharsan, PT, Mythili, Y, Selvakumar, E, Varalakshmi, P. Cardioprotective effect of the pentacyclic triterpene, lupeol and its ester on cyclophosphamide-induced oxidative stress. *Hum Exp Toxicol* 2005; **24**: 313–318.
39 Agarwal, A, Saleh, RA. Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* 2002; **29**: 817–827.
40 Kao, SH, Chao, HT, Chen, HW, Hwang, TI, Liao, TL, Wei, YH. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril* 2008; **89**: 1183–1190.
41 Vernet, P, Aitken, RJ, Drevet, JR. Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* 2004; **216**: 31–39.
42 de Lamirande, E, Gagnon, C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl* 1992; **13**: 379–386.
43 Kurella, EG, Tyulina, OV, Boldyrev, AA. Oxidative resistance of Na/K-ATPase. *Cell Mol Neurobiol* 1999; **19**: 133–140.
44 Said, TM, Agarwal, A, Sharma, RK, Thomas Jr, AJ, Sikka, SC. Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. *Fertil Steril* 2005; **83**: 95–103.
45 Agarwal, A, Said, TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 2003; **9**: 331–345.
46 Moustafa, MH, Sharma, RK, Thornton, J, Mascha, E, Abdel-Hafez, MA, Thomas Jr, AJ, *et al.* Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004; **19**: 129–138.
47 Aguilar-Mahecha, A, Hales, BF, Robaire, B. Chronic cyclophosphamide treatment alters the expression of stress response genes in rat male germ cells. *Biol Reprod* 2002; **66**: 1024–1032.
48 Meseguer, M, Martínez-Conejero, JA, O'Connor, JE, Pellicer, A, Remohí, J, Garrido, N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. *Fertil Steril* 2008; **89**: 1191–1199.
49 Higuchi, H, Nakaoka, M, Katsuda, Y, Kawamura, S, Kato, T, Matsuo, M. Collaborative assessment of optimal administration period and parameters to detect effects on male fertility in the rat: effects of cyclophosphamide on the male reproductive system. *J Toxicol Sci* 1995; **20**: 239–249.
50 Robaire, B, Hales, BF. Mechanisms of action of cyclophosphamide as a male-mediated developmental toxicant. *Adv Exp Med Biol* 2003; **518**: 169–180.
51 Mruk, DD, Cheng, CY. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr Rev* 2004; **25**: 747–806.
52 Sofikitis, N, Giotitsas, N, Tsounapi, P, Baltogiannis, D, Giannakis, D, Pardalidis, N. Hormonal regulation of spermatogenesis and spermiogenesis. *J Steroid Biochem Mol Biol* 2008; **109**: 323–330.
53 Roaiah, MM, Khatab, H, Mostafa, T. Mast cells in testicular biopsies of azoospermic men. *Andrologia* 2007; **39**: 185–189.
54 Cincik, M, Sezen, SC. The mast cells in semen: their effects on sperm motility. *Arch Androl* 2003; **49**: 307–311.
55 Frungieri, B, Weidinger, S, Kohn, M. Proliferative action of mast cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPAR: possiblerevance to human fibrotic disorders. *Proc Natl Acad Sci U S A* 2002; **12**: 15072–15077.
56 Yamamoto, M, Hibi, H, Miyake, K. New treatment of idiopathic severe oligozoospermia with mast cell blocker: results of a single-blind study. *Fertil Steril* 1995; **64**: 1221–1223.
57 Burits, M, Bucar, F. Antioxidant activity of Nigella sativa essential oil. *Phytother Res* 2000; **14**: 323–328.
58 Lambert, RJ, Skandamis, PN, Coote, PJ, Nychas, GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* 2001; **91**: 453–462.
59 Vardar-Unlü, G, Candan, F, Sökmen, A, Daferera, D, Polissiou, M, Sökmen, M, *et al.* Antimicrobial and antioxidant activity of the essential oil and methanol extracts of Thymus pectinatus Fisch. et Mey. Var. pectinatus (Lamiaceae). *J Agric Food Chem* 2003; **51**: 63–67.
60 Slamenová, D, Horváthová, E, Sramková, M, Marsálková, L. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured in vitro. *Neoplasma* 2007; **54**: 108–112.
61 Horvathova, E, Turcaniova, V, Slamenova, D. Comparative study of DNA-damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562. *Neoplasma* 2007; **54**: 478–483.
62 Mezzoug, N, Elhadri, A, Dallouh, A, Amkiss, S, Skali, NS, Abrini, J, *et al.* Investigation of the mutagenic and antimutagenic effects of Origanum compactum essential oil and some of its constituents. *Mutat Res* 2007; **629**: 100–110.
63 Waltner-Law, ME, Wang, XL, Law, BK, Hall, RK, Nawano, M, Granner, DK. Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. *J Biol Chem* 2002; **277**: 34933–34940.
64 Oh, MS, Chang, MS, Park, W. Kim do, R, Bae, H, Huh, Y, et al. Yukmijihwang-tang protects against cyclophosphamide-induced reproductive toxicity. *Reprod Toxicol* 2007; **24**: 365–370.
65 Selvakumar, E, Prahalathan, C, Sudharsan, PT, Varalakshmi, P. Chemoprotective effect of lipoic acid against cyclophosphamide-induced changes in the rat sperm. *Toxicology* 2006; **217**: 71–78.
66 Selvakumar, E, Prahalathan, C, Sudharsan, PT, Varalakshmi, P. Protective effect of lipoic acid on cyclophosphamide-induced testicular toxicity. *Clin Chim Acta* 2006; **367**: 114–119.
67 Vosough-Ghanbari, S, Rahimi, R, Kharabaf, S, Zeinali, S, Mohammadirad, A, Amini, S, *et al.* Effects of Satureja khuzestanica on serum glucose, lipids and markers of oxidative stress in patients with type 2 diabetes mellitus: a double-blind randomized controlled trial. Evid Based Complement Alternat Med 2008 Feb 27 [Epub ahead of print]. doi:10.1093/ecam/nen018.