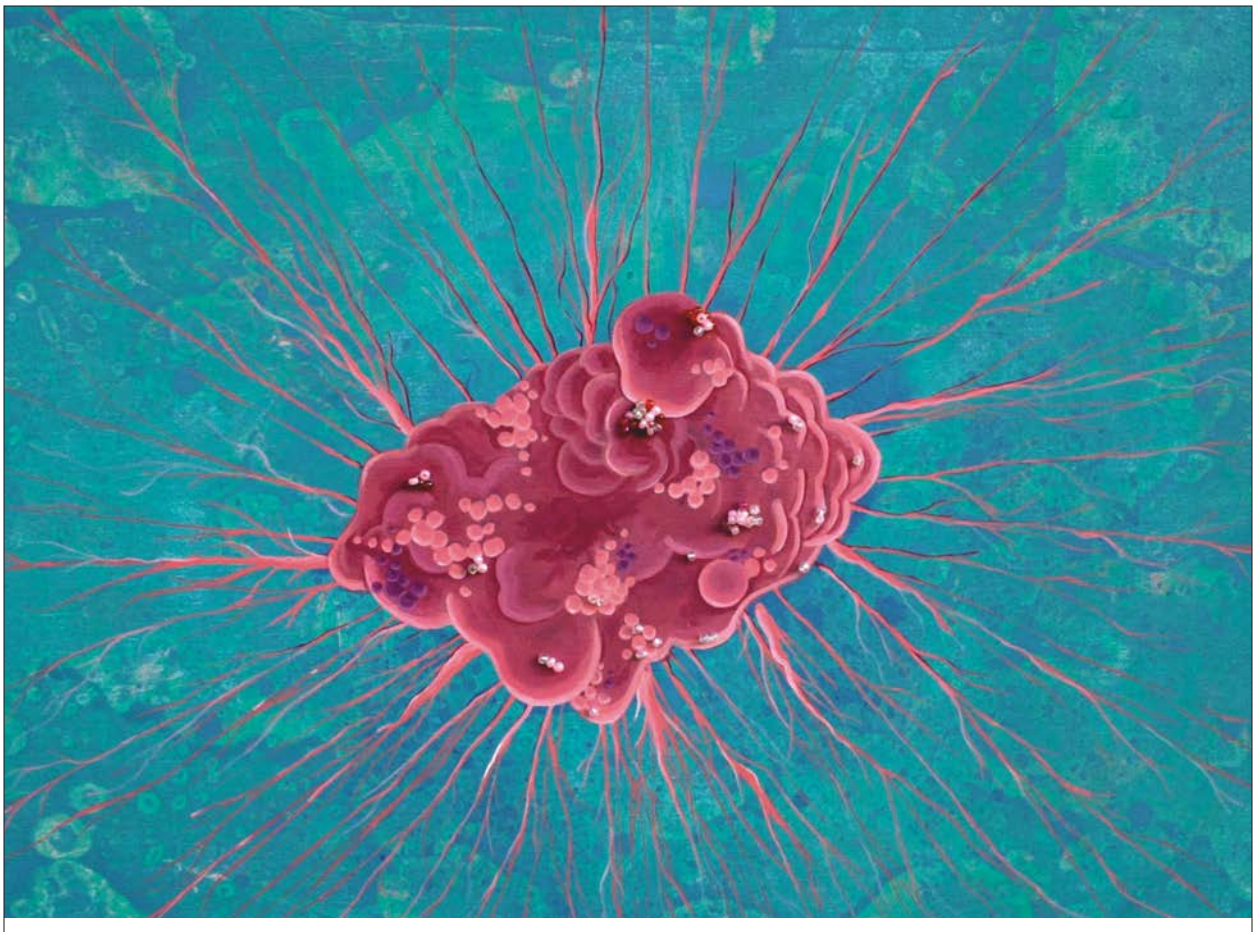

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Early and Delayed Effects of Doxorubicin on Testicular Oxidative Status and Spermatogenesis in Rats

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Abstract: In the present study, we assessed the early and delayed effects of a single dose of DOX on the testicular oxidative status and spermatogenesis in rats. 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 four groups of ten rats each. The control group of rats were given a single dose of normal saline (2.5 mL kg^{-1}) b.wt. intraperitoneally (i.p.) and then were sacrificed two weeks after. Group A animals had 10 mg DOX kg^{-1} b.wt. i.p. as a single dose. These rats were sacrificed two weeks after DOX administration. Group B animals had 10 mg DOX kg^{-1} b.wt. i.p. as a single dose but were sacrificed 8 weeks after. Group C rats had similar treatment as those in group B, except that they were sacrificed at the end of the 16th week after DOX administration. Epididymal sperm characteristics were evaluated. In addition, biomarkers of oxidative stress namely testicular antioxidants and lipid peroxidation in the testicular tissue were determined using spectrophotometric methods. The testicular oxidative status of DOX-treated rats was severely compromised as evidenced by the significant decrease in the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in addition to the significant reduction in the reduced glutathione (GSH) level as well as the significantly enhanced lipid peroxidation measured as malondialdehyde (MDA). There was also a demonstratable worsening of the rat testicular oxidative status and sperm parameters with passage of time following DOX administration. The results indicate that DOX produces persistent damage to the spermatogenic compartment of the testis as well progressive increase in the testicular oxidative stress.

Key words: Doxorubicin, testes, oxidative stress, spermatogenesis

INTRODUCTION

Doxorubicin (DOX) is a quinine-containing anticancer antibiotic that is widely used to treat different types of human neoplastic disease such as hematopoietic, lymphoblastic (Hitchcock-Bryan *et al*., 1986) and solid tumors (Bonadonna *et al*., 1996). However, its clinical value has been limited by dose-limited toxicity (Yeh *et al*., 2007; Berthiaume and Wallace, 2007; Saalu *et al*., 2009).

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Although, a number of potential toxic mechanisms have been identified following exposure to doxorubicin, the major pathogenic mechanism appears to involve the generation of toxic reactive oxygen species (Prahalathan *et al*., 2005; Chularojmontri *et al*., 2005). Several *in vivo* and *in vitro* studies have demonstrated that reactive oxygen metabolites including free radical species, superoxide anion (O_2-•), hydrogen peroxide (H_2O_2) and hydroxyl radical (• OH) are important mediators of tissue injury (Sachdev and Davies, 2008). The involvement of oxygen radical-induced injury of membrane lipids has been reported as the main causative factor for DOX-induced cardiotoxicity (Berthiaume and Wallace, 2007). The cellular and biochemical changes involved in this process have been demonstrated. One-electron reduction of DOX leads to formation of the corresponding semiquinone free radical (Doroshow, 1983). In the presence of oxygen, this free radical rapidly donates its electron to oxygen to generate superoxide anion (O_2•). The dismutation of superoxide yields hydrogen peroxide (H_2O_2) (Pacher, 2007). Under biological conditions, the anthracycline semiquinone or reduced metal ions such as iron reductively cleaves hydrogen peroxide to produce the hydroxyl radical which is the most reactive and destructive chemical species ever known (Panjrath *et al*., 2007). This ultimately leads to lipid peroxidation, causing irreversible damage of membrane structure and function (Kirsi and Timo, 2001).

Mammalian spermatozoan is particularly vulnerable to lipid peroxidation because of the molecular anatomy of its plasma membrane. Unlike somatic cells, mammalian sperm cells present highly specific lipid composition with high content of polyunsaturated fatty acids (PUFA), plasmalogenes and sphingomyelins. This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa's lipids especially PUFA are the main substrates for peroxidation, that may provoke severe functional disorder of sperm (Sachdev and Davies, 2008).

The occurrence of male infertility long after treatment with this anticancer drug is a serious concern (Suominen *et al*., 2003). Quiles *et al*. (2002), Chularojmontri *et al*. (2005) and Prahalathan *et al*. (2005) have attempted to study the effects of this chemotherapeutics drug on the reproductive system. However, most of these studies reported the short term or at most the midterm effects of DOX therapy on the male reproductive organs. The aim of the present study is to evaluate the short, mid and long terms effects of a single dose treatment with DOX on the rat testicular oxidative status and spermatogenesis.

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.

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 the 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 twelve hours 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 four groups of ten rats each such that the average weight difference between and within groups did not exceed ±20% of the average weight of the sample population. Group 1 rats were given were given a single dose i.p. normal saline 2.5 mL kg^{-1} b.wt. and then were sacrificed two weeks after. Group 2 animals had 10 mg DOX kg^{-1} b.wt. 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). These rats were sacrificed two weeks after DOX administration. Group 3 animals had 10 mg DOX kg^{-1} b.wt. i.p. as a single dose but were sacrificed on the 56th day (i.e., 8 weeks after DOX treatment), the duration of spermatogenesis in rat being 51.6-56 days (Heller and Clermont, 1964; Jegou *et al*., 2002). Group 4 rats had similar treatment as those in group 3 except that they were sacrificed at the end of the 16th week after DOX administration.

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.

Sperm Characteristics

The testes from each rat were carefully exposed and removed. They were trimmed free of the epididymides and adjoining tissues.

Epididymal Sperm Concentration

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi (2004). Briefly, the epididymis was minced with anatomic scizzors in 5 mL physiologic saline, placed in a rocker for 10 min and allowed to incubate at room temperature for 2 min. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neuber`s counting chamber (haemocytometer). Approximately 10 μL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 min. This chamber was then placed under a binocular light microscope

using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-celled squares. The sperm concentration was the calculated multiplied by 5 and expressed as $[X]\times10^{6}$ mL^{-1}, where [X] is the number of spermatozoa in a 16-celled square.

Sperm Progressive Motility

This was evaluated by an earlier method by Sonmez *et al.* (2005). The fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 mL with Tris buffer solution. A slide was placed on light microscope with heater table, an aliqout of this solution was on the slide and percentage motility was evaluated visually at a magnification of x 400. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score. Samples for motility evaluation were stored at 35°C.

Sperm Morphology

The cells were examined with the help of a light microscope at x 400 magnification. Caudal sperm were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada). Five hundred sperm from the sample were scored for morphological abnormalities. Briefly, in wet preparations using phase-contrast optics, spermatozoa were as follows: 1) normal head and tail, 2) isolated heads, whether by the head was misshapen or not, 3) head-only defects, that is misshapen head with normal tail, 4) tail defects, that is normal head with abnormal tail or misshapen head with abnormal tail and 5) fused sperm and was expressed as a percentage of morphologically normal sperm.

ASSAY OF TESTICULAR ENZYMATIC ANTIOXIDANTS

Assay of Catalase (CAT) Activity

Catalase activity was measured according to the method of Aebi (1983). The 0.1 mL of the testicular homogenate (supernatant) was pipetted into cuvette containing 1.9 mL of 50 mM phosphate buffer, pH 7.0. Reaction was started by the addition of 1.0 mL of freshly prepared 30% (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of enzyme was expressed as units mg^{-1} protein.

Assay of Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity was measured according to the method of Winterbourn *et al.* (1975) as described by Rukmini *et al.* (2004). The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 2.7 mL of 0.067 M phosphate buffer, pH 7.8, 0.05 mL of 0.12 mM riboflavin, 0.1 mL of 1.5 mM NBT, 0.05 mL 0f 0.01 M methionine and 0.1 mL of enzyme samples. Uniform illumination of the tubes was ensured by placing it in air aluminum foil in a box with a 15 W fluorescent lamp for 10 min. Control without the enzyme source was included. The absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% under the specific conditions. It was expressed as μ mg^{-1} protein.

Assay of Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase activity was measured by the method described by Rotruck *et al.* (1973). The reaction mixture contained 2.0 mL of 0.4 M Tris-HCl buffer, pH 7.0, 0.01 mL of 10 mM sodium azide, 0.2 mL of enzyme. The 0.2 mL of 10 mM glutathione and 0.5 mL of 0.2 mM. H_2O_2. The contents were incubated at 37°C for 10 min followed by the termination of the reaction by the addition of 0.4 mL 10% (v/v) TCA, centrifuged at 5000 rpm for 5 min. The absorbance of the product was read at 430 nm and expressed as nmol mg^{-1} protein.

ASSAY OF TESTICULAR NON- ENZYMATIC ANTIOXIDANT

Assay of Testicular Reduced Glutathione (GSH) Concentration

The GSH was determined by the method of Ellman (1959). The 1.0 mL of supernatant was treated with 0.5 mL of Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The 0.4 mL of distilled water was added. The mixture was thoroughly mixed and the absorbance was read at 412 nm, expressed as nmol mg^{-1} protein.

Estimation of Lipid Peroxidation (Malondialdehyde)

Lipid peroxidation in the testicular tissue was estimated calorimetrically by thiobarbituric acid reactive substances TBARS method of Buege and Aust (1978). A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 mL of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of malondialdehyde which is 1.56×10^5/M/cm and expressed as nmol mg^{-1} protein.

Statistical Analysis

All data were expressed as Mean±SD of number of experiments (n = 5). The level of homogenecity among the groups was tested using Analysis of Variance (ANOVA) as done by Snedecor and Cochran (1980). Where heterogenecity 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

Testicular Oxidative Status

Activities of testicular enzymes-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx): The activities of testicular enzymes, SOD, CAT and GPx are shown in Table 1. Administration of normal saline (control) caused no significant ($p>0.05$) change in testicular SOD, CAT and GPx activities, whereas, DOX provoked statistically significant ($p<0.05$) decrease in SOD, CAT and GPx activities for the animals sacrificed at the end of week 2 and the end of the 8th week when compared to control animals. Furthermore, the rats that were sacrificed 16 weeks post-DOX showed a much more significant ($p<0.001$) reduction in their testicular activities of SOD, CAT and GPx compared to the control.

Testicular Content of Glutathione (GSH) and Malondialdehyde (MDA)

There was a notable reduction ($p<0.05$) in GSH content two weeks after and at the end of the 8th week after DOX challenge. The group that was sacrificed at the end of the 16th

Table 1: Testicular antioxidative enzymes and testicular contents of GSH and MDA

Treatment groups	SOD	CAT	GPX	GSH	MDA
	---------- (μ mg^{-1} protein) ---------		---------- (nmol mg^{-1} protein) ----------		
Control	47.68±4.15	14.75±1.5	0.82±0.05	2.42±0.08	0.76±0.05
A	18.78±4.75*	7.720±3.55*	0.26±0.15*	0.89±0.05*	1.25±0.04*
B	17.54±2.35 *	6.330±2.4*	0.24±0.16*	0.88±0.04*	3.13±0.5**
C	10.40±2.35**	3.250±0.35**	0.15±0.15**	0.52±0.07**	3.93±0.06**

Values are means±S.E.M. n = 10 in each group. *,** Represent significant differences at $p<0.5$ and $p<0.001$, respectively compared to controls. Control group: had Normal saline 2.5 mL kg^{-1} b.wt. i.p. and were sacrificed two weeks after. Group A: had Doxorubicin 10 mg kg^{-1} b.wt. i.p. and were sacrificed two weeks after. Group B: had Doxorubicin 10 mg kg^{-1} b.wt. i.p. and were sacrificed at the end of the 8th week. Group C: had Doxorubicin 10 mg kg^{-1} b.wt. i.p. and were sacrificed at the end of the 16th week

Table 2: Sperm parameters

Treatment groups	Sperm count (x10^6 mL^{-1})	Sperm motility (%)	Sperm progressivity	Sperm morphology	
				Normal	Abnormal
Control	137.5±6.7	99.0±1.0	a1	91.5±1.3	8.40±1.4
A	136.4±5.5	98.3±1.7	a1	92.5±1.2	7.50±1.3
B	50.30±2.8*	35.3±1.9*	b1*	28.7±1.4**	69.3±1.1**
C	15.50±2.6**	10.5±2.7**	b1*	10.2±2.5**	89.1±3.2**

Values are Means±SEM n = 10 in each group. *,** Represent significant differences at $p<0.005$ and $p<0.001$, respectively when compared to the control values. Control Group: had Normal saline 2.5 mL kg^{-1} b.wt. i.p. and were sacrificed after 2 weeks. Group A: had Doxorubicin 10 mg kg^{-1} b.wt.i.p. and were sacrificed after 2 weeks. Group B: had Doxorubicin 10 mg kg^{-1} b.wt. i.p. and were sacrificed at the end of the 8th week. Group C: had Doxorubicin 10 mg kg^{-1} b.wt.i.p. and were sacrificed at the end of the 16th week

week showed even a more significant ($p<0.001$) depletion in their testicular GSH content compared to the controls. The MDA content was significantly ($p<0.05$) increased in the rats that had their testes examined two weeks post- DOX. Furthermore, DOX significantly elevated the testicular MDA by about 5-folds compared to the control value in the groups that were examined at the end of the 8th and 16th weeks (Table 1).

Sperm Parameters

Sperm Count and Sperm Motility

The mean sperm count and motility for the animals treated with only normal saline (the control group) were 137.5±6.7×10^6 mL^{-1} and 99.0±1.0%, respectively.

The DOX-treated Wistar rats that were sacrificed only the next day after DOX therapy did not demonstrate any significant ($p>0.05$) difference in their sperm count and motility when compared to the control values.

The animals that had DOX and were sacrificed at the end of the 8th and 16th week post treatment showed significant ($p<0.05$) and ($p<0.001$) reduction, respectively in both their sperm count and motility as compared to the control group (Table 2).

Sperm Progressivity and Sperm Morphology

The results of the sperm progressivity and morphology parallel that of the sperm count and motility as shown above. The group of one day post DOX sacrifice similarly showed normal sperm progressivity and morphology.

The animals that had DOX and were sacrificed at the end of the 8th and 16th week post treatment showed a significant ($p<0.05$) and ($p<0.001$) reduction, respectively in both their sperm progressivity and normal sperm morphology rates as compared to the control group (Table 2).

In this study a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head.

a_1 = Rapid linear progressive motility, b_1 = Show sluggish linear or non-linear motility

DISCUSSION

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). 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 (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, DNA fragmentation and protein oxidation (Pacher, 2007).

Mammalian spermatozoon is particularly vulnerable to lipid peroxidation because of the molecular anatomy of its plasma membrane. Unlike somatic cells, mammalian sperm cells present highly specific lipid composition with high content of polyunsaturated fatty acids (PUFA), plasmalogenes and sphingomyelins. This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa's lipids especially PUFA are the main substrates for peroxidation, what may provoke severe functional disorder of sperm (Sachdev and Davies, 2008).

This may explain why the testicular oxidative status of DOX alone rats was severely compromised as evidenced by the significant ($p<0.001$) decrease in the activities of SOD, CAT and GPx; in addition to the significant ($p<0.01$) reduction in the GSH level as well as the significantly ($p<0.001$) enhanced lipid peroxidation measured as MDA.

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 and Yel, 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 and apoptosis. Anthracyclines including DOX exert their antitumor 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 (Hrdina *et al.*, 2000). This increase oxidative stress damages the sperm membranes, proteins and DNA (Kirsi and Timo, 2001; Kalender and Yel, 2005). This could explain the significant ($p<0.001$) reduced sperm concentration and sperm motility along with a significantly ($p<0.001$) increased abnormal sperm morphology rates and significantly ($p<0.05$) reduced sperm progressivity as seen in DOX alone group rats when compared to the control groups.

Oxidative stress and lipid peroxidation (self-propagating) mechanism of DOX cytotoxicity could provide an explanation for the progressive worsening of the biomarkers of testicular oxidative stress and spermatogenic parameters following a single dose DOX therapy. Thus the animals sacrificed two weeks post DOX had better testicular oxidative status and sperm characteristics than those that were autopsied 8 weeks post-DOX. Those that were sacrificed 16 weeks after DOX treatment were shown to have the worst testicular cytotoxicity.

CONCLUSION

It is therefore concluded that even a single dose of doxorubicin progressively causes testicular dearrangement. This could be due to its capacity to generate intracellular free radicals and reactive oxygen species. These findings may partly explain the epidemiological observation that male infertility occurs long after cessation of doxorubicin chemotherapy.

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