DOXORUBICIN MEDIATED OXIDATIVE STRESS INDUCED DEGENERATION OF TESTICULAR TISSUES CAUSES MALE STERILITY IN RATS.

MISHRA, M. D. ? AND BHIWGADE, D. A.

Department of Biomedical Sciences, D.Y. Patil Vidyapeeth, Navi Mumbai-400 085, India.
E mail: mdmishra2000@yahoo.com

Received: July 20, 2006; Accepted: August 15, 2006

Abstract: Doxorubicin used for the treatment of reproductive cancer and negatively influences the fertility of cancer patient by still hitherto unknown mechanism. In present study the effect of this drug on cytoarchitectural organization of the cellular components in testicular tissues and its correlation to oxidative stress and other parameters responsible for male fertility has been reported. Tissue exhibiting higher levels of oxidative stress response indicators such as antioxidant enzymes and metabolites showed high degree of degeneration of testicular tissues such as epididymis and testis. Counting of the number of sperms per square area in treated animals and its comparison with non-treated animals showed a significant decrease in the number of sperms, sperm’s head degeneration. The data clearly showed that doxorubicin provoked loss of male fertility in animals occurs due to oxidative stress mediated male reproductive tissues degeneration.

Key words: Doxorubicin, Male sterility, Testicular tissues

INTRODUCTION

Testicular cancer is now one of the solid neoplasms, which could be treated by the use of effective anticancer drugs [1]. These drugs, on one hand, help in the prevention and cure of reproductive cancers, which accounts largely in young patients. On the other hand, they also have serious side effects and patients have risk of losing their fertility [2]. Amongst the anticancer drugs used in chemotherapy, the doxorubicin has been reported to be a highly potent drug used in cancer therapy [1]. However, it also showed the pronounced effects on male fertility. Wattenberg [2] reported an infertile patient, who had bilateral epididymal tumor diagnosed at testis biopsy, develops azoospermia. Lehmann et al. [3] investigated an infertile patient with oligozoospermia, large left varicocele and normal concentrations of testosterone, oestradiol and follicle stimulating hormone. During last decade, several such studies have revealed that chemotherapeutic treatment of reproductive cancers can lead to infertility in patients [4]. Limited efforts have been made to understand the cause of infertility and the mechanism of drugs cytotoxicity that leads to male sterility. In present investigation, we have shown that testicular tissues of the animals treated with increasing doses to doxorubicin (DOX) showed extensive damage of testis and epididymis cells responsible for spermatogenesis, along with significant changes in the levels of antioxidant enzymes and antioxidant metabolites suggesting the induction of oxidative stress in response to drug treatment.

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals used in the study were analytical grade (AR grade) and have the highest purity. They were obtained from Sigma chemicals Co., MERCK chemicals, Sisco Research Laboratory, India LOBA and SD-FINE chemicals. Molecular biology grade chemicals and enzymes were purchased from Bangalore Genie (Pvt.) Limited, Roche Biochemicals, Germany.

Experiments on laboratory animals: The 6-7
weeks old male albino rats (Wistar strain) were used for all the experiments. After acclimatization of the animals to the existing laboratory environment for 10 days, they were divided into two groups of six animals in each. One group of animals was injected normal saline intraperitoneally and considered as control. The other group of animals was intraperitoneally injected DOX at the dose rate of 1.0 mg/kg body weight at the regular intervals of thrice a week as described earlier [5], for 52 days. The rats to be sacrificed were anesthetized with ether and the scrotum of the testis was dissected to isolate testis and epididymis. The tissues were immediately cleaned with ice-cold normal saline and blotted properly with tissue paper. The one side testis and epididymis were used immediately for biochemical assay, the testis and epididymis from the contralateral side were adopted with glutaraldehyde for electron microscopy and also stored in liquid nitrogen for few hours followed by 70 °C for analysis of proteins.

Biochemical studies: The samples were minced and quickly homogenized in 10 ml homogenizing buffer pH 8.0 (100 mM sodium phosphate Buffer pH 8.0, 0.9% NaCl, 1.15% KCl, 4.0 mM EDTA) per gm tissues on ice. The homogenate was centrifuged at 9000 rpm for 20 minutes using a Sorvall SS-34 rotor at 4 °C. The homogenate was centrifuged at 9000 rpm for 90 min. Clear supernatant was stored at -20 °C for the estimation of protein, glutathione, and activities of enzymes such as glutathione peroxidase, glutathione S-transferase and glutathione reductase. The cytochrome P450 was estimated in micro-pellet.

Lipid per-oxidation was measured with control and treated tissue samples by following TBARS methods [6]. Glutathione was estimated from supernatant of treated tissue samples by following TBARS methods [6]. Glutathione reductase [9] and glutathione S-transferase [10] and cytochrome P450 (Cyt P450) was estimated by the method of Omura and Sato [11]. Super-oxide dismutase activity was estimated as per Marklund and Marklund technique (12). Levels of protein carbonylation were measured by Palamanda and Kehrer [13] and total protein by Lowry (14) methods.

Microscopic studies: The light microscopic histology of testis and epididymis were studied adopting paraffin method [15,16]. The sections were stained with haematoxylin followed by eosin as secondary stain. The slides were mounted to using DPX mountant as an adhesive agent.

Statistical analyses: All samples were run in duplicate, inter-graph differences were statistically assessed using student t-Test [17]. The results were expressed as MEAN ± standard error of the mean (mean ± SEM). Graphical representation of biochemical data was made wherever possible to facilitate better comparison.

**RESULTS AND DISCUSSION**

DOX treatment reduces body weight and male organ development in rates: The effect of drug treatment on normal health of the animals was monitored and it was found that the total body weight of the control and DOX treated male albino rats for seven weeks, showed the different pattern (Table 1). Control animals showed the increase in the body weight at the uniform rate of 21.0 ± 2.39 gm/week. In treated animals, though the increase in the body weight was uniform and steady but the rate was nearly four folds lower than control i.e. 5.56 ± 2.27 gm/week. When the growth of the treated animals was almost stopped in fifth week onward, the growth of control animals was continued to increase till measured. This suggested that DOX inhibits the
normal body development.

This study was aimed to investigate the mechanism of action of the drug on male sterility. The effect of DOX on male reproductive organs growth and development and production of viable sperms were measured at the end of treatment. A significant decrease in the weight of male reproductive organs was observed in treated animals as compared to the control samples. Weight of testis has decreased from 1.65 ± 0.0426 grams in control to 0.545 ± 0.0195 gram upon treatment. Similarly, epididymis weight had decreased from 0.56 ± 0.013 gram to 0.34 ± 0.01 gram in control and treated animals respectively. Treated animals showed a significant decrease in sperms counts per gm weight testis. The number of sperm count per unit area has decreased to nearly 10 % in treated samples as compared to control (data not shown). Heavy damage of the sperms head was observed in treated samples. These findings were in agreement with similar studies using a different chemotherapeutic agent [4,18,19]. The results clearly indicated that DOX treatment along with body also affects normal growth and development of male reproductive system and male gametes.

**Drug treatment induced oxidative stress in testicular tissues:** DOX has been known to induce the oxidative stress and thereby alters the antioxidant environment of the cells. DOX induces the generation of reactive oxygen species (ROS) at the expense of the redox potential of the cells and provoked cardiomyopathy [1]. ROS generated inside as well as outside the cells oxidizes biomolecules such as membrane lipids, proteins and nucleic acid bases. The levels of reduced glutathione (GSH), and antioxidant enzymes like glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) combat the oxidative stress effect in the tissues and have been taken an indicator of oxidative stress response in cells [20]. These parameters were measured in testicular tissues of drug treated as well as control animals. The effect of DOX on the levels of these oxidative stress indicators is summarized in Table 2.

The levels of lipid peroxidation and activities of the enzymes such glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) were increased in both testis and epididymis at different levels. However, the change in the levels of reduced glutathione in drug treated samples and control testis and epididymis tissues were insignificant. The Cyt P-450 in testis showed a significant decrease of nearly 16% upon drug treatment in as compared to controls. Lipid per-

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control (n = 6)</th>
<th>Treatment (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>217.50 ± 4.2337</td>
<td>212.50 ± 4.2337#</td>
</tr>
<tr>
<td>1</td>
<td>247.50 ± 3.8196</td>
<td>245.00 ± 4.2826#</td>
</tr>
<tr>
<td>2</td>
<td>290.00 ± 5.1650</td>
<td>258.33 ± 6.2817*</td>
</tr>
<tr>
<td>3</td>
<td>308.33 ± 4.9596</td>
<td>267.50 ± 6.5324*</td>
</tr>
<tr>
<td>4</td>
<td>328.33 ± 6.9135</td>
<td>278.33 ± 6.5418*</td>
</tr>
<tr>
<td>5</td>
<td>358.33 ± 4.4105</td>
<td>293.33 ± 8.9149*</td>
</tr>
<tr>
<td>6</td>
<td>371.67 ± 3.5753</td>
<td>293.33 ± 7.0331*</td>
</tr>
<tr>
<td>7</td>
<td>391.67 ± 3.0738</td>
<td>300.50 ± 5.7385*</td>
</tr>
<tr>
<td>8</td>
<td>416.67 ± 6.0105</td>
<td>307.33 ± 6.1470*</td>
</tr>
</tbody>
</table>

The values are expressed in mean ± SEM. Body weights are represented in gm unit. * Indicates non-significantly different from the control values at (P > 0.05). # Represents significantly different from the control counterparts at (P > 0.05). Number of each group is shown in parentheses.

**Table 1.** Effect of doxorubicin (18 doses of 1.0 mg / kg body weight, thrice a week) treatment on body weight loss of rats.

**Table 2.** Oxidative stress response of doxorubicin on testicular tissues. Different parameters such as antioxidant enzymes, antioxidant metabolites, lipid peroxidation and protein carbonylation were measured in testis and epididymis tissues of control and treated animals. ND not determined.

<table>
<thead>
<tr>
<th>Oxidative response parameters</th>
<th>Testis</th>
<th>Epididymis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Lipid peroxidation a</td>
<td>0.1943 ± 0.0208</td>
<td>0.3304 ± 0.01902</td>
</tr>
<tr>
<td>Reduced GSH b</td>
<td>9.8624 ± 0.6592</td>
<td>8.8823 ± 0.5134</td>
</tr>
<tr>
<td>Glutathione reductase c</td>
<td>1.535 ± 0.0551</td>
<td>1.9937 ± 0.0799</td>
</tr>
<tr>
<td>Glutathione peroxidase d</td>
<td>87.783 ± 6.13</td>
<td>184.23 ± 16.84</td>
</tr>
<tr>
<td>Glutathione-S-transferase c</td>
<td>42.886 ± 2.3435</td>
<td>56.1961 ± 2.1883</td>
</tr>
<tr>
<td>Cytochrome P450 f</td>
<td>753.3936 ± 24.110</td>
<td>630.2248 ± 22.51</td>
</tr>
<tr>
<td>Super-oxide dismutase g</td>
<td>65.67% ± 7.65</td>
<td>37.89% ± 6.98</td>
</tr>
<tr>
<td>Protein carbonylation h</td>
<td>87.65 ± 8.56</td>
<td>112.21 ± 4.53</td>
</tr>
</tbody>
</table>

a. nmole MDA / mg proteins per g of tissue with P>0.05 significance, b. nmole GSH per mg proteins with P>0.05 insignificant, c. µmole of NADPH reduced / min per gm of tissue with P > 0.05 significance, d. nmole of GSH consumed per min / mg protein with P>0.05 significance, e. µmol of CDNB conjugated / min / mg protein with P > 0.05 significance, f. nmole CO bound / mg microsomal protein with P > 0.05 significance, g. U / mg protein, h. nmole carbonyls formed / mg protein with ± mean difference
oxidation has increased significantly to nearly 2.0 and 2.4 fold in the DOX treated testis and epididymis, respectively as compared to control. GPX activity increased significantly (P > 0.05) upon DOX treatment. In testis this increase was nearly two folds while in epididymis the stimulation was 36 % as compared to control. GR levels in the treatment group were found to be increased significantly (P > 0.05) up to 30% in testis and 49% in epididymis as compared to control. GST activity was stimulated by nearly 32% increased significantly in testis as compared to controls. The protein carbonylation was increased by 25-35 % in treated testis and epididymis samples as compared to respective controls. The increase in protein carbonylation and lipid peroxidation was in concurrence with increase in several antioxidant enzymes but super-oxide dismutase (SOD) indicated the induction of a strong oxidative stress by DOX.

Theoretically, a precise balance in different metabolic steps to combat the deleterious effect of ROS is normally required for the development of the cells and normal metabolic process (21). The imbalance in the levels of these enzymes can lead to cellular toxicity. For example too low ratio of SOD relative to GPX and/or catalase could result in an accumulation of O$_2^-$, which is toxic to macromolecules; and on the other hand, too high ratio of dismutase to GPX and/or catalase could lead to increased H$_2$O$_2$ concentration. The higher levels of H$_2$O$_2$ can be converted to OH in the fenton reaction (22) which is more deleterious than superoxide anions and may lead to lipid peroxidation, DNA cross-linking and formation of disulfide bonds in proteins.

These results suggested that DOX induces oxidative stress in testicular tissues, which might affect the membrane integrity and cellular architecture of the testicular cells. The effect of other anticancer drugs like cisplatin and transplatin (23,24) and anthracycline (25,26) on the oxidative stress in other tissues has been reported. Increase in oxidative damage of proteins and higher lipid per-oxidation activity in response to drug mediated oxidative stress further indicated that both lipids and proteins were the target of oxidant produced during drug metabolism.

**Oxidative stress correlates with degeneration of sperm generating tissues:** Histological details of control testis, both in low and high powers are presented in figures 1 and 2. The seminiferous tubules of DOX treated rats showed pronounced shrinkage, and their epithelium was thoroughly disorganized resulting in an increased intertubular space (Fig. 3). The tunica propria and the basement membrane became thick, irregular and folded. At a few places the prominent vacuoles were formed within outer capsule and basal membrane of seminiferous tubules following DOX treatment. The sertoli cells after drug treatment underwent degenerative changes and were different in histological structure as compared to the control animals. The apical cytoplasm showed maximum degeneration and no spermatozoa were found at the apical border of the sertoli cells in DOX treated samples.

The Leydig cells in untreated animals were present in the form of cluster and the space between the seminiferous tubules was filled with connective tissues, peritubular cells, lymphatic vessels and blood capillaries (Fig. 2). In the treated testis, the numbers of Leydig cells were reduced and the cells were atrophied. Their cytoplasm was reduced and the presence of pycnotic nuclei in these cells suggested their loss of functional integrity (Fig 4). The size of these cells was increased due to the vacuolation, and nuclei were shifted towards periphery. The chromatin material was darkly stained indicating the necrosis (Fig. 4).

These results strongly indicated that DOX treatment disrupts the cellular architecture of testicular tissues. The different cells in the testis contribute at different levels in the maturation of sperms (4,18). DOX treatment has destroyed the structural integrity of many of these cells either by necrosis or apoptotic cell death. DOX causes apoptosis in several cell lines in vitro (27). The Leydig cells, which are responsible for the synthesis of testosterone hormones for the maturation of the spermatozoa, were extensively damaged. The secondary spermatocytes, spermatids and spermatozoa were absent in the seminiferous tubules, suggesting that the process of spermatogenesis had stopped at the primary spermatocyte stage only in drug treated cells.

From overall study it is concluded that on one hand DOX, increases free radicals, disturbs glutathione metabolism, increases lipid peroxidation and increase oxidative stress, on the other hand extensively damage the testicular functions. Thus the loss or reduction of male fertility by DOX could be accounted due to oxidative stress mediated testicular
tissue degeneration and cells death leading to the inhibition of fertile sperms maturation.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Hari Misra (BARC, Mumbai) and Dr. Vinod S. Narayane for their help in the data analysis and critical comments during the preparation of manuscript.

REFERENCES