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Protection by doxycycline against doxorubicin-induced oxidative stress and apoptosis in mouse testes

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ABSTRACT

Spermatogenic cells constitute one of the body tissues that are susceptible to doxorubicin-induced oxidative stress and apoptosis. To explore whether doxorubicin toxicity to these male germ cells could be prevented by adjuvant medication, this study was designed to examine the possible ameliorating action of doxycycline, an antibiotic with anti-oxidant property, on doxorubicin-induced oxidative and apoptotic effects in mouse testes. Male mice at 5-week of age were treated with vehicles, doxorubicin alone (3 mg/kg, i.p. every other day for 3 doses), doxycycline alone (2.5 mg/kg, i.p. every other day for 3 doses), or doxycycline plus doxorubicin (each dose given 1 day post-doxycycline). After 28 days, mice treated with doxorubicin alone displayed smaller body and testicular weights, reduced sperm counts, impaired spermatogenic capability (scarcer spermatids and spermatocytes), increased oxidative stress (malondialdehyde levels), decreased anti-oxidant activity (superoxide dismutase and glutathione peroxidase), and elevated apoptotic indexes (upregulation of Bax and Bad, downregulation of Bcl-2 and Bcl-xL, release of cytochrome c from mitochondria to cytosol, activation of caspase-3, and increase of cleaved caspase-3 abundance and TUNEL positive cells), while doxycycline pretreatment could effectively prevent nearly all of these abnormalities. These results provide firm evidence that doxycycline pretreatment would offset the oxidative and apoptotic impact imposed by doxorubicin, and imply doxycycline to be a promising adjuvant agent that may attenuate the toxicity of doxorubicin on testicular tissues in clinical practice.

1. Introduction

Male germ cell development, or spermatogenesis, demands complex chemical, physical, and genetic interactions in the testicular tissue [1]. The obligatory requirement of definite DNA integrity and the brisk activity of cell proliferation render this process very prone to disruption by extrinsic insults, particularly whichever inflict oxidative stress, DNA damage, and apoptosis. Examples include a number of anti-neoplastic agents that, though could suppress the growth of tumor cells

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Abbreviations: Dox, doxorubicin; Dc, doxycycline; DTT, DL-dithiothreitol; DAPI, 4′,6′ diamidino-2-phynylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

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powerfully, may adversely impede the process of spermatogenesis, elicit germ cell degeneration, and cause aspermia and infertility [2,3]. Among these chemotherapeutic agents doxorubicin has long been widely used for its potent efficiency [4], yet remains a drug of great concern for the hazard of undesirable tissue harmful consequences [5–7]. In testes, it can impair motility of sperms [8], induce germ cell apoptosis [9], and result in testicular failure eventually [10]. The exact mechanism of doxorubicin testicular toxicity is still not completely known, but lessons learned from doxorubicin cardiomyopathy implicate that breakage of DNA continuity, overload of oxidative stress, and apoptosis of cells should be largely attributable [11]. Based on this concept, clinical and experimental trials have been directed toward employing various anti-oxidant and anti-apoptotic agents to ameliorate doxorubicin-induced testicular damage, but whether these chemicals could prevent or reverse doxorubicin testicular toxicity remains debated [8,11].

Doxycycline is a tetracycline-derived synthetic antibiotic with potent anti-inflammatory effects [12,13]. Recent researches further documented its anti-oxidant, anti-apoptotic, and anti-fibrotic effects on various tissues stressed by bleomycin or ischemia-reperfusion injury [14], therein implying it to be a promising potential cytoprotective agent against various extrinsic toxic stimuli. To determine whether doxycycline could also antagonize doxorubicin-induced cytotoxicity in testicular cells, this study was designed to investigate the effects of doxycycline on doxorubicin-induced histopathologic, molecular biological, and biochemical changes in mouse testes. The results of this study could shed light on novel solutions to doxorubicin-induced testicular damage, and may help decrease the incidence of serious side effects resulting from doxorubicin-based chemotherapy in clinical practice.

2. Materials and methods

2.1. Materials

Doxorubicin was purchased from Pfizer Italia S.R.L. (Milano, Italy). Anti-cytochrome *c* antibody was from Lab Vision Corp. (Fremont, CA, USA). Anti-prohibitin antibody was from Abcam (Cambridge, MA, USA). All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and chemicals from Sigma (St. Louis, MO, USA) or Fisher Scientific (Fairlawn, NJ, USA).

2.2. Animals and pharmacological treatments

Five-week-old ICR inbred male mice just near sexual maturity were obtained from the National Animal Center, housed under controlled conditioning (25 ± 1 °C constant temperature, 55% relative humidity, 12-h lighting cycle), and fed with standard pelleted diet and water during the study period. These animals were randomly assigned to four groups (10 mice per group) as described below according to the pharmacological treatment they received. Control group (Cont) mice received none but normal saline injection. Doxorubicin group (Dox) mice were given doxorubicin intraperitoneally at the dose of 3 mg/kg every 2 days for totally three shots and accumulated dose of 9 mg/kg [15]. Doxycycline group (Dc) mice received doxycycline 2.5 mg/kg intraperitoneally every 2 days for totally three times and accumulated dose of 7.5 mg/kg [16]. Doxycycline-doxorubicin group (DcDox) mice undertook the same dose of doxycycline followed by identical dose of doxorubicin (each dose given 1 day after doxycycline). The experimental procedures were approved by the Institutional Animal Care and Use Committee at Taichung Veterans General Hospital, and the study design was summarized in Fig. 1.

2.3. Harvest of serum and testicular samples

Mice were sacrificed at day 28 after the first administration of medications. The body and testicular weights of all group animals were measured and compared to their baseline values and between groups. Blood samples were drawn from the caudal vena cava, collected in test tubes containing EDTA, and centrifuged at $1500 \times g$ for 10 min to obtain serum. The testicular tissues were either fixed in 10% formalin for histopathologic examinations or stored at −80 °C in association with the serum samples till later analysis.

2.4. Epididymal sperm counts

The spermatozoa were collected and examined from caudal epididymis of each testicular sample as previously reported

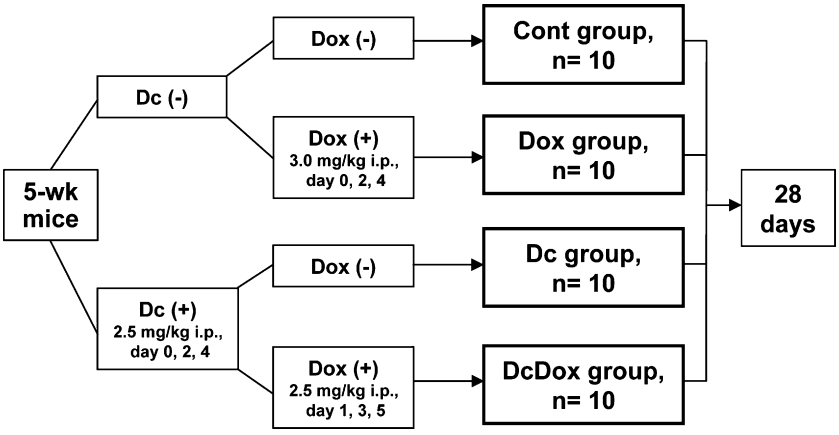

Fig. 1 – Schematic diagram of the experimental protocol.

[11]. Briefly, the epididymal cauda was cut into small pieces in a 35-mm Petri dish and placed in a centrifuge tube containing 3 ml of normal saline to let the sperms swim up for 10 min at 37 °C. After diluted with trypan blue solution, the specimen was transferred to the counting chamber of the hemacytometer and was allowed to stand for 5 min. The cells were then counted under light microscope. The total epididymal sperm numbers obtained from the counting were expressed as the number of sperms per testis ($\times 10^6$).

2.5. Histopathological examinations of spermatogenic structures

Testicular tissues for histopathological examinations were fixed in 10% buffered formalin overnight and then embedded with paraffin. Samples were sectioned at 5 μm and stained with hematoxylin and eosin (H&E). These specimens were examined under bright-field microscopy using a light microscope (Leica, DMR, Bensheim, Germany), and the corresponding digital images were captured for later analysis by a Spot CCD Camera driven by Advanced Spot RT Software version 3.3 (Diagnostic Instruments Inc., MI, USA). The total numbers and the average diameters of the seminiferous tubules were assessed under microscope at 100× magnification.

2.6. Oxidative stress in testicular tissue and in the circulation

Oxidative status in testicular tissues could be estimated from the concentrations of malondialdehyde (MDA) [11] and the activities of two representative anti-oxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) [17,18]. To measure these indicators, testicular tissues frozen at −80 °C were thawed and homogenized in a lysis buffer (0.25 M Sucrose, 10 mM Tris–HCl and 1 mM EDTA adjusted to pH 7.4); the homogenates were centrifugated at 10,000 × *g* for 20 min; the supernatants were saved; and the protein concentrations were determined using Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). The MDA assay was then carried out with the fluorometric method described earlier [20], which detected the reaction of the sample with thiobarbituric acid to quantitate the resultant lipid peroxidation as a surrogate of MDA level. The total SOD activity was estimated from the ability of the tissue to inhibit cytochrome *c* reduction in a xanthine–xanthine oxidase generation system (RANSOD, Randox Laboratories Ltd., Antrim, UK) [21]. The GPx activity was determined based on the UV method [22] using a commercially available kit (RANSEL, RANDOX Laboratories Ltd., Antrim, UK), which estimated the amount of tissue GPx from the reduction of cumene hydroperoxide and oxidation of nicotinadmide adenine dinucleotide phosphate diaphorase (NADPH) to positive nicotinadmide adenine dinucleotide phosphate ($NADP^+$), as reflected by the decrease in absorbance at 340 nm. The activity of Cu/Zn superoxide dismutase (SOD1) in testicular tissue and in the circulation was further analyzed by enzyme-linked immunosorbent assay (ELISA). In short, 96-well polyvinyl plates (Immulon II, Dynatech Labs, Chantilly, VA) were coated with 100 ng of total testicular protein or 10 ng of whole serum in 10 mM sodium carbonate, pH 9.6 and left overnight at 4 °C. Plates were subsequently incubated with 3% BSA for 30 min at room temperature to block nonspecific binding. Dilutions of rabbit anti-Cu/Zn SOD antibody ranging from 1:100 to $1:10^8$ were added and retained at room temperature for 1 h. HRP-conjugated goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA) was then added to react for 1 h. Plates were finally washed extensively and the chromogen orthophenylenediamine (Sigma, St. Louis, MO) was added to demonstrate activity. Plates were read with an ELISA reader (MrX, Dynatech Lab.) at a wavelength of 490 nm. The results were averaged from triplicate extracts of each specimen and expressed by their means.

2.7. Immunoblotting

Extraction of mitochondrial and cytosolic fractions of cellular proteins for analysis of cytochrome *c* was specifically described in Section 2.9. For immunoblotting analysis of other proteins, testicular tissues were lysed with a lysis buffer (50 mM Tris–HCl pH 7.5; 150 mM KCl; 5 mM $MgCl_2$, 0.25 M Sucrose, 0.1 mM dithiothreitol (DTT), 1 mM PMSF; 2 μg/ml leupeptin, 2 μg/ml pepstain A, and 1% NP-40), and were centrifugated at 10,000 × *g* at 4 °C for 20 min to obtain the cellular proteins in the supernatant. The protein concentration was determined by Bradford Protein Assay [19]. Equal amounts of proteins from each sample were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked in blocking buffer (150 mM NaCl in 10 mM Tris, pH 7.5 containing 5% non-fat dry milk) for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4 °C, washed three times (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20), incubated with HRP-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature, washed three times, and then detected with ECL (Pierce Chemical Co., Rockford, IL, USA). The density of each protein bands was scanned using Sciencelab Image-Guage 4.0 Software (Fuijifilm, Tokyo, Japan) and compared by densitometry.

2.8. Immunofluorescence microscopy

Freshly dissected testes were fixed in 4% paraformaldehyde and embedded in OCT compound (Sakura Finetek, Torrance, CA). The cryosections (10 μm in thickness) were mounted on glass slides, washed in PBS, and immersed in 3% BSA for 1 h to block nonspecific binding. These slides were then incubated with primary mouse antibodies against SOD1, Bcl-2 or Bax at dilutions of 1:100 for 18 h at 4 °C, washed twice in PBS/Tween-20 solution, incubated with a fluorescein- or Texas Red-conjugated secondary antibody for 1 h at room temperature, and photographed with a laser scanning confocal microscope (Leica, TCSNT, Bensheim, Germany). Certain sections were incubated with DAPI (Sigma, St. Louis, MO) containing mounting medium for nuclear counterstaining.

2.9. Abundance of mitochondrial and cytosolic cytochrome c

Cytochrome *c* lies primarily in the mitochondrial intermembrane space at resting state. Stimuli that induce release of cytochrome *c* from mitochondria to cytosol trigger a cascade of

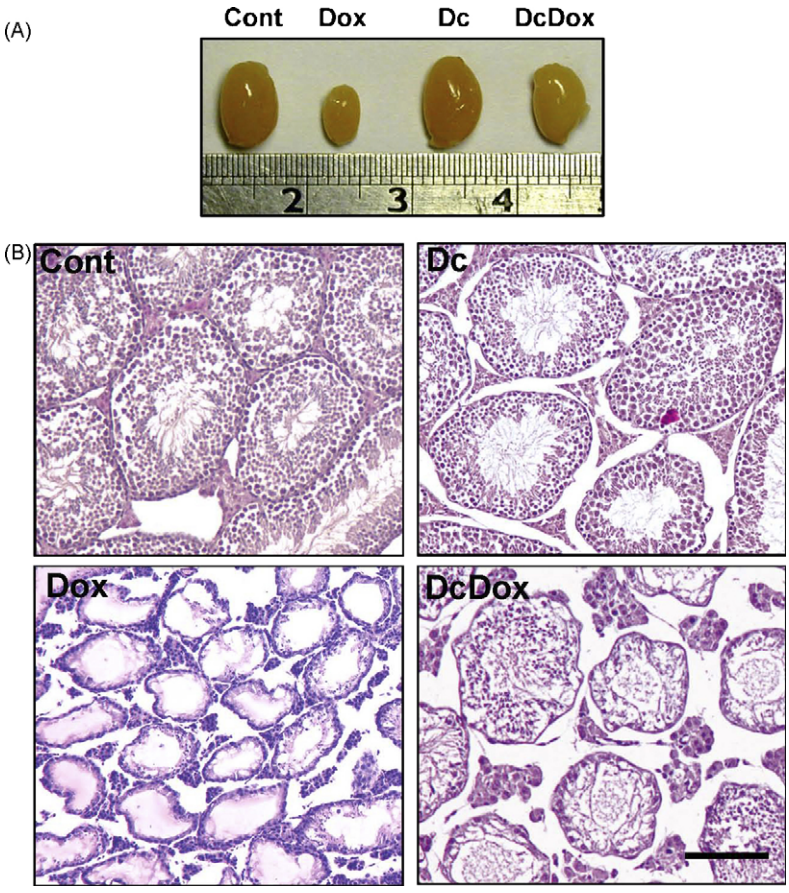

Fig. 2 – Gross and histological morphology of testes from all groups of mice. (A) Illustrations of representative testes in Cont, Dox, Dc, and DcDox groups, respectively. Dox group mice have obviously smaller testes as compared to the other three groups. (B) Cross-sections of testes stained with hematoxylin and eosin. Testes from Cont and Dc group mice exhibit typical features of seminiferous epithelium, while those from Dox group animals display impaired spermatogenesis and diminutive seminiferous tubules. In mice pretreated with doxycycline (DcDox), testes could be prevented from these doxorubicin-induced abnormalities, as characterized by the nearly normal histological morphology in the testicular section. Scale bar = 200 μm.

Table 1 – Effects of doxorubicin and doxycycline on body and testicular weights, sperm counts, and structure of spermatogenic tissues

	Cont	Dox	Dc	DcDox
N	10	10	10	10
Body weights (BW)				
Baseline (g)	27.46 ± 0.46	27.27 ± 0.64	28.06 ± 0.52	28.03 ± 0.36
Final (g)	39.80 ± 1.33	31.28 ± 0.78[§]	38.41 ± 1.25	36.18 ± 0.98[‡,**]
Gain (final vs. baseline, %)	32.30 ± 1.37	14.39 ± 2.07[§]	30.11 ± 0.85	27.24 ± 1.11[‡,#]
Testicular weights (TW)[a]				
Absolute (g)	0.24 ± 0.11	0.11 ± 0.01[§]	0.25 ± 0.11	0.19 ± 0.03[‡]
Relative (vs. final BW, %)	0.56 ± 0.06	0.36 ± 0.03[§]	0.66 ± 0.04	0.52 ± 0.06[†]
Epididymal sperm counts ($\times 10^6$)[a]	2.35 ± 0.27	0.09 ± 0.01[§]	2.45 ± 0.20	1.62 ± 0.02[‡]
Seminiferous tubules				
Number	201.75 ± 7.00	150.50 ± 6.22[†]	211.25 ± 25.52	171.00 ± 15.72
Diameter (μm)	320.05 ± 1.47	192.23 ± 10.67[§]	325.50 ± 9.08	270.01 ± 4.10[§,**]

[a] Sums of right and left testes.
[**] $p < 0.001$ vs. Dox group.
[†] $p < 0.05$ vs. Cont group.
[‡] $p < 0.01$ vs. Cont group.
[§] $p < 0.001$ vs. Cont group.
[#] $p < 0.01$ vs. Dox group.

cell signaling and leads to apoptosis. The activity of this mitochondrion-dependent apoptotic process could be estimated from the ratio of the cytosolic to the mitochondrial cytochrome *c* fractions. To investigate whether doxorubicin and doxycycline modulate this apoptotic factor, testicular tissues were washed with 10 ml of ice-cold PBS buffer and centrifugated at 600 × *g* for 5 min at 4 °C. The supernatants were removed, and the pellets were resuspended on ice with 1 ml of Cytosol Extraction Buffer Mix (BioVision, Mountain View, CA, USA) containing DTT and protease inhibitors. Ten minutes later, these tissues were homogenized by an ice-cold glass Dounce tissue grinder on ice for 15 times, and were then centrifugated at 700 × *g* for 10 min at 4 °C to remove the pellets. The supernatants were further centrifugated at 10,000 × *g* for 30 min at 4 °C to separate the cytosolic (in the supernatant) and the mitochondrial (in the pellets) fractions of the cytoplasmic proteins. The mitochondrial fraction were finally resuspended with Mitochondrial Extraction Buffer Mix (BioVision, Mountain View, CA, USA) containing DTT and protease inhibitors, and vortexed for 10 s to obtain the mitochondrial protein [23]. The purity of the mitochondrial and cytosolic fractions thus obtained was analyzed via immunoblotting for a reliable mitochondrial marker, prohibitin. Demonstration of the existence of this protein in the mitochondrial but not in the cytosolic protein extracts could confirm the purity of these protein fractions. To determine the relative abundance of cytochrome *c* in the compartments of mitochondria and cytosol, 50 μg of proteins from cytosolic and mitochondrial extracts were resolved by 15% SDS-PAGE and underwent immunoblotting analysis as described in Section 2.7.

2.10. Determination of caspase-3 activity

Caspase-3 activity in the testicular tissue was measured with the colorimetric CaspACE assay system (Promega, Madison, WI). Briefly, samples were lysed in 50 μl Cell Lysis Buffer and centrifugated a 10,000 × *g* for 20 min. The supernatants were collected and the protein concentrations determined using Bradford Protein Assay. The protein lysates (50 μg) were added to and incubated with the caspase assay buffer containing 20 mM of caspase-3 substrate Ac-DEVD-pNA at 37 °C for 4 h. Reaction mixture without testis extract was used as negative control. Production of yellow color released from the substrate upon cleavage by caspase-3 was monitored with spectrofluorometry (Hitachi U-1500) at 405 nm, and the amount of yellow color detected was proportional to the caspase-3 activity present in the sample.

2.11. In situ detection of apoptosis

Apoptosis of testicular cells was identified by TUNEL assay using In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). Paraffin sections of testicular tissue (5 μm) were deparaffinized in xyline and rehydrated in a graded ethanol series (100%, 95% and 70%). The slides were incubated with Proteinase K (20 μg/ml) at room temperature for 15 min and then washed with PBS solution. The sections were then treated with equilibrium buffer for 10 min and incubated with a terminal deoxynucleotidyl transferase (TdT) reaction mixture for 1 h at 37 °C in a humidified chamber to catalyze the addition of fluorescein-dUTP labels at free 3′-OH groups in single- and double-stranded DNA breaks. The sections were counterstained with DAPI after twice washing with PBS solution, and the label incorporated at the damaged sites of the DNA visualized by fluorescence microscopy signaled the occurrence of apoptosis.

2.12. Statistical analysis

All experiments were repeated at least 3 times, and one representative from these experiments with similar results

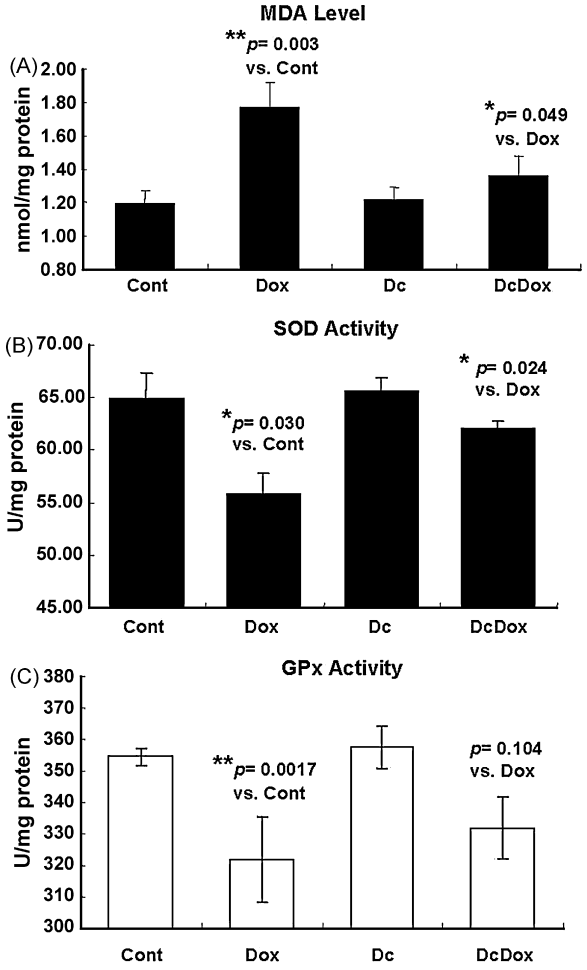

Fig. 3 – Effects of doxorubicin and doxycycline on levels of MDA and activities of anti-oxidants (SOD and GPx) in the testicular tissue. (A) Doxorubicin (Dox) drastically increases the MDA level as compared to the controls (Cont), whereas doxycycline pretreatment (DcDox) significantly alleviates this effect. (B) Doxorubicin (Dox) markedly depresses SOD activity as compared to the controls (Cont), while doxycycline pretreatment (DcDox) could significantly offset this consequence. (C) Doxorubicin (Dox) also notably suppresses GPx activity in the testicular tissue, but the neutralizing effect of doxycycline pretreatment (DcDox) on this impact of doxorubicin on GPx does not reach statistical significance. Values are presented as mean ± S.E.M. ($n = 3$). Significant difference is indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$.

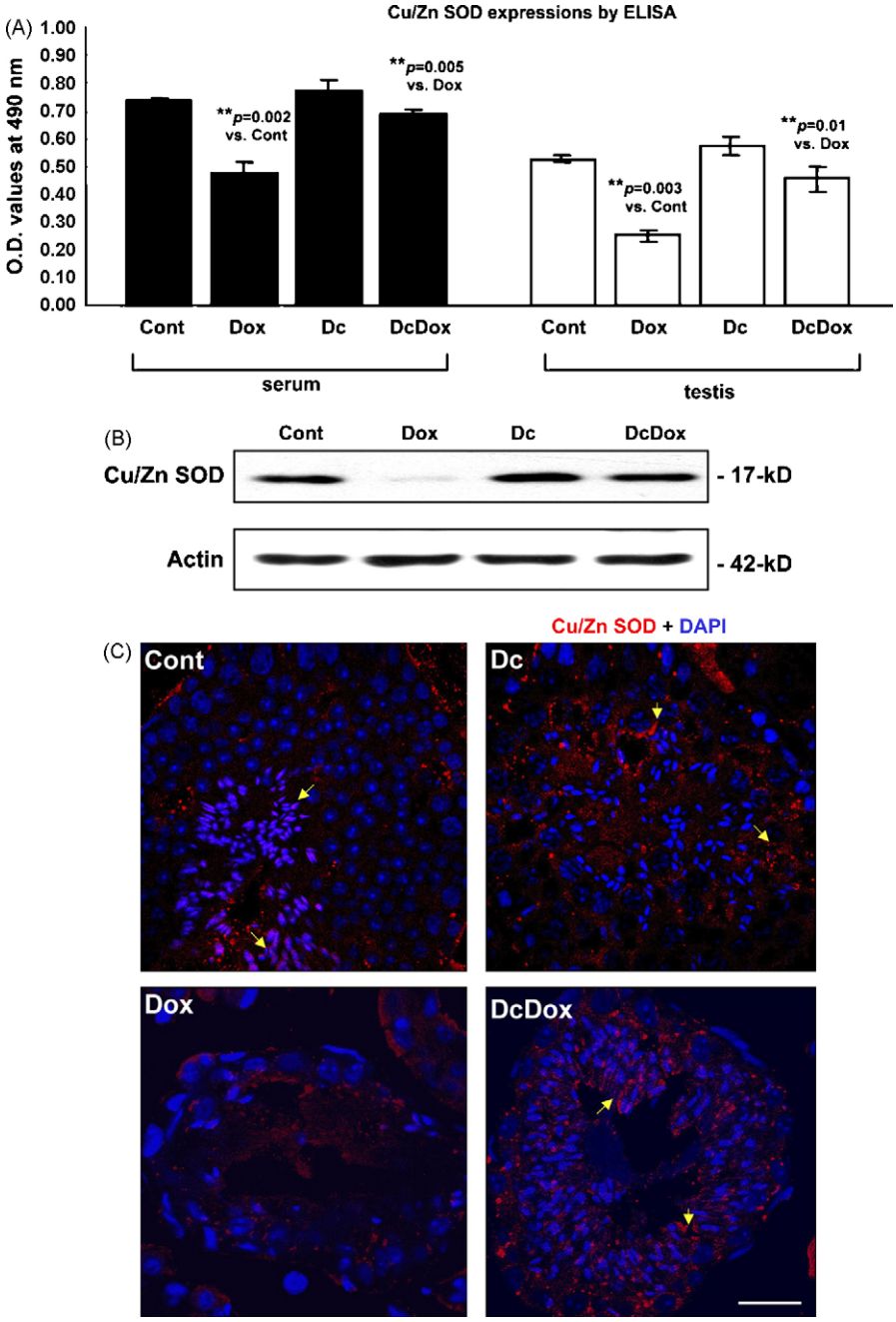

Fig. 4 – Effects of doxorubicin and doxycycline on Cu/Zn SOD (SOD1) expression. (A) ELISA study. Cu/Zn SOD expression is significantly depressed in serum and testicular tissues of doxorubicin group animals (Dox) as compared to the controls (Cont), while doxycycline pretreatment (DcDox) could strikingly offset this doxorubicin effect. OD, optical density. Values are obtained from three independent experiments and expressed as mean ± S.E.M. Significant difference is indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. (B) Immunoblotting study. Abundance of Cu/Zn SOD in testicular extracts of doxorubicin-treated mice (Dox) is markedly diminished unless these animals have been already pretreated with doxycycline (DcDox). (C) Immunofluroscence study of the spatial expression of Cu/Zn SOD protein. Images are phase-contrast micrographs of seminiferous tubules in the testes. Typically, the Cu/Zn SOD-stained signals should exist densely in spermatids near the seminiferous lumen (arrows). In doxorubicin-treated mice (Dox), the Cu/Zn SOD signal intensity is remarkably scarcer compared to controls (Cont). The signal density of this anti-oxidant enzyme is, however, largely preserved in doxycycline-pretreated animals (DcDox). Red fluorescence indicates the location of Cu/Zn SOD, and blue represents testicular sections counterstained with DAPI. Scale bar = 20 μm.

was provided. The quantitative data of continuous variables were expressed as mean ± S.E.M. The statistical significance was tested by Student's *t*-test or ANOVA with post hoc analysis when appropriate. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Body weights, testicular weights, and spermatogenic structures

During the 4-week study period doxorubicin-treated mice displayed progressive physical exhaustion, hair loss, and decreased activity as compared to the rest three groups. These animals also achieved much less weight gain as compared to the controls (14% versus 32%, $p < 0.001$). In contrast, animals in the DcDox group gained significantly more body weight than the Dox group mice (27% versus 14%, $p = 0.04$) though still less than the controls (27% versus 32%, $p = 0.01$), suggesting that pretreatment with doxycycline could partially antagonize the unfavorable effects of doxorubicin on body growth. Similar results could also be demonstrated specifically on the testicular tissues. The gross size of testes from Dox group mice was significantly smaller than the control and the DcDox group animals (Fig. 2A). Microscopically, doxorubicin resulted in dramatic depletion of spermatogonia, spermatocytes and spermatids, but this abnormality was mitigated by doxycycline pretreatment (Fig. 2B). Quantatively, the epididymal sperm counts (0.09×10^6 versus 2.35×10^6, $p < 0.001$), the number of testicular tubules (150.5 versus 201.75, $p = 0.001$), and the diameters of seminiferous tubules (192.23 versus 320.05 μm, $p < 0.001$) were significantly lower in the Dox mice compared to the controls (Table 1). The impact of doxorubicin on testicular growth was even more obvious than the body growth as shown by the significantly lower relative testicular weight to the whole body weight (0.36% versus 0.56%, $p < 0.001$). Again, pretreatment with doxycycline could largely counteract the atrophic effect of doxorubicin on testes and significantly preserve spermatogenic structures and functions.

3.2. Doxycycline prevented doxorubicin-induced upregulation of oxidative stress and downregulation of anti-oxidant enzyme activity in testes

The MDA level reflects the oxidative stress the tissue confronts [11]. Testes from Dox group mice displayed significantly higher MDA levels than the controls ($p = 0.03$), while pretreatment with doxycycline significantly reversed this MDA upregulation (DcDox versus Dox, $p = 0.049$, Fig. 3A), suggesting the antagonistic action of doxycycline on doxorubicin-imposed oxidative burden in testicular tissues.

Activities of anti-oxidant enzymes reflect either the level of oxidative stress the tissue encountered or the opposing capability against oxidative stress the tissue owned [18]. The activities of two representative anti-oxidant enzymes, SOD and GPx, in testes of all groups of mice were illustrated in Fig. 3B and C. Compared with the controls, doxorubicin-treated testes possessed significantly less activity of SOD ($p = 0.03$) and GPx ($p = 0.0017$), whereas doxycycline pretreatment could effectively prevent doxorubicin-induced reduction of SOD activity (versus Dox, $p = 0.024$, Fig. 3B) yet not GPx activity (versus Dox, $p = 0.104$, Fig. 3C).

Since Cu/Zn SOD (SOD1) represents the predominant isoform of SOD in testicular tissue [24], the activity of Cu/Zn SOD in testicular tissues as well as in serum were specifically examined by ELISA. As shown in Fig. 4A, the expression of Cu/Zn SOD in Dox group mice was significantly decreased as compared to controls in both serum ($p = 0.002$) and testis ($p = 0.003$). Doxycycline pretreatment successfully alleviated the reduction of Cu/Zn SOD expression by doxorubicin ($p = 0.005$ for serum and $p = 0.01$ for testis). Similar results could also be demonstrated by immunoblotting and immunofluroscence analyses (Fig. 4B and C).

3.3. Doxycycline suppressed pro-apoptotic effects of doxorubicin through inhibiting cytochrome c release, caspase-3 cleavage, and caspase-3 activity

Apoptosis is a well-known cellular action of doxorubicin. Our immunoblotting and immunofluroscence analyses confirmed that doxorubicin would upregulate the expression of pro-apoptotic proteins (Bax, Bad) as well as decrease the abundance of anti-apoptotic factors (Bcl-2, Bcl-xL) in testicular cells, and pretreatment with doxycycline could largely antagonize these pro-apoptotic effects of doxorubicin (Fig. 5A–C).

Release of cytochrome *c* from mitochondria and subsequent activation of caspase-3 represents a key step in the mitochondrion-dependent apoptotic pathway [25]. To determine whether doxycycline exerts its anti-apoptotic action against doxorubicin via this mechanism, mitochondrial and cytosolic cytochrome *c* abundance, cleaved caspase-3 concentration, and caspase-3 activity were examined. First, the purity of the mitochondrial and cytosolic proteins was affirmed by the exclusive presence of the mitochondrial marker prohibitin [26] in the mitochondrial extracts and the absence of this marker in the cytosolic protein fractions (Fig. 6A). Immunoblotting analysis then showed that cytosolic cytochrome *c* density was markedly elevated from 23 to 56% of the total cellular abundance (cytosolic plus mitochondrial) in doxorubicin-treated animals, whereas pretreatment with doxycycline significantly limited the fractional concentration of cytosolic cytochrome *c* to only 34%, which might otherwise be much greater in response to subsequent doxorubicin treatment (Fig. 6A). Similarly, doxorubicin significantly increased caspase-3 cleavage as reflected by increased concentration of cleaved caspase-3, but pretreatment with doxycycline could largely inhibit this reaction (Fig. 6A). Quantitatively, doxorubicin caused 2.52-fold increase in caspase-3 activity as compared to controls ($p = 0.016$), whereas doxycycline pretreatment effectively suppressed activation of this pro-apoptotic protein (versus Dox, $p = 0.011$) (Fig. 6B).

On TUNEL assay, the Dox group testes contained significantly more positively stained (i.e. apoptotic) cells than the controls, and the vast increase in apoptotic cells could be attenuated by doxycycline pretreatment (Fig. 6C), further affirming the pro-apoptotic action of doxorubicin and the anti-apoptotic role of doxycycline in testes.

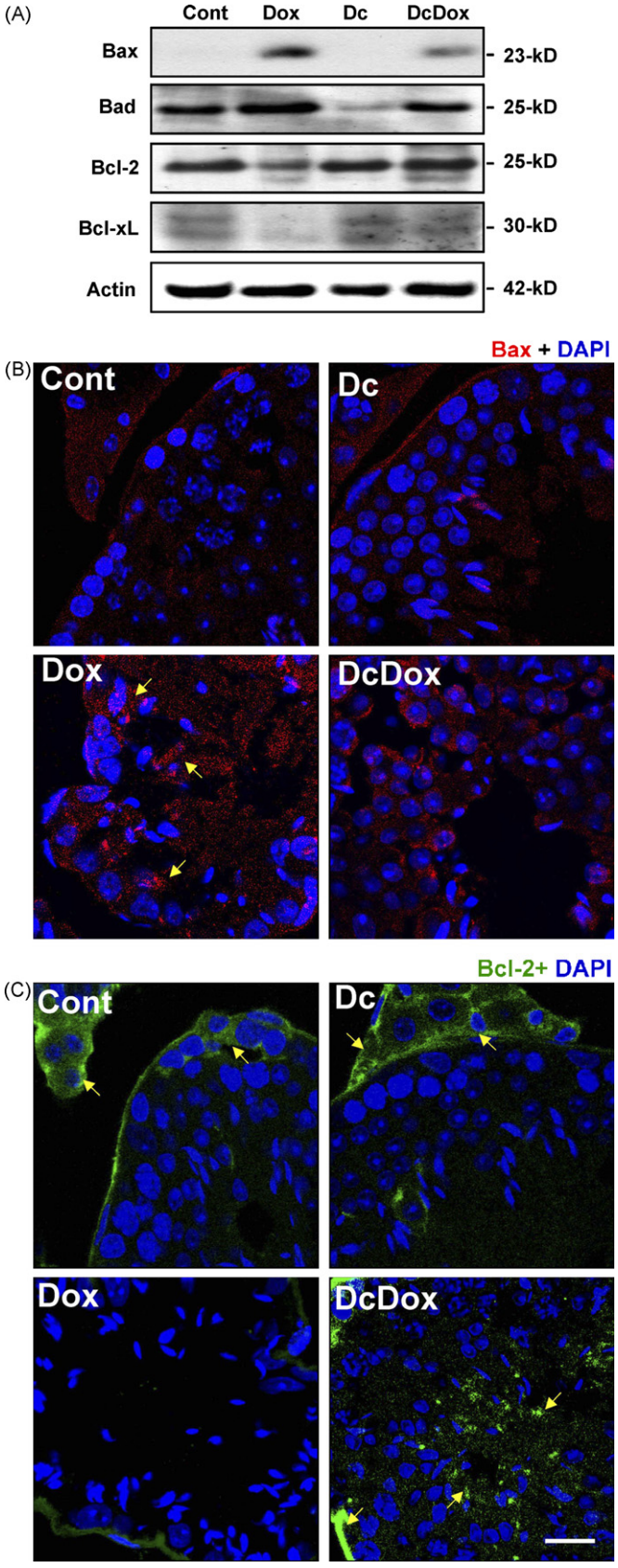

Fig. 5 – Counteracting effects of doxycycline on doxorubicin-modulated expressions of Bcl-2 family proteins in mouse testes. (A) Immunoblotting study. Representative sets of data from three independent experiments are shown. Significant difference is indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. Doxorubicin would substantially upregulate the crucial pro-apoptotic factors (Bax, Bad) and downregulate the key anti-apoptotic proteins (Bcl-2, Bcl-xL) of the Bcl-2 family in testicular tissue,

4. Discussion

Doxorubicin has long been one of the most extensively used chemotherapeutic agents for treatment of various cancers. The clinical application of this drug is, however, complicated by its potential toxicity to the heart [5], liver [6], and kidney [7]. Recent researches also demonstrate its serious disturbing impact on spermatogenesis [11,27]. The present study further provides evidence that doxorubicin retards testicular growth and impairs spermatogenic function mainly through eliciting oxidative stress and cell apoptosis, while pretreatment with the tetracycline-derived antibiotic doxycycline could effectively prevent these toxic effects. Since chemotherapy with doxorubicin carries the risk of severe testicular damage and even infertility, introduction of doxycycline as an antagonist against doxorubicin in this study may raise this drug a potential therapeutic adjuvant that could protect the testicular tissue from doxorubicin-induced cytotoxicity.

4.1. *Effects of doxorubicin on testicular germ cells*

The major cytotoxic action of doxorubicin to eradicate malignant tumors is to intercalate the DNA backbone of rapidly growing cells and to interfere with cell division [4]. However, its dose-related side effects, chiefly production of reactive oxygen species and induction of cell apoptosis, could adversely damage various non-target tissues that are not the goal of treatment [4,11,28]. Findings from this study that testes exposed to doxorubicin are subject to retardation of growth, degeneration of spermatogenic structures, and depressed yields of sperms implicate that spermatogenic cells represent another vulnerable target of doxorubicin. Though the mechanism for the exceedingly susceptibility of testes to doxorubicin toxicity remains unclear, ample evidence has implied that the fragile characteristics of the sperm cells in response to oxidative stress should be largely attributable. The spermatozoa membranes are uniquely rich in polyunsaturated fatty acids so are liable to lipid peroxidation [29]. The sperm DNA is exceptionally prone to damage from reactive oxygen species that may attack the integrity of DNA and accelerate the process of germ cell apoptosis [30]. Besides, the anti-oxidant defense system in testicular tissue is inherently deficient in a potent component of the SOD family, Mn-SOD (SOD2) [24]. Moreover, our data illustrate that at least two important members of the endogenous anti-oxidant enzymes responsible for converting superoxide radicals and protecting cells from oxidative injury, i.e. SOD and GPx, are markedly downregulated by doxorubicin in testes. These factors together well render the testes extraordinarily inclined to doxorubicin toxicity. Based on this concept, application of anti-oxidant agents to counteract doxorubicin-related side effects has been widely studied [21,31]. These researches open the window that anti-oxidants could possibly protect the testes from doxorubicin injury.

Apoptosis is a delicately controlled process of programmed cell death that could be completed through either mitochondrion-dependent or mitochondrion-independent pathway. Doxorubicin is a well-known pro-apoptotic agent, and our results demonstrate that it can induce apoptosis of testicular cells by stimulating at least the mitochondrion-dependent pathway, since the key trigger step responsible for this mechanism (cytochrome *c* release) was activated in doxorubicin-treated tissues. The markedly reduced sperm counts in testes exposed to doxorubicin are therefore deemed the consequences of both increased destruction (apoptosis of sperm cells) and decreased production (injury of spermatogenic structures) of sperm cells. These results emphasize the importance of neutralizing sperm cell apoptosis to lessen doxorubicin toxicity, as shown in researches employing anti-apoptotic agents on various tissues to oppose doxorubicin adverse effects [32,33].

4.2. *Protection by doxycycline against doxorubicin cytotoxicity in testes*

Doxycycline can reduce oxidative burst of neutrophils in patients with acute myocardial infarction and modulate post-infarction left ventricular remodeling [34,35]. It can also decrease cytokine-induced NO production and ameliorate systemic inflammation [36]. Results from our study further demonstrate that doxycycline could effectively counteract doxorubicin-induced oxidative injury in testicular tissues through ameliorating overall oxidative stress as well as activating SOD activity, therein confirming the helpful role of doxycycline as a promising anti-oxidant against doxorubicin toxicity in testes. Additionally, although this study failed to reveal statistically significant beneficial effect of doxycycline on GPx activity, the relatively small animal number might be responsible for this negative result; therefore, the influence of adjuvant doxycycline on doxorubicin-induced GPx depression might merit reassessment in further researches of larger series.

Importantly, the findings that doxycycline pretreatment prevents doxorubicin-induced diminishment of anti-apoptotic Bcl-2/Bcl-xL, augmentation of pro-apoptotic Bax/Bad, release of mitochondrial cytochrome *c*, production of cleaved caspase-3, and increase of TUNEL-positive testicular cells clearly depict that doxycycline could effectively protect the testicular cells from doxorubicin apoptotic injury through interfering in at least the mitochondrion-dependent pathway. As doxycycline could also effectively lessen the extent of oxidative stress, another important trigger factor of apoptosis

while these apoptosis-provoking reactions could be considerably counteracted by doxycycline pretreatment. (B) Immunofluroscence analysis of the spatial expression of Bax. Increased expression of Bax (red signals) could be observed in testicular sections from doxorubicin-treated mice (arrows), and doxycycline pretreatment (DcDox) greatly suppresses this phenomenon. (C) Immunofluroscence analysis of the spatial expression of Bcl-2. The signal density of Bcl-2 protein (green fluorescence) typically exists in the seminiferous epithelium (arrows) but is greatly diminished in the Dox group testes. Again, doxycycline pretreatment (DcDox) could immensely save its expression. The blue color represents testicular sections counterstained with DAPI. Scale bar = 10 μm.

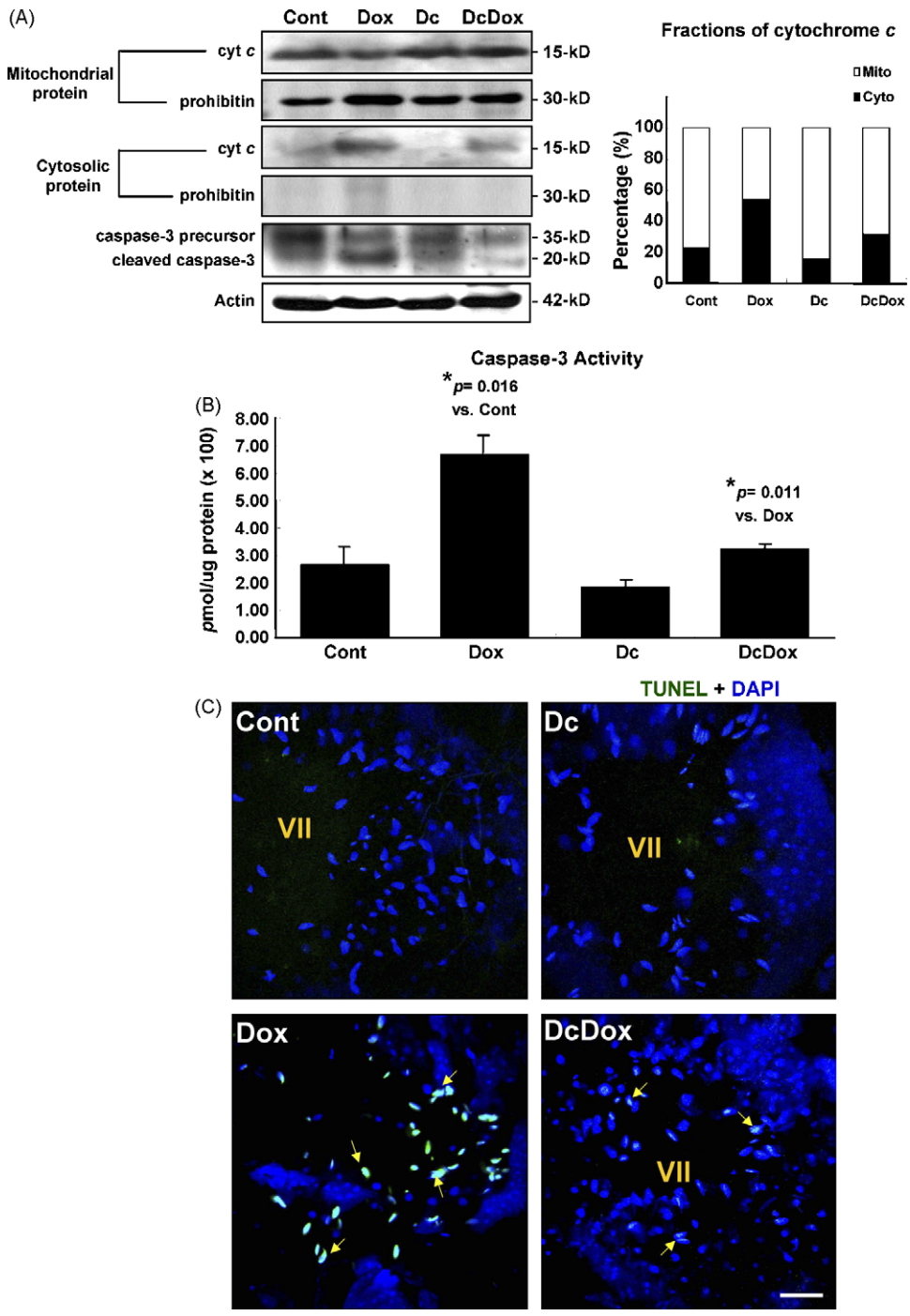

Fig. 6 – Protection by doxycycline against doxorubicin-induced cytochrome c release, caspase-3 cleavage, and TUNEL index increase in mouse testes. (A) Immunoblotting study. The abundant presence of the mitochondrial marker, prohibitin, in the mitochondrial extracts and the absence of this marker in the cytosolic fractions (columns 2 and 4) confirms the reliable purity of both protein fractions analyzed in this study. Expressions of cytosolic cytochrome c and cleaved caspase-3 are profoundly increased by doxorubicin, but this intensity is ameliorated by doxycycline pretreatment (columns 3 and 5). Quantatively, the cytosolic fraction of cytochrome c increases from 23 to 56% of the total contents in doxorubicin-treated mice, yet only to 34% when pretreated with doxycycline (bar chart). Mito, mitochondrial; Cyto, cytosolic. (B) Statistical analysis. Doxorubicin soundly elevates caspase-3 activity by 2.52-fold, and doxycycline pretreatment effectively prevents activation of this apoptotic factor. Values were obtained from three independent experiments and represented as mean ± S.E.M. Significant difference is indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. (C) TUNEL assay to identify apoptotic cells in testicular sections at stages VII seminiferous cycle. Doxorubicin-treated testes contain significantly more TUNEL positive (green color) apoptotic cells, and the increase in apoptotic cells is significantly attenuated by doxycycline pretreatment (arrows). Blue color represents testicular sections counterstained with DAPI. Scale bar = 10 μm.

through not yet clarified mechanism [37], it can be rationally deduced that doxycycline could potently suppress possibly two major upstream apoptotic signals transduced by doxorubicin (one mitochondrion-dependent and the other oxidative stress-related). Whether doxycycline could also directly block the mitochondrion-independent pathway through inhibiting specific upstream apoptotic receptors or downstream caspase enzymes awaits further study to clarify.

In conclusion, this study provides firm evidence that doxorubicin can adversely damage the testicular tissue and significantly reduce sperm production through imposing oxidative stress and inducing apoptosis, while doxycycline pretreatment could effectively prevent these adverse effects. Since doxorubicin-based chemotherapy is still indispensable for treatment of various cancers yet the resultant drug-induced infertility is devastating and often unavoidable, our results raise the hope that co-administration of doxycycline with doxorubicin may be a promising solution to this otherwise very serious complication of doxorubicin in testes.

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