Thrombopoietin Protects Against In Vitro and In Vivo Cardiotoxicity Induced by Doxorubicin

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**Background**—Doxorubicin (DOX) is an important antineoplastic agent. However, the associated cardiotoxicity, possibly mediated by the production of reactive oxygen species, has remained a significant and dose-limiting clinical problem. Our hypothesis is that the hematopoietic/megakaryocytopoietic growth factor thrombopoietin (TPO) protects against DOX-induced cardiotoxicity and might involve antiapoptotic mechanism exerted on cardiomyocytes.

**Methods and Results**—In vitro investigations on H9C2 cell line and spontaneously beating cells of primary, neonatal rat ventricle, as well as an in vivo study in a mouse model of DOX-induced acute cardiomyopathy, were performed. Our results showed that pretreatment with TPO significantly increased viability of DOX-injured H9C2 cells and beating rates of neonatal myocytes, with effects similar to those of dexrazoxane, a clinically approved cardiac protective agent. TPO ameliorated DOX-induced apoptosis of H9C2 cells as demonstrated by assays of annexin V, active caspase-3, and mitochondrial membrane potential. In the mouse model, administration of TPO (12.5 μg/kg IP for 3 alternate days) significantly reduced DOX-induced (20 mg/kg) cardiotoxicity, including low blood cell count, cardiomyocyte lesions (apoptosis, vacuolization, and myofibrillar loss), and animal mortality. Using Doppler echocardiography, we observed increased heart rate, fractional shortening, and cardiac output in animals pretreated with TPO compared with those receiving DOX alone.

**Conclusions**—These data have provided the first evidence that TPO is a protective agent against DOX-induced cardiac injury. We propose to further explore an integrated program, incorporating TPO with other protocols, for treatment of DOX-induced cardiotoxicity and other forms of cardiomyopathy. (Circulation. 2006;113:2211-2220.)

**Key Words:** apoptosis ■ cardiomyopathy ■ doxorubicin ■ echocardiography ■ thrombopoietin

Doxorubicin (DOX), an anthracycline drug, is one of the most active antineoplastic agents developed to date for the treatment of solid tumors and hematologic malignancies. However, its clinical use is limited by acute and chronic cardiotoxicities, which are dose related, cumulative, and essentially irreversible. It has been estimated that the normal heart can compensate for a lifetime dose of 400 to 550 mg/m² of DOX.1,2 Beyond this level, severe injury, including cardiomyopathy and congestive heart failure, might occur. Heart failure in DOX-treated patients may take place many years after treatment cessation, as reported by Steinherz et al3 (1991). Analyzing a group of 630 patients with breast carcinoma and small-cell lung carcinoma, Swain et al4 reported that an estimated 26% of patients would experience DOX-related congestive heart failure at a cumulative dose of 550 mg/m².

The pathogenesis of DOX-induced cardiotoxicity is not entirely clear. It has been suggested that the anticancer effects and cardiotoxicity of DOX do not follow identical mechanisms.5,6 Available laboratory evidence shows that DOX induces generation of reactive oxygen species. The increase in oxidative stress and depletion of endogenous antioxidants trigger the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes.5,7 Other outcomes include disturbance of myocardial adrenergic function, intracellular calcium overload, and release of cardiotoxic cytokines. Numerous signal molecules, such as cytochrome c, superoxide dismutase, creatine kinase, nitric oxide, Bcl-2, Bax, p53, and Fas, have been indicated in the reactive oxygen species–induced apoptotic pathways of cardiomyocytes.6,8

**Clinical Perspective**

Failure in DOX-treated patients may take place many years after treatment cessation, as reported by Steinherz et al3 (1991). Analyzing a group of 630 patients with breast carcinoma and small-cell lung carcinoma, Swain et al4 reported that an estimated 26% of patients would experience DOX-related congestive heart failure at a cumulative dose of 550 mg/m². The pathogenesis of DOX-induced cardiotoxicity is not entirely clear. It has been suggested that the anticancer effects and cardiotoxicity of DOX do not follow identical mechanisms.5,6 Available laboratory evidence shows that DOX induces generation of reactive oxygen species. The increase in oxidative stress and depletion of endogenous antioxidants trigger the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes.5,7 Other outcomes include disturbance of myocardial adrenergic function, intracellular calcium overload, and release of cardiotoxic cytokines. Numerous signal molecules, such as cytochrome c, superoxide dismutase, creatine kinase, nitric oxide, Bcl-2, Bax, p53, and Fas, have been indicated in the reactive oxygen species–induced apoptotic pathways of cardiomyocytes.6,8
Thrombopoietin (TPO) is an established cytokine for promoting early hematopoietic progenitor cells, the megakaryocytic/platelet lineage, angiogenesis, and antiapoptosis.\textsuperscript{10,11} We hypothesize that TPO may protect against cardiotoxicity induced by DOX. This is based on the rationale that TPO possesses antiapoptotic functions mediated by the Akt prosurvival axis in hematopoietic stem cells and megakaryocytes.\textsuperscript{11,12} The Akt pathway has been known to exert survival protections in cardiomyocytes.\textsuperscript{13} In addition, TPO and erythropoietin (EPO) have strong sequence homology, with 20% identity and 25% similarity in their receptor-binding regions.\textsuperscript{14} It was shown that TPO may exert its mitogenic effects by binding to EPO receptors.\textsuperscript{15} EPO is reported to protect and promote cardiomyocytes.\textsuperscript{16,17} To test the hypotheses, we performed 2 in vitro investigations as well as 1 in vivo study in a mouse model of DOX-induced cardiomyopathy.

**Methods**

Details of methodology are described in the online-only Data Supplement.

**In Vitro Model of Myocytes**

**Rat H9C2 Myoblast Cell Line**

This embryonic line (American Type Tissue Collection, Manassas, Va; catalog No. CRL-1446) was maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum and cultured in 5% CO\textsubscript{2} at 37°C. All media and culture reagents were products of Gibco (Grand Island, NY) unless specified otherwise. H9C2 cells were seeded at 2 × 10\textsuperscript{4} cells per well (24 well plates) for 24 hours, with or without preincubation with TPO (5, 10, 50, or 100 ng/mL, overnight; Peprotech, Rocky Hill, NJ) or dexrazoxane (DEX; 50 µg/mL, 30 minutes). DOX (5 µg/mL; Ebewe Pharma Ges, Austria) was added to the system for another 24 hours. Cell viability was measured by the MTT assay by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (250 µg/mL; Sigma, St Louis, Mo) for 4 hours, and the optical density was read at 570 nm. Apoptotic cell death was analyzed by assays of annexin V, active caspase-3, and mitochondrial membrane potential (ΔΨm) with the use of flow cytometry.

**Primary Neonatal Rat Cardiomyocytes**

Spontaneously beating cells from heart ventricles of neonatal rat were cultured\textsuperscript{18} and subjected to treatment with DOX (1 µmol/L or 0.38 µg/mL) with or without pretreatment (1 hour before) with TPO (50 or 100 ng/mL) or dexrazoxane (5.8 µg/mL). Changes in beating rates were captured by video camera.

**In Vivo Mouse Model of DOX-Induced Cardiotoxicity**

Male Balb/c mice (Laboratory Animal Services Centre, The Chinese University of Hong Kong, Hong Kong) at 9 to 10 weeks of age were randomly divided into 4 groups. The control group was given 3 doses of normal saline intraperitoneally (IP) on alternate days. At day 0, the DOX group received a single dose of DOX dissolved in 0.9% NaCl at 20 mg/kg. The DOX + TPO group was given a single dose of DOX (20 mg/kg IP) and 3 doses of TPO (12.5 µg/kg, dissolved in normal saline) 1 day before (day −1) and 1 and 3 days after DOX injection. The TPO group received 3 doses of TPO but no DOX treatment. Animal viability was recorded daily for 5 days. In an independent experiment, the viability was recorded for 8 days in 4 groups of animals receiving the same treatments but not subjected to any sample collection or daily manipulation. All procedures were approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong.

![Figure 1. Viability of H9C2 cells by MTT assay. H9C2 cells were cultured in the presence of DOX (5 µg/mL) with or without pretreatment with TPO (5, 10, 50, 100 ng/mL, overnight) or dexrazoxane (DEX; 50 µg/mL, 30 minutes). Results are expressed as percentage of control cells (without DOX) as mean ± SD. The cell viability was increased with pretreatment of TPO at 50 ng/mL and 100 ng/mL (Mann-Whitney test; n = 4; *P = 0.010 and *P = 0.016, respectively).](http://circ.ahajournals.org/)

**Echocardiography**

Transthoracic echocardiography (Sonos 7500, Philips Ultrasound, Bothell, Wash) was performed with the use of a linear array 6- to 15-MHz transducer at baseline (day −1) and day 5 on animals maintained at a conscious state.\textsuperscript{19} All echocardiographic images were transmitted to the Xcelera image management workstation (Philips Medical Systems, Nederland BV, the Netherlands) for later offline measurements.

**Blood Cell Count and Histopathology**

Peripheral red blood cells, white blood cells, and platelets were counted at baseline and day 5. On day 5, after measurement of body weight and echocardiographic parameters, all mice were killed under anesthesia with ketamine and xylazine. The heart tissue was fixed in 4% formaldehyde, and 5-µm-thick paraffin sections were stained with hematoxylin-eosin for histological examination. The frequency and severity of DOX-induced myocardial damage were evaluated by a blinded investigator using semiquantitative light microscopic analysis of the sections. The severity of the damage was scored from 0 to 3 according to the percentage of vacuolization and myofibrillar loss in 8 randomly assigned areas of each section and 2 sections per heart.\textsuperscript{20}

**Terminal Deoxynucleotidyltransferase–Mediated Nick-End Labeling Assay**

An independent experiment was performed on 4 groups of mice (n = 5) treated according to the same protocol except that the animals were euthanized at day 3 after DOX treatment and had received 2 doses of TPO (day −1 and day 1). The terminal deoxynucleotidyltransferase–mediated nick-end labeling assay was used for microscopic detection of apoptosis.

**Statistical Analysis**

Results on in vitro studies were analyzed with the Kruskal-Wallis ranking test or Friedman test, and post hoc comparisons were performed by Wilcoxon rank sum test or the Mann-Whitney test, respectively. The levels of significance were adjusted by Bonferroni adjustment of multiple comparisons (Figures 1 to 5; eg, P = 0.017 for 3 comparisons on DOX versus control, DOX + TPO versus DOX, and TPO only versus control). For in vivo studies, heart function parameters and blood cell counts in same animals at day −1 and day 5 were compared with the Wilcoxon signed rank test, and differences between groups at day 5 were compared by the Mann-Whitney test (Table 1). The survival rates of mice were compared by the log rank test. Effects of TPO on trends of daily heart rate were examined by multilevel modeling, and the likelihood ratio test was used to assess the significance of estimates at the 5% level. Cardiomyopathy scores...
of experimental animals were analyzed by the Kruskal-Wallis ranking test and Mann-Whitney test (Table 2).

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

TPO Increased Viability of DOX-Treated H9C2 Cells

Results of MTT assay on the H9C2 cell line demonstrated that DOX (5 \( \mu g/mL \), 24 hours) significantly reduced cell viability by 26.2\( \pm \)1.1\% (Figure 1; \( n=4 \); \( P<0.001 \)). These cells responded to TPO at a dose-dependent manner, and cell viability was recovered to 85.6\( \pm \)4.4\% (100 ng/mL TPO; \( P=0.016 \)). Dexrazoxane at 50 \( \mu g/mL \) increased the cell viability to 82.8\( \pm \)2.0\%.

TPO Protected Against DOX-Induced Apoptosis of H9C2 Cells: Annexin V/PI Staining, Active Caspase-3 Expression, and Damage of Mitochondrial Membrane Potential in DOX-Treated H9C2 Cells

Our data demonstrated that apoptotic cells (R2) and total dead cells (R1+R2), as identified by annexin V and PI stainings, were significantly increased in DOX-treated H9C2 cells (\( *P=0.016; n=7 \)) (Figure 2). The addition of
TPO reduced the proportion of these populations (\(P=0.016; n=7\)) to near control levels. TPO alone had no noticeable effect on the apoptosis of H9C2 cells.

The expression of active caspase-3, a downstream effector protein of apoptosis, was significantly increased in DOX-treated cells both in terms of the proportion of cells expressing the protein and the mean fluorescence intensity of expression (both \(P=0.016\)). Pretreatment with TPO significantly decreased caspase-3 expression, from 79.3±7.9% (DOX group) to 38.5±16.9% (DOX+TPO group) of the cell populations (\(P=0.016\)), and the relative mean fluorescence channel from 49.8±12.7 to 22.2±8.0 (\(P=0.016\)) (Figure 3, flow cytometry histogram). However, total recovery from DOX-induced caspase-3 activation was not achieved by TPO treatment. TPO alone (without DOX) had no effect on the level of active caspase-3.

There were significant differences on the JC-1 status among treatment groups (\(P<0.002\), Friedman test). For paired comparisons, DOX treatment increased the proportion of cells containing JC-1 monomers (R1+R2, green fluorescence), indicating a trend in the drop of \(\Delta \Psi_m\) (17.9±9.5% versus 7.5±3.6%; \(P=0.028; n=6\)) (Figure 4). This population of apoptotic cells was decreased in cultures pretreated with TPO (11.3±7.2%; \(P=0.028\)). The addition of TPO to H9C2 without DOX did not significantly alter the status of \(\Delta \Psi_m\). A similar trend was observed when the R1 and R2 populations were independently analyzed, the former representing a population at the transition from JC-1 aggregates to monomers (early apoptosis) and the latter being cells containing depolarized mitochondria membrane and JC-1 monomers (late apoptosis).

**TPO Exerted Cardioprotective Effects on Primary Rat Neonatal Myocytes**

Treatment with DOX for 24 hours significantly decreased beating rates of cardiomyocyte colonies (Figure 5). The rates declined further to 15.2±16.9% at 48 hours (\(P<0.001\) for both time points compared with control cultures; \(n=10\),...
compartmentalization from JC-1 aggregates to monomers) and late (R2, cells showing trends of increased JC-1 monomers (green fluorescence in FL-1, R1+R2) in the DOX-treated group and reduced fluorescence in FL-1, R1) in the DOX+TPO group (n=6; both P<0.028; Wilcoxon rank sum test). TPO alone did not change the cellular Δψm status. Similar results were observed in the early (R1, cells at transition from JC-1 aggregates to monomers) and late (R2, cells containing monomers) apoptotic populations.

despite the fact that the cultures were replaced with fresh medium without DOX at 24 hours. Cultures pretreated for 1 hour with dexrazoxane or TPO at 50 or 100 ng/mL before the addition of DOX had compromised beating rates measured at 24 hours, but the rates were significantly increased to 69.2±30.4%, 43.2±16.8%, and 47.8±22.9% at 48 hours, respectively (*P=0.001, *P=0.002, and *P=0.002, respectively, compared with DOX-only cultures). The addition of dexrazoxane or TPO to cardiomyocytes in cultures without DOX did not alter the beating rates of these cells.

TPO Protected Against DOX-Induced Cardiotoxicity In Vivo

Animal Body Weight and Survival
At day 5, DOX-treated animals had reduced body weights (18.8±2.1 g; n=14) compared with control animals (24.0±1.4 g; n=13) (P<0.001). Treatment with TPO (DOX+TPO group) did not increase body weights of animals (18.5±1.7 g; n=13). The TPO alone group had body weights (23.3±0.5 g; n=8) similar to those of the control animals. Heart weight/body weight ratio, however, remained similar in all 4 groups. DOX treatment did not alter the heart weight/body weight ratio (5.9±0.7 mg/g in control animals versus 5.9±0.9 mg/g in DOX-treated animals). There was no difference in the dry weight of the heart on day 5 among the 4 groups.

The survival rates of animals in the control group and TPO alone group were consistently 100% in all series of experiments. At day 5, DOX-treated animals had a compromised survival rate (66.7%; n=39), and there was a trend of increased survival in DOX+TPO-treated animals (83.3%; n=36) compared with the DOX-treated group (P=0.097).

In an independent and prolonged experiment in which treated animals were not subjected to any disturbance of blood collection and echocardiography monitoring (n=20 in each group), animal mortality was first observed at day 4 (Figure 6). At day 8, only 20% of DOX-treated animals survived. TPO treatment significantly increased the survival rate to 50% (P=0.018).

Blood Cell Counts
Baseline (day −1) measurements of blood cell parameters were similar in all 4 groups of animals (Table 1). At day 5, red blood cell, white blood cell, and platelet counts were significantly decreased in animals treated with DOX compared with those measured at baseline from same animals (all *P<0.01). In the DOX+TPO group, an increase of platelet counts was observed in day 5 (*P<0.001). The TPO alone group also had raised platelet counts at day 5 (*P=0.008).

Heart Function Parameters by Echocardiography
The heart rate, fractional shortening, and cardiac output were similar among the 4 groups of animals at baseline. With the progression of time from day −1 to day 5 (Figure 7), gradual reductions in heart rates were observed in the DOX-treated group (n=14) (quadratic trend P=0.038) compared with control animals (n=13). Increased heart rates were demonstrated in the DOX+TPO-treated animals (n=13) (P=0.001). At day 5, significant compromises in

Figure 4. Mitochondrial membrane potential (Δψm) changes in H9C2 cells. Flow cytometric dot-plot analysis of H9C2 cells showed trends of increased JC-1 monomers (green fluorescence in FL-1; R1+R2) in the DOX-treated group and reduced damage in the DOX+TPO group (n=6; both P=0.028; Wilcoxon rank sum test). TPO alone did not change the cellular Δψm status. Similar results were observed in the early (R1, cells at transition from JC-1 aggregates to monomers) and late (R2, cells containing monomers) apoptotic populations.

Figure 5. Beating rates of primary cardiomyocytes in culture. Neonatal rat cardiomyocytes were cultured with or without DOX (1 μmol/L) or pretreatment with dexrazoxane (5.8 μg/mL) or TPO (50 and 100 ng/mL). Results are expressed as percent change (mean±SD) in beating rate of the same colony with response to treatment. DOX significantly decreased beating rates (Mann-Whitney test; #P<0.001; n=10) compared with those of control cultures. The addition of dexrazoxane (#P=0.001), TPO at 50 ng/mL (#P=0.002), or TPO at 100 ng/mL (#P=0.002) significantly increased beating rates of DOX-treated cells at 48 hours. The light microscopic image of a beating cell colony is shown on the right.
Fractional shortening were observed in the DOX-treated group compared with that of day -1 (*P<0.001). Fractional shortening was also reduced in the DOX+TPO group (day -1 versus day 5, P=0.006), but the level was significantly higher than that observed in the DOX-only group at day 5 (†P<0.001) (Table 1). The left ventricular diastolic dimension was significantly lower in DOX- and DOX+TPO-treated groups at day 5 (both *P<0.001) than in those at day -1, at which left ventricular systolic dimension was not different among all groups of animals.

Again, DOX-induced cardiac dysfunction was revealed in the decreased cardiac outputs (both *P<0.001 in DOX and DOX+TPO groups) at day 5. Nevertheless, some improvement in cardiac outputs was observed in the DOX+TPO-treated animals compared with DOX-treated animals at day 5 (†P=0.008). Animals treated with only TPO had cardiac functions at day 5 similar to those observed at baseline. Representative echocardiograms of experimental mice at day 5 are shown in Figure 8.

**Gross Anatomic Changes and Pathology of the Myocardium**

A noticeable finding on opening the chest of mice in the DOX group was the smaller heart size compared with the control group. There was no pleural effusion, lung edema, or ascites. The myocardial pathology associated with DOX treatment included myofibrillar loss and cytoplasmic vacuolization (Figure 9). The incidence, severity, and statistical significance of the myocardial pathology found in the 4 groups of animals are shown in Table 2. The lesion scores from all animals who survived to day 5 were included. The mice that died before day 5 were only dissected for gross anatomic changes, and the heart histology was not studied because of extensive postmortem changes. No cardiomyocyte pathology was detected in the control group. The DOX-treated group showed significantly more severe lesions than the control group (*P<0.001), and the DOX+TPO-treated group had reduced scores of cardiotoxicity (†P<0.001) compared with those in the DOX-treated animals. The TPO-treated animals had normal myocardial morphology.

**Apoptotic Nuclei Determined by Terminal Deoxynucleotidyltransferase-Mediated Nick-End Labeling Assay**

Data from this semiquantitative assay showed that DOX treatment significantly increased the number of apoptotic nuclei in heart tissue sections (*P<0.001; n=5). Apoptotic activity was reduced in the DOX+TPO group compared with the DOX-treated animals (†P=0.019). TPO alone had no effect on cardiomyocyte apoptosis in vivo (Figure 10).

**Discussion**

Our data demonstrated the efficacy of TPO in reducing cardiotoxicity in in vitro models of H9C2 cell line and spontaneously beating cardiomyocytes. The cardioprotective function of TPO was further confirmed in the in vivo model of DOX-induced acute cardiomyopathy in mice. The observed cardiotoxic effects of DOX on H9C2 and primary neonatal cardiomyocytes are in agreement with reported data.\textsuperscript{21,22} The mechanism of DOX on these cells has been attributed to the induction of apoptosis mediated by the intrinsic signaling cascade, resulting in mitochondrial dysfunction and myofibrillar degeneration.\textsuperscript{21,23–25} We have demonstrated DOX-induced apoptosis in H9C2 cells, as indicated by the increase in annexin V staining and expression of active caspase-3. Pretreatment with TPO at 50 ng/mL or 100 ng/mL, doses commonly reported as effective in promoting hematopoietic cells in culture,\textsuperscript{26,27} significantly reduced DOX-induced toxicity in H9C2 cells. The protective effect of TPO was again demonstrated in

### TABLE 1. Blood Cell Counts and Echocardiographic Parameters at Baseline and Day 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=13)</th>
<th>DOX (n=14)</th>
<th>DOX+TPO (n=13)</th>
<th>TPO (n=8)</th>
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<tbody>
<tr>
<td></td>
<td>Day -1</td>
<td>Day 5</td>
<td>Day -1</td>
<td>Day 5</td>
</tr>
<tr>
<td>WBCs, ×10^9/L</td>
<td>7.5±1.5</td>
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<td>8.3±1.8*</td>
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<td>RBCs, ×10^12/L</td>
<td>8.7±0.8</td>
<td>8.9±0.9</td>
<td>9.7±1.2*</td>
<td>9.7±1.2</td>
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<tr>
<td>Platelets, ×10^9/L</td>
<td>934±55</td>
<td>964±81</td>
<td>916±107*</td>
<td>967±102*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>58.1±3.3</td>
<td>59.6±3.9</td>
<td>57.9±5.2*</td>
<td>57.5±4.9*</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>9.6±3.0</td>
<td>10.6±2.5</td>
<td>10.3±2.2*</td>
<td>11.7±3.6*</td>
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<td>LVDD, mm</td>
<td>2.84±0.29</td>
<td>2.86±0.23</td>
<td>2.83±0.03*</td>
<td>2.87±0.28*</td>
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<td>LVSD, mm</td>
<td>1.18±0.15</td>
<td>1.15±0.12</td>
<td>1.20±0.19</td>
<td>1.22±0.11</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Data at baseline (Day -1) and Day 5 were compared. There were no significant differences between treatment groups at baseline on any parameters. *P<0.01, Day -1 versus Day 5 in the same treatment group (Wilcoxon rank sum test). †P<0.01, DOX group versus DOX+TPO group on Day 5 (Mann-Whitney test).

### TABLE 2. Cardiomyopathy Scores of Experimental Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Cardiomyopathy Score</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>13</td>
</tr>
<tr>
<td>DOX*†</td>
<td>14</td>
</tr>
<tr>
<td>DOX+ TPO†</td>
<td>13</td>
</tr>
<tr>
<td>TPO</td>
<td>8</td>
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</tbody>
</table>

The Kruskal-Wallis test showed that there were differences among treatment groups (P<0.001). There was a significant increase of cardiomyopathy score in DOX-treated animals compared with control animals (Mann-Whitney test, *P<0.0001) and reduction of score in DOX+TPO-treated animals (†P=0.002) compared with the DOX group.
primary cardiomyocytes by partially recovering the beating rate. We speculate that the gain of contractile activity might also involve the antiapoptotic function of TPO. The effects of TPO on cell viability and beating rates were comparable to those observed with dexrazoxane, a clinically approved cardioprotective agent, added at a recommended dose of 10 times that of DOX.28

The cardioprotective activity of TPO has been found to be applicable in the animal model. The responses of animals to a single dose of DOX administered at 20 mg/kg were in accordance with those reported, in terms of reduced heart weight, unaltered heart/body weight ratio, low blood cell count, myocyte lesions, apoptotic cell death, cardiac dysfunction, and animal mortality.7,29–32 In agreement with reports on DOX-induced acute cardiotoxicity of experimental animals,32,33 our data showed that heart rates of mice decreased progressively, and the heart size was smaller 5 days after DOX administration. Notably, these observations are in contrast to chronic cardiomyopathy observed in clinical situations during which repeated smaller doses of DOX are given over a period of time. In this study, we used the M-mode Doppler method to assess heart functions. This method is accurate and is easier to perform than telemetric ECG recording. However, one

![Figure 6](image6.png)

**Figure 6.** Cumulative survival of experimental animals. The Kaplan-Meier plot of animal survival showed significant increase of survival rate in the DOX+TPO group (n=20) compared with DOX-treated animals (P=0.018, log rank test). There was no mortality in the control group and TPO group.

![Figure 7](image7.png)

**Figure 7.** Daily heart beating rates of experimental animals. No significant difference in heart rates was demonstrated among the 4 groups of animals at baseline. Analysis by the multilevel modeling and likelihood ratio test on trend effects of treatments showed significant differences in the heart rates of DOX-treated animals (n=14) compared with the control group (n=13; quadratic trend P=0.038) or the DOX+TPO-treated group (n=13; P=0.001). Administration of TPO alone did not alter the trend of heart rates (n=8).

![Figure 8](image8.png)

**Figure 8.** Representative echocardiograms of experimental animals. DOX treatment resulted in significant left ventricular cardiac impairment as indicated by 2-dimensional M-mode tracings of wall motion from control and DOX-administered mice at day 5 after treatment. The DOX+TPO group had significantly improved cardiac functions. TPO alone had no effect on the uninjured heart.
technical limitation is that less information about electrophysiological change of the heart could be obtained. Our results consistently showed that cardiac functions, including heart rate, fractional shortening, and cardiac output, were severely compromised in the DOX-treated group.\(^{30,31}\) The animal mortality and impaired cardiac functions during this acute phase, however, might not be contributed exclusively by the DOX-induced cardiotoxicity. Toxicity to other organs as well as the heart might also lead to severe anorexia and poor oral intake. Dehydration might have occurred, leading to decrease of body weight and heart weight.

Significantly, the administration of TPO at a relatively low dose of 12.5 μg/kg\(^{34}\) for 3 alternate days was effective in protecting the animals against DOX-induced cardiotoxicity. This was consistently manifested in all morphological and functional parameters assessed in the heart. In the clinical setting, various measures have been taken to reduce DOX-induced cardiotoxicity, which include using meticulous dosing schedules to lower the DOX peak plasma concentration, identifying and monitoring high-risk patients, and preparing less toxic forms of DOX. Cardioprotective agents, such as dexrazoxane, have been developed to protect patients who undergo DOX therapy. However, dexrazoxane could not provide absolute cardioprotection, as shown by studies that 7% to 14% of breast cancer patients treated with dexrazoxane experienced cardiac complications.\(^{35}\) A higher dose of dexrazoxane has been known to cause neutropenia in cancer patients.\(^{36}\) Myelosuppression is a common complication in patients who receive chemotherapy. TPO is known to promote hematopoietic stem cells, particularly the megakaryocytic lineage. In our animal model, we observed recovery of platelet counts in animals that received TPO treatment. Various clinical trials on cancer patients have demonstrated beneficial effects of TPO for treating chemotherapy-induced severe thrombocytopenia.\(^{37-40}\) Vadhan-Raj et al\(^ {39} \) (2003) suggested that the timing of TPO administration (1 dose before and 1 after chemotherapy) was important for optimal effects on platelet recovery in cohorts of sarcoma patients. The application of TPO for reduction of cardiotoxicity might therefore provide additional beneficial outcomes by ameliorating neutropenia and thrombocytopenia in patients receiving chemotherapy and dexrazoxane.

In summary, using in vitro models of H9C2 cell line and primary neonatal rat cardiomyocytes in culture, we demonstrated the protective effects of TPO against DOX-induced myocardial injury, possibly mediated by antiapoptotic activity. The efficacy of TPO was confirmed in the in vivo mouse model of DOX-induced cardiotoxicity, as demonstrated by morphological, antiapoptotic, and functional parameters. Most outcomes in the TPO-treated groups of these models, although consistently significant, were in some way inferior to those of the respective control groups. We thus propose to further explore an

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**Figure 9.** Histopathology of myocardium from experimental animals. Representative images of left ventricular histology from experimental animals are shown (hematoxylin-eosin staining; bar=20 μm). A, Control mouse had normal myocardium morphology. B, TPO alone did not alter the morphology of myocardium. C, Heart tissue damage in DOX-treated mouse as indicated by increased cytoplasmic vacuolization and myofibrillar loss. D, Heart myocardial lesions were significantly reduced in animals that received TPO treatments.

**Figure 10.** Myocardium apoptosis in vivo by terminal deoxynucleotidyltransferase–mediated nick-end labeling assay. Representative heart tissue sections from animals treated with DOX (A) and DOX+TPO (B) demonstrated apoptotic nuclei (green fluorescence; original magnification ×400). The plots showed the density of apoptotic nuclei in the 4 groups of animals (n=8; 6 sections counted per heart). A significant increase in the number of apoptotic nuclei was demonstrated in DOX-treated hearts compared with the control group (Mann-Whitney test, \( ^{*}P<0.001 \)). The DOX+TPO groups had fewer apoptotic nuclei compared with the DOX group (\(#P=0.019\)).
integrated program, incorporating TPO with other protective reagents/protocols (eg, dexrazoxane and EPO) against DOX-induced cardiac dysfunction. Our data have provided the first evidence on the potential of TPO as a protective agent against DOX-induced apoptosis and cardiotoxicity. The clinical applications of TPO in this category of patients and other forms of cardiomyopathy deserve further investigation.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Anthracine drugs are highly effective antineoplastic agents for the treatment of solid tumors and hematologic malignancies. However, their clinical use is limited by delayed cardiotoxicity, which is dose related, cumulative, and essentially irreversible. In the clinical setting, various measures have been taken to reduce anthracycline-induced cardiotoxicity, including dosing schedules to lower peak plasma concentrations, identifying and monitoring high-risk patients, and preparing less toxic forms of the drugs. Cardioprotective agents, such as dexrazoxane, have been developed to protect patients who undergo anthracycline therapy. However, dexrazoxane treatment could not provide absolute cardioprotection, and a high dose might result in neutropenia. The present study has presented the first evidence that thrombopoietin (TPO), a known hematopoietic growth factor, provided protection against doxorubicin-induced cardiotoxicity in in vitro and in vivo models. The mechanism was possibly associated with antiapoptotic activities. The results indicate the potential of using TPO as a protective agent against doxorubicin-induced cardiac injury. The administration of TPO to patients may provide additional benefits by ameliorating neutropenia and thrombocytopenia resulting from chemotherapy and dexrazoxane treatment. We thus propose to further explore an integrated program, incorporating TPO with other protective reagents/protocols (eg, dexrazoxane and erythropoietin) against doxorubicin-induced cardiac dysfunction in cancer patients.
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