

## Protective effect of tetrandrine on doxorubicin-induced cardiotoxicity in rats

Meng Xu, Lianghe Sheng, Xinhai Zhu, Shibin Zeng, Dexiang Chi, and Guo-jun Zhang

Department of Oncology, The First Affiliated Hospital, Jinan University, Guangzhou 510630, China

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### ABSTRACT

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**Aims and background.** Doxorubicin (Dox) is effective in curative and adjuvant chemotherapy of malignant tumors. Cardiotoxicity is the chief toxic effect that limits the clinical use of Dox. We studied the effects of tetrandrine (Tet) on doxorubicin-induced cardiotoxicity in rats and its protective activity.

**Materials and methods.** Sprague-Dawley rats were randomly divided into the following 4 groups: a control group (received only saline), Dox group (received only Dox), Tet/Dox group (received Tet plus Dox), and Tet group (received only Tet). Rats were injected intravenously with 2 mg/kg Dox once a week for 7 weeks and 50 mg/kg Tet was administered intraperitoneally weekly for 7 weeks. Measurements of cardiac contractile parameters including LSVP, +dP/dt max and -dP/dt max, and assessment of electrocardiograms were carried out. Mitochondrial oxidation and phosphorylation state 3 (S<sub>3</sub>) and state 4 (S<sub>4</sub>) respiration were measured. Respiration control rate (RCR) and the ADP/O ratio were calculated. Cardiac ultrastructure was examined by electron microscopy.

**Results.** Dox induced significant cardiotoxicity in this rat model. The values of LSVP, +dP/dt max, and -dP/dt max in the Tet/Dox group increased as compared to the Dox group ( $P < 0.05$ ). The cardiac contraction and relaxation improved on Tet administration. Tet inhibited the prolonged QT interval on the electrocardiogram in Dox-treated rats. Compared to the Dox group, the values of S<sub>3</sub>, RCR, and ADP/O increased by more than 28%, 48%, and 27%, respectively, in the Tet/Dox group. Significant cardiac morphological protection was observed in the Tet/Dox-treated rats.

**Conclusion.** Tet can improve the reduced cardiac function caused by Dox treatment and prevent Dox-induced mitochondrial impairment in rat cardiotoxicity. Free full text available at [www.tumorionline.it](http://www.tumorionline.it)

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### Introduction

The anthracycline antibiotic doxorubicin (Dox) is used widely in the chemotherapy of breast cancer, liver carcinoma, and non-Hodgkin's lymphoma. Dox is effective in curative and adjuvant chemotherapy and in the palliation of symptoms of malignancy in middle-to-late stage disease. However, cardiotoxicity is the chief toxic effect that limits the clinical use of Dox<sup>1,2</sup>. The onset of cardiac dysfunction often occurs after a total dose of 450-550 mg/m<sup>2</sup> Dox has been injected. Combination chemotherapy with other chemotherapeutic drugs, such as paclitaxel, and mediastinal radiotherapy may aggravate Dox-induced cardiomyopathy, making it potentially lethal<sup>3</sup>.

The mechanisms of Dox-induced cardiotoxicity have been investigated<sup>4,5</sup>. Dox can lead to increased lipid peroxidation and oxidative damage to cardiac muscle. Free radical formation and cardiac DNA and membrane injuries are major aspects of Dox-induced cardiotoxicity<sup>6</sup>. Treatment schedules that include cardioprotectants have been developed to ameliorate Dox-induced toxicity.

**Key words:** doxorubicin, cardiotoxicity, oxidative phosphorylation, tetrandrine.

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**Correspondence to:** Dr Meng Xu, Department of Oncology, The First Affiliated Hospital, Jinan University, Guangzhou 510630, China. Tel +86-20-38688645; fax +86-20-38680000; e-mail [xug@fimmu.com](mailto:xug@fimmu.com)

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Tetrandrine (Tet) was isolated from *Stephania tetrandra* S. Moore, and possesses notable pharmacological properties inducing antiinflammatory, antiedemic, antihypertensive, antiarrhythmic, antifibrotic, and antitumor effects<sup>7</sup>. Tet is an effective multidrug resistance (MDR) modulator for reversing p-glycoprotein-mediated MDR cancers. In our previous research, Tet was found to exhibit significant efficiency in combination treatment of lung cancer with chemotherapy<sup>8</sup>. It appeared that Tet may be a potential protective agent.

The aim of this study was to examine the cytoprotective effects of Tet in preventing Dox-induced cardiotoxicity in a rat model.

## Methods

### *Reagents and chemicals*

Dox was purchased from Hisun Pharmaceutical Inc. (Zhejiang, China). Tet was purchased from Kanghong Chemical Co. (Chendu, China). Adenosine diphosphate (ADP), ethylene glycol tetraacetate acid (EGTA), and the routine chemicals for the isolation of heart mitochondria were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *Experimental animals*

Adult male Sprague-Dawley rats weighing 180-200 g were purchased from Southern Medical University (Guangzhou, China). The rats were kept in specific pathogen-free animal rooms and fed standard laboratory chow and tap water. The rats were randomly divided into the following 4 groups: a control group (received only saline,  $n = 8$ ), Dox group (received only Dox,  $n = 8$ ), Tet/Dox group (received Tet plus Dox,  $n = 8$ ), and Tet group (received only Tet,  $n = 8$ ). Rats were injected intravenously with 2 mg/kg Dox once a week for 7 weeks and 50 mg/kg Tet was administered intraperitoneally weekly for 7 weeks. All rats were anesthetized with sodium pentobarbital on day 7 after the last Dox injection and subjected to experimental evaluation. All procedures and protocols were conducted in accordance with the guidelines of the Laboratory Animal Center of Jinan University. Experiments were approved by the Committee on Laboratory Animal Research of Jinan University.

### *Measurements of cardiac contractile parameters*

The rats were anesthetized and the right carotid artery was exposed. A catheter filled with heparinized saline was inserted into the artery and left ventricle. Left ventricular pressure was recorded by a pressure transducer (PowerLab/4SP, ADInstruments, UK). The left ventricular systolic pressure (LSVP) was monitored. The maximal value recorded for the contraction rate (+dP/dt max) was used as the inotropism index, and

the minimal value (-dP/dt max) served as the relaxation index.

### *Assessment of electrocardiogram (ECG)*

The electrocardiogram system, which consisted of mini-electrodes, transmitters and a receiver, was obtained from Data Sciences International (DSI, St. Paul, MN, USA). The data acquisition system consisted of a MacLab, which was connected to an Apple Macintosh computer with the Chart MacLab program. For interpretation of the ECG, 3 consecutive complexes were analyzed in detail. The PR segment, QT interval, and RR interval were determined. The rate meter from the program Chart was used for automatic calculation of the heart rate from the ECG input.

### *Mitochondrial oxidation and phosphorylation*

Hearts were removed and placed into ice-cold isolation medium (280 mM sucrose, 5 mM Tris-HCl, and 0.5 mM EGTA), homogenized using a homogenizer, and centrifuged (2,000 rpm; Beckman J2-21 rotor; 10 min). The supernatant was recentrifuged (9,000 rpm; 10 min) and the resulting pellet was resuspended in the isolation medium without EGTA. Mitochondrial oxygen consumption was measured polarographically in a closed reaction vessel. After a brief equilibration period, state 3 ( $S_3$ ) respiration was induced by addition of 280 nmol ADP. After all the added ADP was phosphorylated to adenosine triphosphate (ATP), state 4 ( $S_4$ ) respiration was measured. The ratio of oxygen consumption in the presence of ADP to that in its absence (respiration control rate, RCR) and the ADP/O ratio were calculated as indices of oxidative and phosphorylative mitochondrial function. RCR = oxygen consumption in  $S_3$ /oxygen consumption in  $S_4$ . ADP/O = mol of ATP formed from ADP per atom of oxygen consumed. State 3 and  $S_4$  respiratory rates are reported as ng atoms of oxygen per mg mitochondrial protein per min.

### *Detection of cardiac ultrastructure by electron microscopy*

Cardiac samples were fixed with 2.5% glutaraldehyde and then with 1% osmic acid. Using standard embedding techniques, dehydration, osmosis, epoxy resin, ultrathin sectioning with a LAB-2088V ultramicrotome, and double staining with uranium acetate and lead citrate, samples were observed and photos taken using an electron microscope (JEM-1200EX, JEOL, Japan).

### *Statistical analysis*

Results are reported as mean  $\pm$  standard error of mean (SEM). Statistical significance between 2 measurements was determined by the 2-tailed unpaired Student's *t*-test. *P* values  $<0.05$  were considered statistically significant.

## Results

### Effects of Tet on cardiac function of Dox-induced cardiotoxicity

The values of LSVP, +dP/dt max, and -dP/dt max (Table 1) decreased significantly in the Dox group as compared to the control group ( $P < 0.05$ ), indicating that cardiac function in the left ventricle and cardiac contractility were damaged, whereas the values of LSVP, +dP/dt max, and -dP/dt max in the Tet/Dox group increased as compared with the Dox group ( $P < 0.05$ ). Tet could improve the reduced cardiac function induced by Dox, which showed that cardiac contraction and relaxation were improved. The QT interval recorded by ECG was calculated as the duration between the Q wave and the apex of the T wave, indicating the late repolarization phase. The QT interval in the Dox group was more prolonged than that in the control group (Figure 1), which showed that the duration between depolarization and repolarization in the ventricle increased. However, Tet inhibited the prolonged QT interval change in the ECGs of Dox-treated rats.

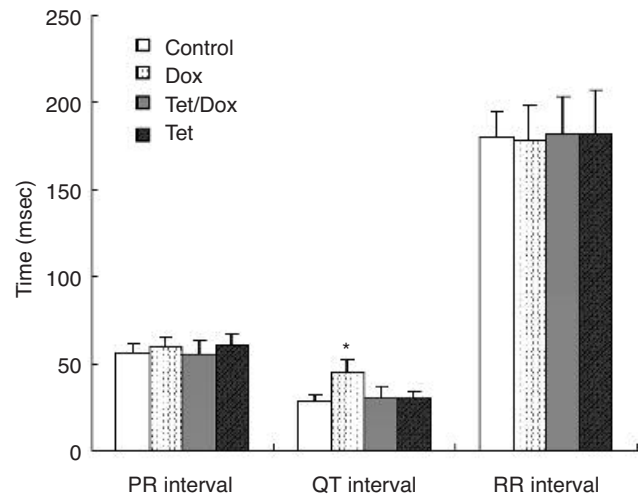
### Changes in mitochondrial oxidative phosphorylation after treatment with Tet and Dox

As shown in Table 2, Dox produced the inhibition of  $S_3$  respiration from  $205 \pm 36$  to  $135 \pm 17$  ng atoms of O/min/mg protein in the Dox group; under the same experimental conditions,  $S_4$  respiration increased from  $41 \pm 5$  to  $53 \pm 8$  ng atoms of O/min/mg protein. RCR provided an index of mitochondrial integrity. The combination of decreased  $S_3$  and increased  $S_4$  respiration produced a reduced RCR and loss of respiratory control after treatment with Dox. The ADP/O ratio represents the number of ADP molecules phosphorylated/mol of oxygen atoms consumed and is used as an index of mitochondrial efficiency. Compared to the Dox group, the values of  $S_3$ , RCR, and ADP/O increased by more than 28%, 48%, and 27%, respectively, in the Tet/Dox group. The phosphorylating capacity, expressed as ADP/O, indicated that cardiac mitochondria in the Tet/Dox group maintained a better ability to couple oxidation with phosphorylation. This enhanced oxidative phosphorylation function occurs to

**Table 1 - Cardiac function changes in Dox- and Tet-treated rats**

	LSVP (mmHg)	+dP/dt max (mmHg/s)	-dP/dt max (mmHg/s)
Control	154.45 ± 18.73	9432.59 ± 1137.85	8596.27 ± 799.59
Dox	107.51 ± 12.58*	5581.45 ± 897.34*	6091.56 ± 631.72*
Tet/Dox	130.47 ± 15.12**	7639.21 ± 942.17**	7162.42 ± 850.07**
Tet	149.78 ± 20.55	8900.51 ± 1411.21	8100.27 ± 687.04

\*  $P < 0.05$ , compared to the control group; \*\*  $P < 0.05$ , compared to the Dox group



**Figure 1 - Electrocardiogram alterations in Dox- and Tet-treated rats.** \* $P < 0.05$ , compared to other groups.

**Table 2 - Effect of Dox and Tet treatment on mitochondrial oxidative phosphorylation in the cardiac tissues of rats**

	Rate of respiration (ng atoms of O/min/mg protein)			
	$S_3$	$S_4$	RCR	ADP/O
Control	205 ± 36	41 ± 5	5.1 ± 0.6	2.6 ± 0.3
Dox	135 ± 17*	53 ± 8*	2.5 ± 0.2*	1.8 ± 0.1*
Tet/Dox	168 ± 20**	45 ± 6	3.7 ± 0.4**	2.3 ± 0.2**
Tet	217 ± 19	43 ± 6	5.2 ± 0.3	2.4 ± 0.3

\*  $P < 0.01$ , compared to the control group; \*\*  $P < 0.05$ , compared to the Dox group.

meet the increased cellular energy demand after treatment with Tet. There was a significant difference between the Tet/Dox and Dox groups in cardiac mitochondrial respiratory function. Thus, we conclude that Tet can prevent Dox-induced impairment of mitochondrial oxidative phosphorylation.

### Cardiac morphological changes after treatment with Tet and Dox

Intracellular edema, mitochondria, nuclei, and morphological changes in the myofibril ultrastructure were examined by transmission electron microscopy at 5000-fold magnification. As shown in Figure 2, cytoplasmic vacuolization, myofibril loss, mitochondrial swelling, and pyknotic nuclei of myocytes were observed in the Dox group. Mild cardiac damage was observed in the Tet/Dox group. This shows significant protection against cardiac morphological damage in the group of Tet/Dox-treated rats.

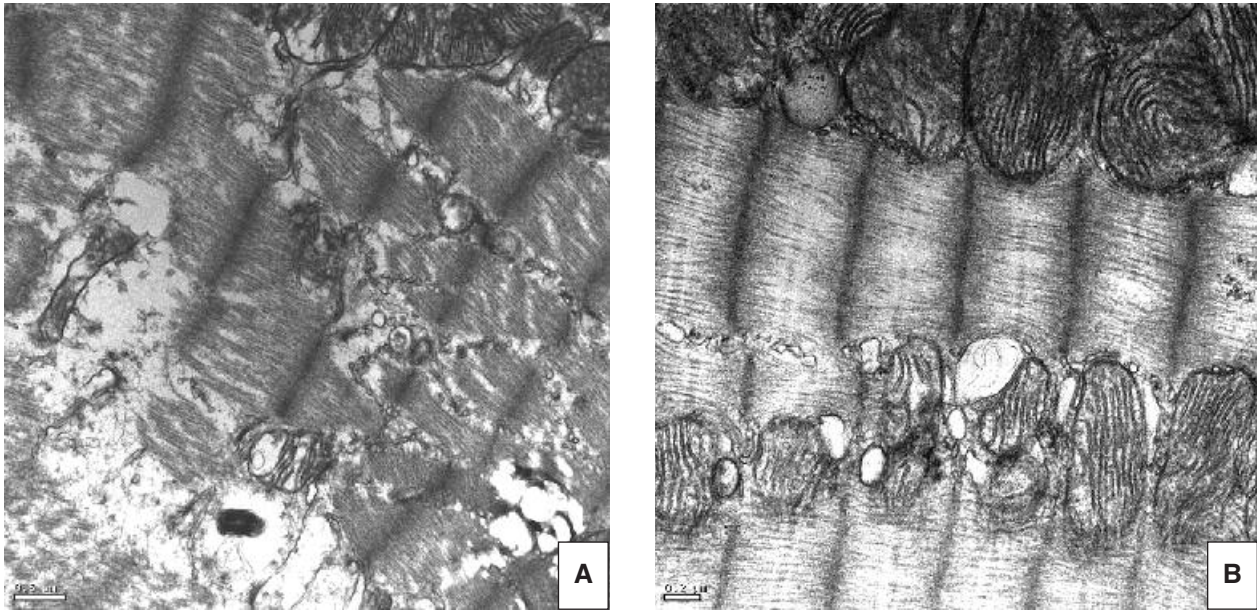


Figure 2 - Representative electron micrographs of cardiac tissue in Dox-treated and Tet-treated rats. A) In the Dox group, cytoplasmic vacuolation, myofibrillar disorganization, and mitochondrial damage can be seen. B) In the Tet/Dox group, cardiac myocyte damage was significantly improved ( $\times 5000$  magnification).

## Discussion

Impaired cardiac contractility and relaxation due to Dox have been reported previously<sup>9</sup>. Signs of Dox-induced cardiotoxicity include decreased ejection fraction, ECG alterations, congestive heart failure, and cardiomyopathy. To study Dox-induced cardiotoxicity, cardiac histology is often used to distinguish the severity of myocardial injuries, because Dox induces specific damage<sup>10</sup>. At the end of this experimental period, after a total dose of 14 mg/kg chronic Dox administration to rats, the cardiac damage in the rats was similar to the changes observed in patients with Dox-induced cardiotoxicity<sup>11</sup>. Mitochondria of myocytes appeared to have electron density reductions, swollen and broken cristae, cytoplasmic vacuolation, and myofibril disorganization in the Dox group. The myotome of cardiac muscle fibers was unclear. These abnormal alterations induced by Dox were inhibited by treatment with Tet. The detailed effects of Tet against Dox-induced cardiotoxicity were studied in a rat model.

Beneficial effects of Tet include induction of apoptosis in tumor cells<sup>12</sup>, reversal of MDR, sensitization of the tumor to radiation, reduction of radiation damage to granulocytes, and inhibition of angiogenesis. Prolonged treatment with Tet may markedly reduce blood pressure, inhibit regression of left ventricular hypertrophy, and decrease the collagen content of cardiac tissue in a hypertensive rats model. Tet reverses vascular media thickness and collagen deposition, improves the reactivity of microvessels, and decreases vessel resistance<sup>13</sup>. Tet has

attracted more attention for its role as a  $\text{Ca}^{2+}$  channel blocker. It directly blocks both T-type and L-type calcium current in the ventricular and vascular smooth muscle cells, which may be useful in the treatment of angina, arrhythmias, and other cardiovascular disorders<sup>14</sup>. Additionally, functional and biochemical parameters have been studied in Dox-induced cardiotoxicity<sup>15</sup>. Dox binds to the intracellular iron, forming complexes toxic to cardiolipids. Dox may also inhibit the respiratory chain and mitochondrial phosphorylation. Reactive oxygen radical production and dysfunction in mitochondria may be an important mediator of Dox-induced cardiotoxicity. Cardiac muscle is the specific organ target for Dox toxicity because of the relatively low antioxidant defenses in cardiac tissues<sup>16</sup>. Fortunately, Dox-induced cardiotoxicity can be reversed by decreasing myocardial concentrations of Dox or concurrently administering other drugs that can reverse or block its toxic effects. For example, liposomal encapsulation of Dox may reduce the incidence of cardiotoxicity by reducing peak plasma concentrations<sup>17</sup>. The introduction of cardioprotective agents, such as dexrazoxane, has significantly reduced cardiotoxicity in cancer patients treated with Dox chemotherapy<sup>18-20</sup>. However, dexrazoxane may cause myelotoxicity and chemical phlebitis. Thus, it seems that an effective cardioprotective drug should scavenge hydroxyl radicals, reach therapeutic levels in the myocardium, and have less toxicity in normal tissues.

Protective effects of Tet against Dox-induced cardiotoxicity were observed in our study. The values of LSPV,  $+dP/dt$  max, and  $-dP/dt$  max increased in the

Tet/Dox group. Tet can inhibit the cardiac function damage induced by Dox and can improve cardiac function significantly<sup>9</sup>. ECG changes are one of the most reliable parameters for assessing Dox-induced cardiotoxicity. Significant changes in the ECG, such as widening of the QRS complex, bradycardia, ST-segment widening, progressive and irreversible prolongation of the QT interval, and T-wave flattening, have been reported in chronic cardiotoxicity *in vivo*. The severity of the ECG changes correlate with Dox-induced cardiotoxicity. Dox specifically prolongs the QT phase by disturbing the ion flux across the myocellular membrane, which is related to the morphological injuries caused by Dox. Tet inhibited the prolonged QT interval change on ECGs in Tet/Dox-treated rats. Disruption of mitochondrial function after early introduction of Dox, an uncoupling effect on mitochondrial function was observed, together with decreased RCR, increased  $S_4$  respiration, and a reduced ADP/O ratio. These changes may cause severe damage to cardiac cells, suggesting a degenerative and necrotizing process that is characteristic of cardiac cellular ischemia. A late phase with damage to mitochondrial ATP synthesis also occurred. Mitochondrial dysfunction was likely to be associated with ischemic damage. We found that the changes in  $S_3$  and  $S_4$  induced mitochondrial RCR alterations, resulting in Dox-induced cardiac injury. Tet can reduce adriamycin-induced mitochondrial impairment. Dox might intercalate with DNA<sup>10</sup>, interact with a unique consensus sequence located in upstream regulatory sequences, and interact with genetic effects, affecting cardiac gene expression. Our results indicated that Dox can induce upregulation of AdRK $\beta$  and AT<sub>2</sub>R<sub>2</sub> gene expression and Tet can inhibit the expression of AdRK and AT<sub>2</sub>R<sub>2</sub> in cardiac tissue (data not shown).

In summary, Tet could improve reduced cardiac function and prevent Dox-induced mitochondrial impairment in rat cardiotoxicity. Further studies are necessary to determine the appropriate combination of Tet with Dox to reduce Dox-induced cardiotoxicity.

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