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Grapefruit Seed Extract Moderates Morphologic, Functional and Biochemical Evidences of Epidoxorubicin-Induced Testicular Toxicity

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The study investigated the moderating role of Grapefruit Seed Extract (GSE) on Epidoxorubicin (EPI)-induced testicular injury. Thirty rats weighing 200-250 g were divided into three treatment groups A, B and C. Group A (Control) received a single dose intraperitoneal injection of 1 mL physiological saline following treatment with 0.5 mL peanut oil (vehicle) for 2 weeks. Groups B and C each received a single dose intraperitoneally of 10 mg kg⁻¹ body weight EPI following 2 weeks gavage administration of 10 mg kg⁻¹ body weight ethanol extract of grapefruit seed and 0.5 mL peanut oil respectively. All animals were sacrificed 4 days after EPI or physiological saline injections by decapitation. Results showed that GSE attenuated testicular weight loss resulting from EPI treatment. EPI-induced reduction in sperm motility and epididymal sperm concentration as well as increase in total abnormal sperm rates were all normalized in the group pretreated with GSE. Similarly histopathological changes in the testis following EPI administration were effectively reverted by GSE pretreatment. Testicular lipid peroxidation as reflected by malondialdehyde level was significantly more in group C than group B (p<0.05). Lipid peroxidation in Group B was higher but not significantly different from the control. There was however no significant difference in the plasma testosterone levels in all the groups. We conclude that pretreatment with grapefruit seed extract may attenuate epidoxorubicin-induced testicular toxicity.

Key words: Testicular toxicity, epidoxorubicin, grapefruit seed extract, rat

INTRODUCTION

The principle of clinical cancer chemotherapy is based on the necessity to achieve a balance between maximal destruction of the neoplastic cells and an acceptable burden of toxic effects. The strategy is usually not only to tailor the drug doses to the physical state of the individual but also to ameliorate the toxic effects with other compounds (Mycet *et al.*, 1997).

EPI is a cytotoxic (anthracycline) antibiotic used for leukemias, lymphomas, breast cancer and other malignancies. It is a 4 demethoxy analog of daunorubicin. It is cell cycle specific with its maximal effects in the S and G₂ phases. The Biochemical mechanism by which EPI causes cytotoxicity is currently unknown. However inhibition of DNA Synthesis and the formation of oxygen radicals following lipid peroxidation in the tissues have been implicated (Kirsi and Timo, 2001). The therapeutic usage of EPI in long term treatment is however limited due to its toxicity to various organs. In particular the occurrence of infertility in treatment with anticancer drugs is a serious concern (Sikka, 1996; Kalender and Yel, 2005). In order to protect the testis from oxidative damage during anthracycline antibiotic chemotherapy, many antioxidants such as carotenoids, selenium, melatonin and lipoic acid have been attempted (Atessahin *et al.*, 2006; Prahalaathan *et al.*, 2005; Kalender and Yel, 2005; Teicher *et al.*, 1994).

Grapefruit Seed Extract (GSE) from grapefruit (*Citrus paradisi*) contains high levels of vitamin C, vitamin E and bioflavonoids (naringenin). These compounds are powerful antioxidants individually and collectively. GSE is therefore now regarded as the most powerful natural antioxidant available (Sachs, 1997). The aims of the present study were to study the effect of short term administration of EPI on the testicular function and histochemistry and to investigate the attenuating effects of GSE on these parameters.

MATERIALS AND METHODS

Chemicals: Ellence[®] Pfizer (Epidoxorubicin) was obtained from Juli pharmacy Ikeja Lagos.

Plant materials: The ripe grapefruits were obtained at Bariga Market Lagos. The plant material was identified and authenticated at the Forestry Research Institute of Nigeria where a voucher specimen with herbarium number F.H.I 106998 was deposited. The air dried seeds were

reduced to a powdered substance by grinding. The sample was exhaustively extracted with ethanol 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 when required.

Animals and interventions: In this study, 30 healthy adult male Sprague-Dawley rats, weighing 200-250 g were used. They were obtained from LASUCOM Animal House and were kept in the Animal House of Department of Anatomy, LASUCOM, Ikeja under standard laboratory conditions (12 h light and 12 h dark). They were allowed unrestricted access to water and food. They were randomly divided into 3 groups of 10 each. Group A (control) where given Intraperitoneal (Ip) physiological saline 1 mL (single dose) following gavage administration of peanut oil (vehicle) 0.5 mL daily for 2 weeks. Group B had Ip EPI 10 mg kg⁻¹ b.w. (single dose) following gavage administration of ethanol extract of Grapefruit Seed (GSE) 10 mg kg⁻¹ b.w. daily for 2 weeks. Group C also had single dose Ip EPI 10 mg kg⁻¹ b.w but following administration of peanut oil 0.5 mL by gavage daily for 2 weeks. The dosage of EPI given is well documented to induce testicular damage in rats (Howell and Shalet, 2001).

Sampling: All animals were sacrificed by decapitation on the fourth day after EPI or saline injections. Blood samples were collected into tubes containing 2% sodium oxalate and centrifuged. The testes weights of each animal were estimated along with the sperm characteristics. One of the testes was fixed in 10% formal aldehyde for histological examination. Plasma and the other testes were stored at -25°C for biochemical assay.

Sperm characteristics: The testes from each rat were carefully exposed and removed. They were trimmed free of the epididymides and adjoining tissues. From each separated epididymis, the cauda part was removed and placed in a beaker containing 1 cc physiological saline solution. Each section was quickly macerated with a pair of sharp scissors and left for a few minutes to liberate its spermatozoa into the saline solution. Sperm motility, concentration and progressive motility were determined as earlier described (Saalu *et al.*, 2006). Semen drops were placed on the slide and two drops of warm 2.9% sodium citrate were added. The slide was covered with a cover slip and examined under the microscope using X40

objective for sperm motility. Sperm count was done under the microscope using improved Neubauer haemocytometer.

Morphological abnormal sperm rate was determined using the method described by Atessahin *et al.* (2006). Briefly, slides were prepared with Indian ink. A total of 300 sperm cells were counted on each slide under light microscope at 400x magnification.

Estimation of plasma levels of testosterone: Plasma testosterone concentrations were estimated using the Enzyme Immunology Assay (EIA). The EIA kits used were obtained from Immunometrics (London U.K.) and contained testosterone EIA substrate reagents and EIA quality control samples. A quality control sample was run for the hormone at the beginning and at the end of the assay to ascertain acceptability with respect to bias and within assay variation. The EIA kit used had a sensitivity level of 0.3 nmol L⁻¹ (0.1 ng mL⁻¹). The intra and inter assay variations were 11.00 and 10.10%, respectively.

Histological investigations: Fixed testes tissue samples on 10% formal aldehyde were embedded in paraffin sectioned at 5 µm and were stained with Hematoxylin and Eosin (H and E). Evaluations were done using light microscope.

Malondialdehyde (MDA) assay: Testis tissue homogenates was first prepared with a buffer containing 1.5% potassium chloride to obtain 1:10 w/v whole homogenate. The assay was then done using NWLSS™ Malondialdehyde Assay Kit which is based on the reaction of MDA (a product of lipid peroxidation) with thiobarbituric acid (TBA); forming a MDA-TBA adduct that absorbs strongly at 532 nm (Ohkawa *et al.*, 1979).

Statistical analyses: Data are expressed as mean±SEM. One-way Analyses of Variance (ANOVA) were performed to determine the differences among all groups in the parameters. p<0.05 was accepted as significant.

RESULTS

Testicular weight: There was a statistically significant decrease (p<0.05) in the weight of the testis of EPI alone group compared to the control group. No statistically significant difference was observed in the GSE pretreated group as compared to the control group (Table 1).

MDA in testis tissue: EPI alone group had statistically significant higher MDA levels compared with control

group (p<0.05). The group pretreated with GSE however had significantly lower levels of MDA that EPI alone group (p<0.05) (Table 1).

Epididymal sperm characteristics: The group treated with EPI alone had significantly lower sperm concentration (p<0.05) and % sperm motility (p<0.01) than the control group. The sperm count and % sperm motility in the group pretreated with GSE even though lower but not significantly lower than the control.

EPI alone animals had significantly higher (p<0.01) % of abnormal sperm forms compared to the control. Pretreatment with GSE was seen to have an ameliorating effect on EPI-induced increase in sperm abnormalities (Table 2).

Testosterone levels in plasma: There was no significant (p>0.05) difference between the control rats and other groups, which also did not significantly (p>0.05) differ from each other (Table 3).

Testis histology: Light microscopy evaluation showed severe generation of the germinal epithelium and widening of the lumen due to hypospermatozoa formation in the EPI-alone group compared with the controls (Fig. 1 and 2).

However in the group pretreated with GSE, there were only minimal degenerative changes in the seminiferous tubules (Fig. 3).

Table 1: Testis weight and MDA concentration

	Control	EDX with GSE	EDX alone
Testis weight (g)	1.62±0.13	1.43±0.18	0.69±0.07 ^a
MDA (nmol mL ⁻¹)	0.78±0.07	0.88±0.09	1.63±0.24 ^a

Note: Values are mean±S.E.M. ^ap<0.05 control vs EPZ alone

Table 2: Sperm characteristics in all groups

Characteristics	Treatment Groups		
	Control	EPI alone	GSE+EPI
Sperm concentration (X 10 ⁶)	63.0±6.1 ^a	20.4±3.7 [*]	54.90±8.2 ^a
Sperm motility (%)	74.0±500 ^a	18.9±6.07 ^{**}	68.36±4.33 ^a
Progressivity	a ₁	b ₁	b ₁
Normal (%)	94.3±1.45 ^a	63.8±2.56	90.2±1.24 ^a
Morphology %abnormal ^f	5.7±2.33 ^b	36.19±1.60 ^{**}	9.8±2.55 ^b

^fIn this study a spermatozoon has considered abnormal morphologically if it had one or more of the following features: Rudimentary tail, round head and detached head, a₁ = rapid linear progressive motility, b₁ = Show sluggish linear or non-linear motility, ^ameans along each row with same superscript are not significantly different, ^{*}significantly lower than control value (p<0.05), ^{**}significantly lower than control value (p<0.01)

Table 3: Mean ±SE of testosterone levels in all groups (ng mL⁻¹)

Groups	Testosterone level
Control	2.8±0.32
EPI alone	2.8±0.52
GSE+EPI	2.9±0.37

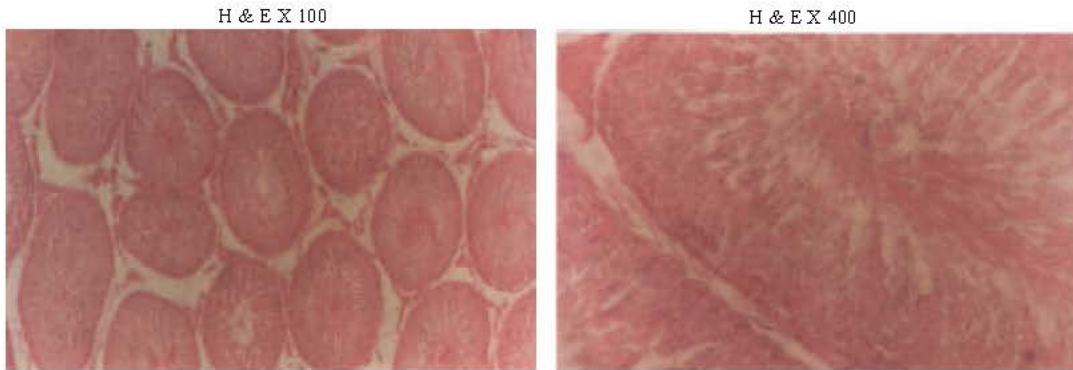


Fig. 1: Section through testis of rats of control

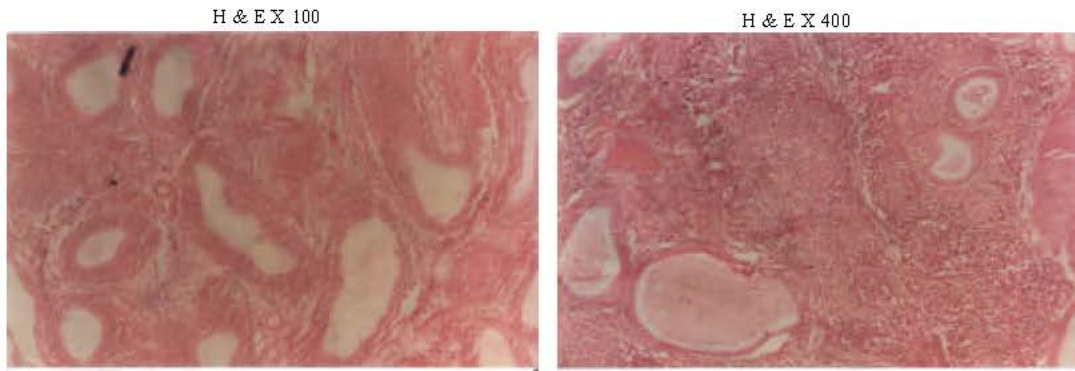


Fig. 2: Section through testis of rats treated with EPI only

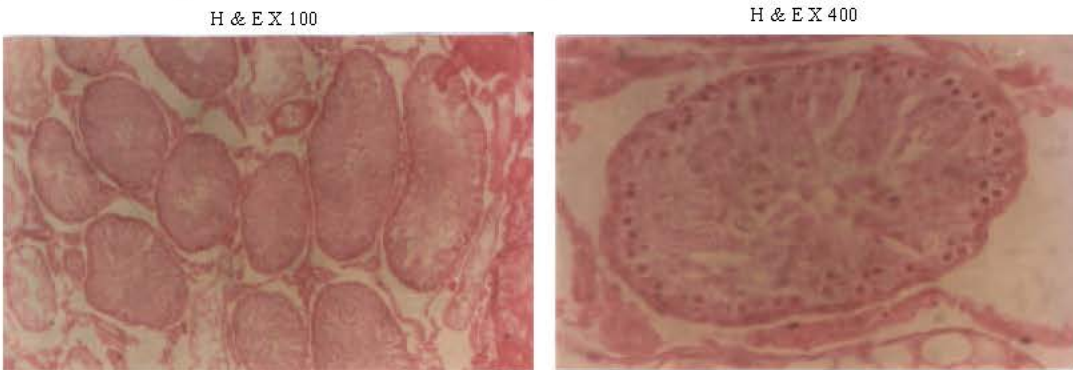


Fig. 3: Section through testis of rats treated with EPI and GSE

DISCUSSION

In this study the magnitude of Epidoxorubicin-induced testicular injury in the rats was assessed with the estimation of testicular weight, epididymal sperm characteristics, plasma testosterone, histopathology and testicular MDA. Our results demonstrated a disruption of the seminiferous epithelium and testicular interstitial of rats administered EPI only. This agrees with findings of many investigators (Endo *et al.*, 2003; Kato *et al.*, 2001; Atessahin *et al.*, 2006) who provide well documented evidence of anthracycline antibiotics-induced testicular

toxicity in animal models. Atessahin *et al.* (2006), reported that administration of EPI can decrease testicular weights of rats. Endo *et al.* (2003), did not report any significant weight changes with EPI administration. However in this study testis weights of EPI alone group were significantly lower than control group. This could be due to severe parenchyma atrophy in the seminiferous tubules after EPI administration. The group pretreated with GSE however showed remarkably normal testis weights.

Male infertility associated with EPI chemotherapy has been shown to be as a result of alterations in sperm characteristics (Howell and Shalet, 2001). EPI exerts its

antitumor effects as well as other organ toxicity by generation of free radicals and reactive oxygen species. This increased oxidative stress damages the sperm membranes, proteins and DNA (Kalender and Yel, 2005; Sikka, 1996). This explains the reduced sperm concentration and sperm motility with accompanying increase in abnormal sperm rates as seen in EPI alone rats. Pretreatment with GSE, powerful antioxidant and free radical scavenging agent resulted in a remarkable normalization of these parameters. Our findings in this regard are in consonant with an earlier result obtained by Dembinski and Konturek (2004), using an ischaemic/reperfusion experimental model in rats.

Studies by Quiles *et al.* (2002), Suominen *et al.* (2003) and Sikka (1996), have shown that anthracycline antibiotics administration resulted in direct oxidative injury to DNA and generates lipid peroxidation. Our study showed a statistically significantly increase in levels of MDA (a product of lipid peroxidation) in 4 days following EPI alone treatment. Administration of GSE prior to EPI helps to overcome the oxidative stress as shown by the normalized MDA concentration.

The non-significant difference between the groups in plasma testosterone suggests that although all cell types of the testes may be affected by EPI toxicity, the most prominent damage occurred in the seminiferous epithelium. Thus the Leydig cells, which secrete testosterone may have been only minimally affected EPI-induced toxicity. This agrees with earlier findings which indicate that the germinal epithelium is far more sensitive to the effects of cytotoxic drugs and oxidative stress than the Leydig cells (Sonmez *et al.*, 2005; Saalu *et al.*, 2006).

In conclusion, the present study demonstrated clearly that EPI treatment resulted in testicular oxidative stress and morphological impairment. It also showed that pretreatment with GSE might attenuate this injury in rats. GSE may therefore have a potential for clinical application as a testiculo-protectant during cancer chemotherapy with anthracycline antibiotics.

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