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The Delayed Testicular Morphologic Effects of Doxorubicin and the Rejuvinating Role of Grapefruit Seed Extract

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Abstract: Doxorubicin (DOX), one of the anthracycline antibiotic drugs isolated from the soil fungus *Streptomyces peucetius caesius*, which has been widely used to treat cancer effectively. It has also been known to induce reproductive abnormalities in males. The implication of natural phenolic compounds in the prevention of many pathologic diseases has been reported. Herein, the ability of polyphenolic-rich Grapefruit Seed Extracts (GSE), to protect rat testis against DOX-induced histomorphometric impairment was investigated. Four groups of Wistar rats were used; The GSE- alone group received intraperitoneal (i.p.) Normal Saline (NS) 2.5 mg kg^{-1} b.wt. followed by orally GSE 10 mg kg^{-1} b.wt. daily for 16 weeks. The DOX-alone group were given i.p., DOX 20 mg kg^{-1} b.wt. as a single dose. The GSE plus DOX-group were similarly given DOX, but also had oral GSE 10 mg kg^{-1} b.wt. post-treatment for 16 weeks. Another group of rats were each given orally 2.5 mL kg^{-1} b.wt. peanut oil (vehicle) daily for 16 weeks, after 2.5 mL kg^{-1} b.wt. normal saline was given as a single dose i.p., to serve as the control. The animals were sacrificed 16 weeks after DOX or NS injections. The testicular toxicity induced by DOX was assessed by histologic and stereologic evaluation of the testis. Present results demonstrated that post-treatment with GSE was capable of reversing the reduction of body and testicular weights as well as the testicular histomorphometric evidences of high dose and delayed DOX toxicity in the animals. The GSE was therefore shown to exert testicular cytorejuvinative effects on rats challenged with DOX.

Key words: Doxorubicin, testis, histology, rat

INTRODUCTION

Doxorubicin (DOX) also known as hydroxydaunorubicin is a drug used in cancer chemotherapy. It is one of the earliest compounds derived from an antibiotic group of chemotherapy known as anthracycline. It is highly effective in many human tumours and is currently the first line anti-cancer drug in many chemo-responsive tumours such as ovarian cancers, breast cancers and lymphomas (Atessahin *et al*., 2006). The clinical use of DOX can be viewed as a sort of double-edged sword. On the one hand, DOX plays an undisputed key role in the treatment of many neoplastic diseases; on the other hand, chronic administration of DOX induces organ toxicity particularly testicular injury (Rubin *et al*., 2008).

The biochemical mechanism by which DOX causes cytotoxicity is currently unclear. Several mechanisms have been postulated to account for the effects of DOX, both in terms of anticancer potential and adverse effects. It is widely accepted that DOX-induced organopathy resides for the most part on oxidative stress arising from excess production of free radicals (Quiles *et al*., 2002; Chularojmontri *et al*., 2005; Prahalathan *et al*., 2005). The DOX is known to generate free radicals either by the enzymatic pathway of redox cycling between a semiquinone form and a quinone form or by the non-enzymatic pathway of forming a DOX-Fe^{3+} complex (Davies and Doroshow, 1986; Armstrong and Lipsy, 1993). In both pathways, molecular oxygen is reduced to superoxide anion (O_2^-), which is converted to other forms of reactive oxygen species such as hydrogen peroxide (H_2O_2) and the more toxic hydroxyl radical (OH•). These free radicals could then cause membrane and macromolecule damage by three basic mechanisms; lipid peroxidation, deoxyribonucleic acid (DNA) fragmentation and protein oxidation (Pacher, 2007).

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Numerous free radical scavengers, such as probucol, amifostine and dexrazoxane, have been shown to protect the organs against DOX-induced cytotoxicity (Siveski-Iliskovic *et al*., 1995; Samelis *et al*., 1998; Nazeyrollas *et al*., 1999). Indeed, a wealth of studies has emerged incorporating a plethora of non-enzymatic antioxidants with doxorubicin in an attempt to prevent or attenuate its testicular toxicity (Sikka, 1996; Quiles *et al*., 2002; Prahalathan *et al*., 2005).

Unfortunately, most of these scavengers have pronounced clinical disadvantages. Probucol, a lipid-lowering antioxidant, confers significant protection against DOX-induced cytotoxicity (Siveski-Iliskovic *et al*., 1995). However, concerns about its high-density lipoprotein-lowering property discourage its application in cancer patients. The cytoprotective drug amifostine possesses less than optimal potency (Herman *et al*., 1994). Similarly, dexrazoxane, an iron chelator interferes with the antitumor activity of anthracycline antibiotics (Sehested *et al*., 1993) and also potentiates the hematotoxicity of DOX (Koning *et al*., 1991).

Grapefruit Seed Extract (GSE) from grapefruit (*Citrus paradisi* Macfad) contains high levels of vitamin C, E and bioflavonoids (Sachs, 1997; Halliwell, 2008). These compounds are powerful antioxidants individually and collectively. Bioflavonoids form a class of benzo-gamma-pyrone derivatives that have high pharmacological potency. A great interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activity of these polyphenolic compounds (Diplock *et al*., 1998). This is due primarily to their radical-scavenging and iron-chelating properties (Cook and Samman, 1996). They are found naturally in the leaves, bark, roots, flowers and seeds of plants (Cook and Samman, 1996).

One bioflavonoid with especially potent antioxidant capabilities is naringenin (Lee *et al*., 2002). It is the aglycone of the natural glycoside naringin present abundantly in grapefruit and which constitute its bitter principle. The antioxidant activities of bioflavonoids complement, extend and sometimes synergize the antioxidant activities of vitamin C, E and carotenoids, making them an important nutritional component in the body's defenses against free radical damage (Ho, 1994). Indeed GSE with its pharmacological contents taken together has since been touted as one of the most powerful natural antioxidant available (Sachs, 1997).

Further, unlike most other antioxidants that have been linked with antichemotherapeutic effects, naringenin (one of the active components of GSE) has been reported to potentiate the antitumour effect of DOX (Hossamm *et al*., 2005). In spite of this inviting scenario, to the best of our knowledge, there has not been any documented report on the use of GSE to protect the testis and indeed any other organ in the body from DOX-induced cytotoxicity.

The realization that DOX-induced testicular injury is principally mediated through the oxidative pathway and that GSE possesses potential antioxidative effect by scavenging free radicals, has prompted us to address in the current study the possible rejuvinative effects of GSE on the testicular histomorphometric evidences of delayed and high dose DOX-induced toxicity using simple but efficient stereological tools.

MATERIALS AND METHODS

Chemicals: Doxorubicin hydrochloride (®Adricin) Korea United Pharm. Inc., Chungnam, Korea was obtained from Juli Pharmacy, Ikeja, Lagos State, Nigeria in the month of May, 2008.

Plant material and its extraction: In the first week of May, 2008, fresh parts of grapefruit (*Citrus paradisi)* tree were collected from a cultivated farmland within the deciduous forest of Odorasanyi District, Ijebu-Igbo, Ogun state, Nigeria. The plant material was collected at this site because Adeneye (2008) had earlier collected plant material at same site which was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen with herbarium number FHI 107460 was deposited. Plant taxonomy was done by Mr. T. K. Odewo, Chief Superintendent Officer, Taxonomy Section, FRIN, Ibadan, Oyo state, Nigeria. Plant authentication was done by Dr. A.B. Kadiri, the Herbarium, Botany and Microbiology Department, the University of Lagos, Akoka, Lagos State, Nigeria.

Twenty eight grapefruits were cut into pieces and the seeds were separated out. These were thoroughly but gently rinsed in distilled water. The seeds were completely dried at room temperature for three weeks. The air dried seeds weighing 140 g were reduced to a powdered substance by grinding. The sample was exhaustively extracted with 99.8% ethanol (BDH scientific supplies Ltd., Lagos, Nigeria) by means of a Soxhlet apparatus and the extract was evaporated *in vacuo*. The residue was processed to give 10 g (7.5% yield) of dark yellowish solid crude mass, which was stored at 4°C for the study. Fresh solution of the extract was prepared in peanut oil as vehicle when required. The concentration of the extract in peanut oil was 5 mg mL^{-1}.

Animals and interventions: Experimental procedures involving the animals and their care were conducted in

conformity with International, National and institutional guidelines for the care of Laboratory Animals in Biomedical Research and use of Laboratory Animals in Biomedical Research as promulgated by Canadian Council of Animal Care (1985). Further, the animal experimental models used conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and use of Animals (American Physiological Society, 2002).

The rats were procured from a breeding stock maintained in the Animal House of Lagos State University College of Medicine (LASUCOM). The animals were housed in well ventilated wire-wooden cages in the Animal Facility of the Department of Anatomy, LASUCOM, Ikeja, Lagos. An approval was sought and obtained from the Departmental ad-hoc Ethical Committee. The rats were maintained under standard natural photoperiodic condition of 12 h of light alternating with 12 h of darkness (i.e., L:D; 12:12) with room temperature of between 25 to 26°C and humidity of 65±5%. They were allowed unrestricted access to water and rat chow (Feedwell Livestock Feeds Ltd., Ikorodu, Lagos, Nigeria). They were allowed to acclimatize for 28 days before the commencement of the experiments. The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiments and twice within a week throughout the duration of the experiment, using an electronic analytical and precision balance (BA210S, d = 0.0001 g) (Satorius GA, Goettingen, Germany).

Forty male adult (11 to 13 weeks old) Wistar rats weighing 185-210 g were used for this research work. The rats were randomly divided into 4 groups of 10 rats each such that the average weight difference between and within groups did not exceed ±20% of the average weight of the sample population. First group, received 2.5 mL kg^{-1} b.wt. normal saline as a single dose i.p. Then 24 h after, they were given the vehicle 2.5 mL kg^{-1} b. wt. peanut oil daily by gastric gavage for 16 weeks. These animals served as control. Ten animals were each given a single dose normal saline 2.5 mL kg^{-1} b.wt., i.p. Then 24 h after they were each given reference dose (Heggers *et al*., 2002) 10 mg kg^{-1} b.wt. 99.8% ethanol extract of crude GSE by gastric gavage daily for 16 weeks. The third group of rats was each given 2.5 mL kg^{-1} b.wt. peanut oil by gastric gavage daily for 16 weeks, after they were initially each given 20 mg kg^{-1} b.wt. DOX intraperitoneally (i.p.) as a single dose. This dosage being well documented to cause testicular damage in rats (Howell and Shalet, 2001; Atessahin *et al*., 2006). Another group of rats received each 10 mg kg^{-1} b.wt. 99.8% ethanol GSE per kg body weight by gastric gavage daily for 16 weeks, after they were initially each given 10 mg kg^{-1} b.wt. DOX i.p., as a single dose. All the animals were sacrificed 16 weeks after DOX or NS injections.

Animal sacrifice and sample collection: The rats were at the time of sacrifice first weighed and then anaesthesized by placing them in a closed jar containing cotton wool sucked with chloroform anaesthesia. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Then the testes were excised and trimmed of all fat. The testes weights of each animal were evaluated .The testes were weighed with an electronic analytical and precision balance (BA 210S, d = 0.0001- Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation.

The testes were fixed in 10% formol-saline and histological slides prepared. However, prior to embedding, it was ensured that the sections were orientated perpendicular to their long axis and designated as vertical sections.

Determination of morphometric parameters: For each testis, five vertical sections from the polar and the equatorial regions were sampled (Qin and Lung, 2002) and an unbiased numerical estimation of the following morphometric parameters was determined using a systematic random scheme (Gundersen and Jenson, 1987):

- **Diameter (D) of seminiferous tubules:** The diameter of seminiferous tubules with profiles that were round or nearly round were measured for each animal and a mean, D, was determined by taking the average of two diameters, D_1 and D_2 (Perpendicular to one another). D_1 and D_2 were taken only when $D_1/D_2 = 0.85$
- **Cross-sectional area (A_C) of the seminiferous tubules:** The cross-sectional areas of the seminiferous tubules were determined from the formula $A_C = \pi D^2/4$, (where, π isequivalent to 3.142 and D the mean diameter of the seminiferous tubules)
- **Number of profiles of seminiferous tubules in a unit area of testis (N_A):** The Number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame proposed by Gundersen (1977). Using this frame, in addition to counting profiles completely inside the frame we counted all profiles with any part inside the frame provided they do not touch or intersect the forbidden line (full-drawn line) or exclusion edges or their extension

- **Numerical Density (N_V) of seminiferous tubules:** This is the number of profiles per unit volume and was determined by using the modified Floderus equation (Gilliland *et al.*, 2001):

$$N_V = N_A / (D + T)$$

where, N_A is the number of profiles per unit area, D is the diameter and T the average thickness of the section.

The evaluation of the diameter was done with calibrated eyepiece and stage grids mounted on a light research microscope. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto whom a graph sheet was superimposed and on which slides were projected from a research light microscope.

Statistical analysis: All data were expressed as Mean±SD of number of experiments (n = 10). The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA) as done by Snedecor and Cochran (1980). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). A value of $p<0.05$ was considered to indicate a significant difference between groups (Duncan, 1957).

RESULTS AND DISCUSSION

Body weight changes: Figure 1 shows that rats in control and GSE groups significantly increased in weight when compared to their initial mean live weight. Both DOX- administered groups lost weights when compared with their initial weights. However, the weight loss by the DOX-administered alone rats was higher than the losses by the group received GSE post-treatment after DOX challenge.

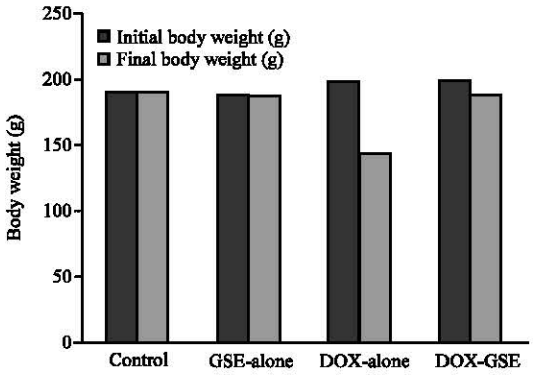

Fig. 1: Initial and final body weights of rats

Weights and volume of testes mean: The testicular weights, testis weight/body weight ratio and volumes of the DOX-alone rats were the least, being significantly lower compared to the mean testicular weights, testis weight/body weight ratio and volumes of the DOX rats that in addition had GSE, which in turn were also lower but not significantly lower than those of the control and GSE-alone rats (Fig. 2).

Testes histo-morphometry: The cross-sections of the seminiferous tubules of control rats were moderately circular or oval in outline with normal seminiferous epithelium and numerous spermatozoa within their lumen (Fig. 3). Rats that were given DOX alone showed degenerative changes in their seminiferous tubules.

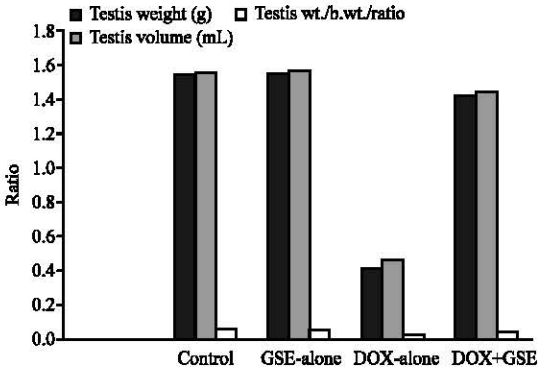

Fig. 2: Testis weights, testis volume and testis b.wt. ratio of rats

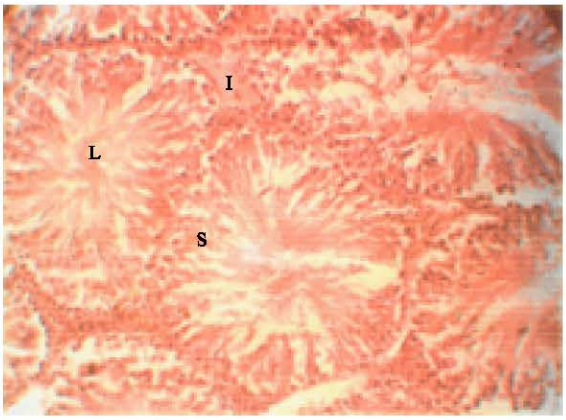

Fig. 3: Cross-section of testis of rat treated with normal saline (2.5 mg kg^{-1}) i.p., before peanut oil 2.5 mg kg^{-1} daily, orally for 16 weeks. Sacrificed after 16 weeks. (CONTROLS). Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

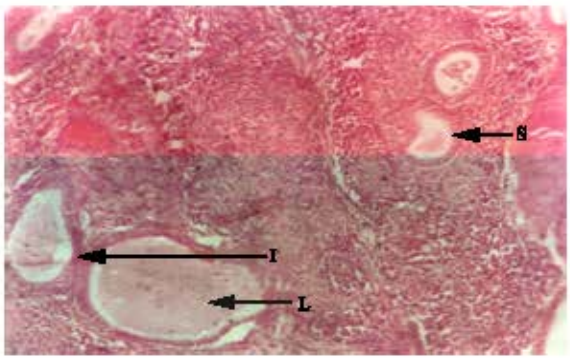

Fig. 4: Cross-section of testis of rat treated with Doxorubicin (20 mg kg^{-1}) i.p. and then sacrificed at the end of the 16th week. Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

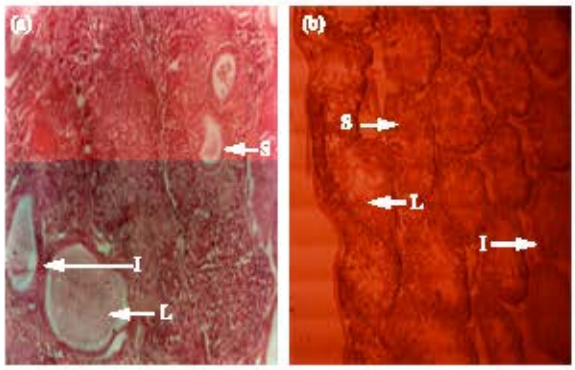

Fig. 5: Micrograph of a cross-section of testis of rat treated with peanut oil (2.5 mL kg^{-1}, gastric gavage) for 16 weeks, after Doxorubicin (20 mg kg^{-1}, i.p.) and then sacrificed at the end of week 16 (a) compared with; Micrograph of a cross-section of testis of rat treated with grapefruit seed extract (10 mg kg^{-1}, gastric gavage for 16 weeks, after doxorubicin (10 mg kg^{-1}, i.p.) and then sacrificed at the end of week 16 (b). Figures showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stain: haematoxylin and eosin; Magnification: x400

The animals demonstrated marked testicular atrophy. The seminiferous tubules having been replaced mainly by interstitial cells (Fig. 4). The rats that had GSE post-treatment after DOX challenge showed a remarkable preservation of their seminiferous epithelium post DOX treatment. The lesions of seminiferous epithelial cells were therefore only trifling in rats that had GSE post-treatment when compared with those animals that did not have GSE post-treatment (Fig. 5a, b).

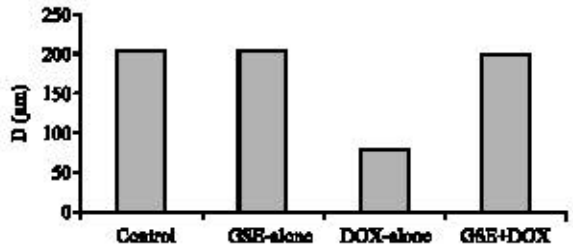

Fig. 6: The diameter (D) of seminiferous tubules of rats

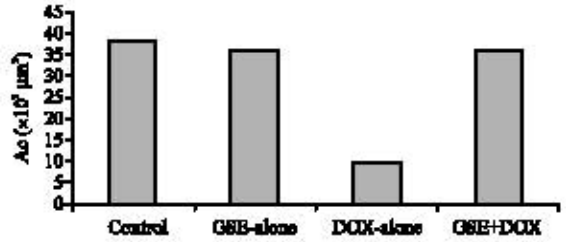

Fig. 7: The cross-sectional area (Ac) of the seminiferous tubules of rats

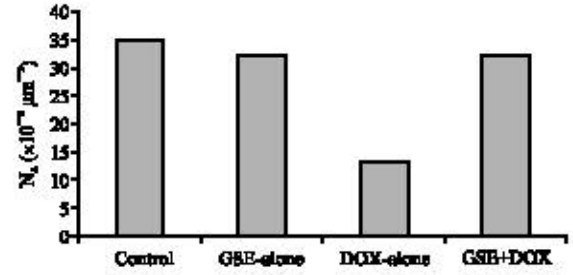

Fig. 8: The No. of seminiferous tubular profiles per unit area (N_A) in rats

There was a statistically significant reduction in the tubular diameter, the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the animals that had DOX alone. However, the Wistar rats that were post-treated with GSE had only a non-significant reduction in the tubular diameter, the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules when compared to the controls (Fig. 6-9).

Doxorubicin, one of anthracycline antibiotic drugs isolated from the soil fungus Streptomyces peucetius caesius, has been widely used to treat cancer effectively (Atessahin *et al*., 2006). Nevertheless, the clinical utilization of DOX is greatly limited due to its adverse toxic side effects mainly in cardiac and testicular tissues (Priestman, 2008; Saalu *et al*., 2009a, b). The molecular

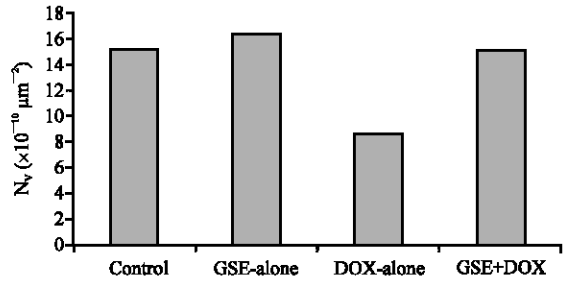

Fig. 9: The mean numerical density of seminiferous tubules (N_V) of rats

mechanism involved in biochemical and molecular pathophysiology to protect against doxorubicin-induced testicular insult is still under investigation.

The results from this study indicate that administration of DOX in a dose of 20 mg kg^{-1} b.wt. i.p., decreased the absolute testicular weights, testicular weight/body weight ratio and testicular volumes of rats. This agrees with the findings by many investigators (Kato *et al*., 2001; Endo *et al*., 2003; Atessahin *et al*., 2006). Prahalathan *et al*. (2005) on the other hand, did not report any significant testicular weight changes with DOX treatment. This could be due to the shorter post exposure time before sacrifice model established in that study. The alterations in these gross anatomical parameters could be attributed to severe parenchyma atrophy in the seminiferous tubules following DOX challenge. The group of rats that were given GSE after DOX however had near normal testis weights, testis weight/body weight ratio and testis volumes.

A derangement in the seminiferous epithelium of the testis was observed in group of rats that received DOX alone. This agrees with the findings by many investigators (Kato *et al*., 2001; Endo *et al*., 2003; Atessahin *et al*., 2006), who provide well documented evidences of testicular morphologic and morphometric impairment following DOX challenge in animal models. As was the case with the weights and for probably similar reasons, post-treatment with GSE showed a remarkable improvement in the histomorphometric parameters.

The occurrence of sterility in testicular cancer and Hodgkin`s lymphoma after treatment with anthracycline antibiotics is well documented (Suominen *et al*., 2003; Endo *et al*., 2003; Kalender *et al*., 2005). Howell and Shalet (2001) showed that the occurrence of male infertility following DOX chemotherapy is due to alterations in the sperm parameters. Spermatogenic cells constitute one of the body tissues that are susceptible to DOX-induced oxidative stress. Anthracyclines including DOX exert their antitumour effects as well as other organ toxicity by intracellular generation of free radicals and reactive oxygen species along with intercalation with DNA and subsequent inhibition of topoisomerase (Mornparler *et al*., 1976; Hrdina *et al*., 2000). This increase oxidative stress damages the sperm membranes, proteins and DNA (Kirsi and Timo, 2001; Kalender *et al*., 2005).

Studies by Sikka (1996), Quiles *et al*. (2002) and Suominen *et al*. (2003) have shown that DOX therapy results in direct oxidative injury to DNA. The biochemical mechanism by which DOX causes cytotoxicity is currently unclear. Several mechanisms have been postulated to account for the effects of DOX, both in terms of anticancer potential and adverse effects. It is widely accepted that DOX-induced organopathy resides for the most part on oxidative stress and the production of free radicals (Quiles *et al*., 2002; Chularojmontri *et al*., 2005; Prahalathan *et al*., 2005).

Post-treatment with GSE helped to overcome the oxidative stress. GSE, a potent antioxidant (Sachs, 1997) could have attenuated the DOX testicular derangement through a reduction of free radicals dependent lipid peroxidation.

The present study has provided an addition to the body of evidence that doxorubicin chemotherapy induces histomorphometric impairments of the testis of Wistar rats even 16 weeks after a single dosage. It has also been demonstrated in this report that post-treatment with grapefruit seed extract containing powerful antioxidant vitamins and citrus bioflavonoids, exerted a potent testiculorejuvinative activity against doxorubicin- induced testicular injury.

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