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ORIGINAL ARTICLE

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Amelioration of doxorubicin-induced cardiac and renal toxicity by pirfenidone in rats

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Abstract *Purpose*: Doxorubicin (DXR) is an anthracycline glycoside with a broad spectrum of therapeutic activity against various tumors. However, the clinical use of DXR has been limited by its undesirable systemic toxicity, especially in the heart and kidney. This study was designed to test the effectiveness of dietary intake of pirfenidone (PD) against DXR-induced cardiac and renal toxicity. *Methods*: Male Sprague Dawley rats were placed into four treatment groups: saline injected intraperitoneally (i.p.) plus regular diet (SA + RD); DXR i.p. plus regular diet (DXR + RD); saline i.p. plus the same diet mixed with 0.6% PD (SA + PD); and DXR i.p. plus the same diet mixed with 0.6% PD (DXR + PD). The animals were fed regular or regular plus PD diets 3 days prior to i.p. injections of either saline or DXR and continuing throughout the study. A total dose of DXR (16.25 mg/kg) or an equivalent volume of saline was administered in seven injections (2.32 mg/kg per injection) three times per week with an additional dose on the 12th day. At 25 days following the last DXR or saline injection, some animals were anesthetized for the measurement of cardiac and pulmonary function, and others were killed by an overdose of pentobarbital. At the time the animals were killed, abdominal fluid was collected. Kidney and heart were removed, weighed, fixed with 10% formalin or frozen in liquid nitrogen. The fixed tissues were used for histological examination and the frozen tissues were used for biochemical studies. *Results*: The average volumes of abdominal fluid in the DXR + RD and DXR + PD groups were 9.42 ml and 3.42 ml and the protein contents of abdominal fluid in the DXR + RD and DXR + PD groups were 218 mg and 70 mg, respectively. A 12.5% mortality occurred in the DXR + RD group as compared to 0% in DXR + PD group. There were no changes in any of the cardiac or pulmonary physiological parameters in any of the four groups. The changes in the heart and kidney of the DXR + RD group included reduction in organ weight, increase in hydroxyproline content of heart, increase in hydroxyproline, and lipid peroxidation in the kidney and plasma, and increase in protein concentration in urine as compared to rats in the control, SA + RD and SA + PD groups. Treatment with PD abrogated the DXR-induced increases in hydroxyproline content in the heart and kidney, lipid peroxidation of the kidney and plasma, and protein content of the urine in the DXR + PD group. DXR treatment alone caused disorganization of cardiac myofibrils, vacuolization of the myofibers, and renal tubular dilation with protein casts in both the cortical and medullary regions. Treatment with PD minimized the DXR-induced histopathological changes of heart and kidney in the DXR + PD group. *Conclusions*: Treatment with PD reduced the severity of DXR-induced toxicity as assessed by reduced mortality, diminished volume of recovered fluid in the abdominal cavity, and severity of cardiac and renal lesions at both the biochemical and morphological levels. These results indicate that PD has the

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potential to prevent DXR-induced cardiac and renal damage in humans on DXR therapy.

Keywords Doxorubicin · Pirfenidone · Antioxidant · Heart · Kidney

Introduction

Doxorubicin (DXR) is an anthracycline glycoside with a broad spectrum of therapeutic activity against a variety of human neoplasms [7, 12]. However, the clinical use of DXR has been limited by its undesirable side effects, especially cardiac and renal toxicity [8, 9, 12]. Cardiac toxicity usually appears in the form of congestive heart failure and develops within 1 to 6 months after initiation of therapy in humans [8, 12]. DXR-induced cardiac damage is characterized by a progressive degeneration of heart muscle and is dependent on the total amount of the drug administered [16]. Cardiac and renal damage has also been reported in laboratory animals following treatment with DXR [6, 24, 29, 30, 42]. Nephropathy has also been reported in cancer patients on DXR therapy, as well as in experimental animals treated with DXR [9, 39, 42, 49]. However, it is not yet clear whether DXR-induced cardiac toxicity precedes nephrotoxicity or follows nephrotoxicity. Similarly, how DXR treatment differentially affects the cardiac and renal function is also not known.

It is believed that biochemical mechanisms for DXR-induced heart and kidney damage involve the generation of reactive oxygen species (ROS) as a consequence of redox cycling [19]. ROS initiate free-radical-mediated chain reactions resulting in the conversion of membrane unsaturated fatty acids into lipid peroxide [10, 39, 49]. In addition, inactivation of creatine kinase [14], and iron regulatory proteins that modulate the fate of mRNAs for transferrin receptor and ferritin have also been implicated in DXR-induced cardiotoxicity [36].

Pirfenidone (PD, Deskar, 5-methyl-1-phenyl-2-(1H) pyridone) is a new investigational drug. This compound has been demonstrated in several laboratories including our own to have anti-inflammatory and antifibrotic effects in animal models [25, 26, 28], and these effects are attributed to its ability to directly scavenge ROS including O_2^-, H_2O_2, and ·OH, thereby preventing lipid peroxidation both in vitro and in vivo [17, 38]. The present study was designed to test the effectiveness of dietary intake of PD in reducing DXR-induced cardiac and renal toxicity in rats at structural, biochemical and functional levels.

Materials and methods

Animals

Male Sprague Dawley rats (Charles River, Boston, Mass.), 7–8 weeks old, were used in this study. The average initial body weight of the animals was 207 ± 1 g (± SE). The rats were housed individually in polycarbonate cages in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. The animals were acclimatized to laboratory conditions for 1 week prior to starting the experiments. A 12 h/12 h light/dark cycle was maintained in the housing facilities. The animals were weighed and distributed among the various groups based on their body weight to attain approximately the same mean weight in all groups. The animals had free access to water and food that consisted of pulverized Rodent Laboratory Chow 5001 (Purina Mills, St. Louis, Mo.) or the same pulverized food mixed with 0.6% PD (w/w) ad libitum. PD was generously donated by Marnac (Dallas, Tx.). DXR solution (2 mg/ml) was purchased from Pharmacia & Upjohn (Milan, Italy), and all other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.) with a purity of > 99%.

The rats were placed into four treatment groups: saline injected intraperitoneally (i.p.) plus regular diet (SA + RD); DXR i.p. plus regular diet (DXR + RD); saline i.p. plus the same diet mixed with 0.6% PD (SA + PD); and DXR i.p. plus the same diet mixed with 0.6% PD (DXR + PD). The animals were fed either regular diet or regular diet plus PD 3 days prior to i.p. injections of either saline or DXR and continuing throughout the entire course of the study. The regimen of DXR administration was approximately the same as reported by Mostafa et al. [40]. Briefly, DXR hydrochloride (2.32 mg/kg) or an equivalent volume of sterile isotonic saline was injected i.p. three times per week for 2 weeks with one additional dose on the 12th day. The animals were weighed two or three times per week and their daily food intake was monitored for the entire 37 days of the study.

Sample collection

After 25 days following the last DXR or saline injection, the rats used for biochemical and histological evaluations were killed by an overdose of sodium pentobarbital (70 mg/kg i.p.), and those used for the measurement of cardiovascular and pulmonary functions were anesthetized with a mixture (4 ml/kg i.p.) of α-chloralose/urethane (0.1 g/kg α-chloralose/10 g/kg urethane). After anesthesia, urine was collected from the bladder and then ascitic fluid, if present, from the peritoneal cavity. Blood was obtained by cardiac puncture or via the abdominal aorta. Immediately after collecting the blood, the left and right kidneys were removed and weighed individually. The left kidney was cut into four cross-sections using a sharp razor blade and placed in 10% buffered formalin. The right kidney was flash-frozen in liquid nitrogen and stored at −80°C. The heart was also removed, weighed and placed in 10% formalin or frozen in liquid nitrogen. The fixed tissues were used for histological studies. The frozen tissues were used for the determination of hydroxyproline and lipid peroxidation content.

Measurement of cardiovascular and pulmonary functions

After anesthetizing the rats with the α-chloralose/urethane mixture, a cannula was placed in the trachea and in both femoral arteries. The left femoral artery was cannulated with a 1.5 FR temperature probe (Columbus Instruments, Columbus, Ohio) and advanced so the tip was approximately at the level of the descending aorta. The right artery was cannulated with PE50 tubing and this line was used for the measurement of blood pressure and blood gases. A thermistor (Physitemp Instruments, Clifton, N.J.) was placed in the rectum for the measurement of body temperature. Blood pressure was measured using a Spectramed DTX pressure transducer (Oxnard, Calif.). Blood gases were measured using a RapidLab 248 pH/blood gas analyzer (Chiron Diagnostics). The animal was then placed into a whole-body plethysmograph for the measurement of breathing pattern and pulmonary function utilizing the methods described by Mansoor et al. [34]. After completing the pulmonary function measurement, the animal was prepared for cardiac output measurements. The right external jugular vein was cannulated for

the measurement of cardiac output utilizing the thermodilution technique. Cardiac output was measured using a Cardiotherm-500 thermodilution cardiac output analyzer (Columbus Instruments).

Pathological evaluation of the heart and kidney

The pathological changes in the hearts and kidneys of six rats from each group were characterized. Kidneys and hearts were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at a thickness of 5–6 μm. The sections were stained with hematoxylin and eosin for histological evaluation [35]. The frequency and the severity of lesions in the hearts and kidneys were assessed semiquantitatively by light microscopy on a scale of 0 to 3 as follows: score 0 was assigned normal, score 1 mild, score 2 moderate, and score 3 severe. The scoring criteria for myocardial lesions included degree of myocyte vacuolization with respect to size and number of vacuoles and loss of myofibrils. The criteria for kidney lesions were degree of tubular regeneration and dilation, and size and frequency of protein casts in the cortical and medullary regions. The tissue sections were read independently by two of the authors (M.A.A. and F.CM., a veterinary pathologist who was not familiar with the treatment protocol) both of whom arrived at the same conclusions with respect to histopathological changes found in the hearts and kidneys.

Biochemical measurements

Frozen right kidneys and hearts were first thawed and homogenized individually in Tris buffer (0.01 *M*, pH 7.4). The homogenate was used for the measurement of hydroxyproline content of heart and kidney by the colorimetric method of Woessner [48]. Lipid peroxidation levels of heart, kidney and plasma were determined by measuring the malondialdehyde equivalent (MDAE) as an index of lipid peroxidation according to the method of Ohkawa et al. [41]. The protein content of the abdominal fluid and urine samples was determined by the method of Lowry et al. [33].

Analysis of PD in plasma

PD concentrations in the plasma of 4 rats in the SA + RD group, 7 rats in the SA + PD group and 12 rats in the DXR + PD group were measured by high performance liquid chromatography (HPLC). The PD standard was prepared by adding a known concentration of PD in water to 0.5 ml control rat plasma. Protein in the plasma samples was precipitated by adding 0.5 ml acetonitrile. The samples were vortexed and stored at 4°C overnight and then centrifuged at 6000 rpm for 20 min. The supernatant was separated and then evaporated to dryness using a vacuum-centrifuge. The residue was dissolved in 0.25 ml acetonitrile and then centrifuged at 6000 rpm for 10 min. An aliquot of the supernatant (0.2 ml) was analyzed by HPLC (model Waters 712 WISP) using a C18 column (250×4.6 mm) at a wavelength of 310 nm (Water 2487 UV column monitor). The area was calculated using a computer with the Waters Millennium program. The PD peak appeared at 61 min. Ten standard samples that ranged from 5 to 2500 ng per injection were analyzed. The following equation was generated for the PD standard curve: PD (ng) = (area + 56475)/2113, $r^2 = 0.9975$. This equation was used to calculate the PD plasma concentrations.

Presentation and statistical analysis of data

The biochemical data are expressed per gram of wet tissue. The data for all groups are presented as means ± SE and were analyzed using ANOVA followed by pair-wise comparisons using the Newman-Keuls test. However, the lesion score data were analyzed by nonparametric statistical tests. P values < 0.05 were considered to be the minimum level of statistical significance.

Results

Body weight, feed intake, and mortality

The average percentages of initial body weights (207 ± 1 g) for rats in each group at different times of the study are summarized in Fig. 1. The rats in the SA + RD and SA + PD groups had gained 196% and 195% of their initial body weight at 37 days, respectively. However, the gains in body weight for rats in the DXR + RD and DXR + PD groups were significantly reduced to 154% and 155% of their initial body weight at that time, respectively. The average daily food intakes for rats during the study period for each group are summarized in Fig. 2. The average food intake on the first day after the first injection of saline or DXR for rats in the control the SA + RD and SA + PD groups ranged from 20 to 21 g, but in the DXR + RD and DXR + PD groups it was markedly reduced to 5 and 7 g, respectively. The average daily food intake 1 day after the last injection of saline or DXR for the rats in the SA + RD and SA + PD groups was 25 g each and for rats in the DXR + RD and DXR + PD groups was 12 and 16 g, respectively. Also the rats in the DXR + PD group consumed a significantly higher quantity of food than the rats in the DXR + RD group.

The daily food intake per rat for the individual groups 1 day prior to the day the animals were killed averaged 21 g, 26 g, 18 g and 23 g for the SA + RD, SA + PD, DXR + RD, and DXR + PD groups, respectively. The food intake on this day for rats in the DXR + RD group was significantly less than for the rats in the SA + RD group. However, there was no difference in food intake between the SA + PD and DXR + PD groups on this day, suggesting the beneficial effect of PD against DXR-induced reduced food intake. This is also reflected by the total amount of food consumed per rat in each group during the entire period of the study starting from the first day after the injection: this averaged 885.4 ± 34.5 g, 916.7 ± 34.0 g, 604.3 ± 44.9 g and 688.7 ± 45.1 g in the SA + RD, SA + PD, DXR + RD, and DXR + PD groups, respectively. The statistical analysis of the total food consumption revealed that the rats in both control groups (SA + RD and SA + PD) consumed a higher amount of food than the rats in the DXR + RD and DXR + PD groups. However, rats treated with PD (DXR + PD group) consumed a significantly higher quantity than rats treated with DXR without PD (DXR + RD group).

DXR treatment caused 12.5% mortality in the DXR + RD group. However, there was no mortality in the DXR + PD group during the study period. None of the animals died during the study period in the control (SA + RD and SA + PD) groups.

Measurement of physiological functions

A number of parameters of cardiovascular and pulmonary functions were evaluated in rats in all groups in

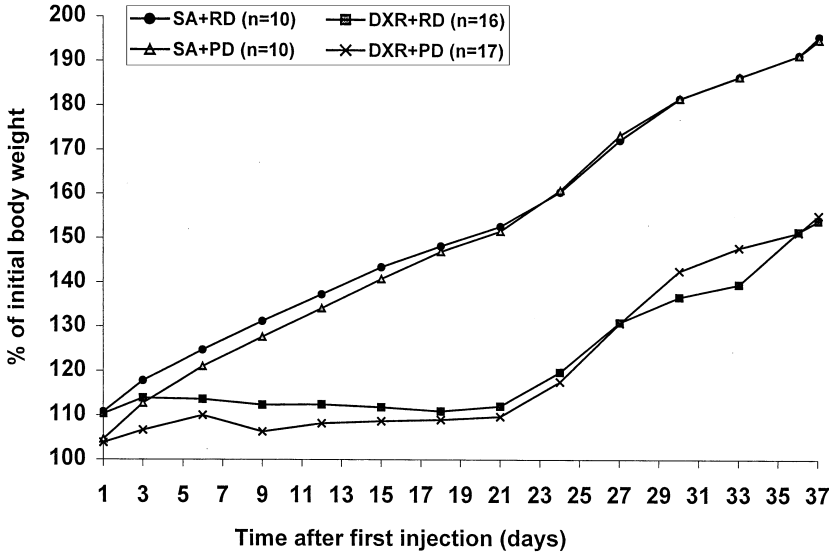

Fig. 1 Effects of PD on DXR-induced changes in body weight expressed as percentage of initial weight. DXR (2.32 mg/mg) or an equivalent volume of saline was injected three times per week for 2 weeks with one additional dose on the 12th day. The animals were weighed two to three times per week for the entire 37 days of the study (*SA + RD* saline i.p. on regular diet, *DXR + RD* DXR i.p. on regular diet, *SA + PD* saline i.p. on the same diet mixed with 0.6% PD, *DXR + PD* DXR i.p. on the same diet mixed with 0.6% PD). Each point represents the mean percentage of the initial average weight (207 ± 1 g) for 10 rats in the SA + RD group, 14 rats in the DXR + RD group, 10 rats in the SA + PD group and 14 rats in the DXR + PD group

order to determine whether DXR treatment had any effects on these functions and whether PD treatment reversed these effects. These parameters included: heart rate, mean arterial pressure, cardiac output, cardiac index, stroke volume, total peripheral resistance, breathing frequency, lung tidal volume, airway resistance, lung compliance, and arterial O_2 saturation. It is interesting that the regimen of DXR treatment used in the present study failed to alter any of the cardiovascular or pulmonary function parameters because there was no difference in any of the measured parameters among the four groups (Table 1).

Measurement of abdominal fluid volume

The volume of fluid accumulated in the abdominal cavity was measured at the end of the experiment. Rats treated with DXR in the DXR + RD group had 9.42 ± 2.07 ml of ascites, whereas in the DXR + PD group, the volume of fluid was significantly ($P < 0.05$) reduced to 3.42 ± 1.06 ml. The total protein content of ascites in the DXR + RD group was 218 ± 105 mg as compared to a significant ($P < 0.05$) reduction to 70 ± 20 mg in the DXR + PD group. The volume of fluid accumulated in the abdominal cavity of rats in both control (SA + RD and SA + PD) groups was not measurable.

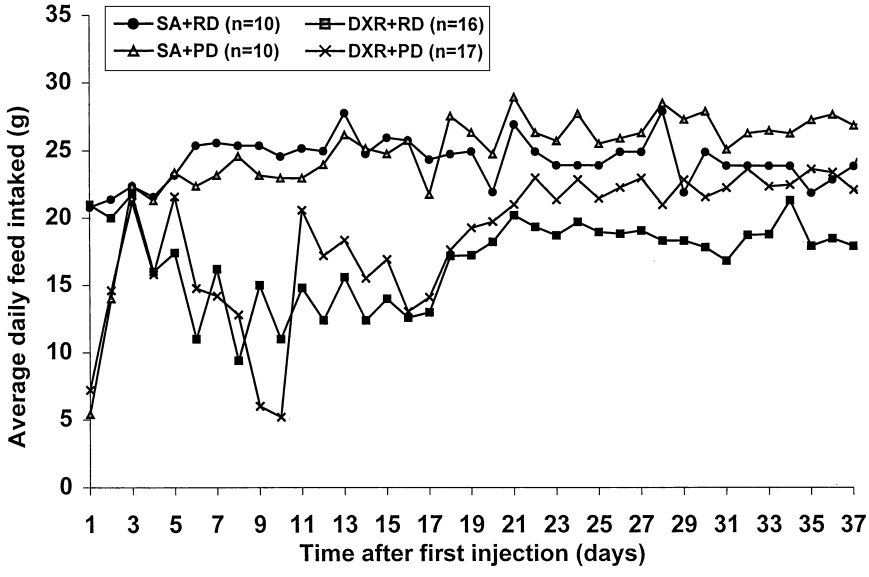

Fig. 2 Effects of PD on DXR-induced changes in food intake. The average daily food intake for each group was measured for the entire period of the study. See the legend to Fig. 1 for experimental details and explanations of the abbreviations and the number of animals in each group. Each point represents the average daily food intake in grams

Table 1 Cardiac and pulmonary functions in the various treatment groups measured 25 days after the last injection of saline or DXR prior to killing the animals. Values are means ± SE

	Treatment group			
	SA + RD ($n=7$)	DXR + RD ($n=9$)	SA + PD ($n=7$)	DXR + PD ($n=10$)
Heart rate (beats/min)	367 ± 35	361 ± 23	337 ± 18	398 ± 14
Mean arterial pressure (mm Hg)	66.1 ± 5.5	105 ± 7.3	88.9 ± 4.0	110 ± 6.5
Cardiac output (ml/min)	174 ± 16	159 ± 11	185 ± 16	173 ± 17
Cardiac index (ml/min/100 g BW)	0.42 ± 0.04	0.49 ± 0.03	0.46 ± 0.04	0.53 ± 0.04
Stroke volume (ml)	0.50 ± 0.08	0.44 ± 0.04	0.54 ± 0.06	0.44 ± 0.04
Total peripheral resistance (mm Hg/ml/min)	0.42 ± 0.09	0.67 ± 0.09	0.49 ± 0.04	0.66 ± 0.05
Breathing frequency (breaths/min)	58.9 ± 5.8	57.7 ± 2.7	60.3 ± 4.5	58.3 ± 2.6
Lung tidal volume (ml)	1.94 ± 0.12	1.57 ± 0.07	1.97 ± 0.16	1.87 ± 0.12
Airway resistance (cm H_2O/ml/s)	0.58 ± 0.01	0.60 ± 0.01	0.58 ± 0.01	0.58 ± 0.02
Lung compliance (ml/cm H_2O)	6.72 ± 0.69	5.06 ± 0.43	6.34 ± 0.50	5.81 ± 0.42
Arterial O_2 saturation (%)	87.0 ± 5.4	91.0 ± 1.2	91.8 ± 1.2	94.6 ± 1.1

Table 2 Effects of pirfenidone on DXR-induced biochemical changes in the heart. Values are means ± SE

	Treatment group			
	SA + RD ($n=7$)	DXR + RD ($n=7$)	SA + PD ($n=7$)	DXR + PD ($n=11$)
Weight (g)	1.5 ± 0.08*	1.14 ± 0.07	1.36 ± 0.03*	1.17 ± 0.05
Hydroxyproline (μg/g)	702.8 ± 43.0	911.6 ± 66.2**	621.6 ± 30.5	711.3 ± 23.1
MDAE (nmol/g)	105.8 ± 3.7*	66.3 ± 8.9	96.9 ± 6.2*	55.2 ± 7.3
Plasma MDAE (nmol/ml)	3.78 ± 0.26	8.69 ± 1.85**	3.33 ± 0.36	4.34 ± 0.29

*$P < 0.05$ vs DXR + RD and DXR + PD groups
**$P < 0.05$ vs all other groups

Table 3 Effects of pirfenidone on DXR-induced biochemical changes in the right kidney. Values are means ± SE

	Treatment group			
	SA + RD ($n=10$)	DXR + RD ($n=14$)	SA + PD ($n=10$)	DXR + PD ($n=14$)
Weight (g)	1.61 ± 0.06*	1.26 ± 0.03	1.64 ± 0.05*	1.37 ± 0.04
Hydroxyproline (μg/g)	703 ± 15.9	887.8 ± 29.6**	803.8 ± 12.4	805.4 ± 20.2
MDAE (nmol/g)	149.9 ± 9.6	235.5 ± 13.1**	165.7 ± 9.1	194.8 ± 10.7***
Urine protein (mg/ml)	15.8 ± 2.9	25.3 ± 3.1**	12.4 ± 3.4	8.2 ± 1.9

*$P < 0.05$ vs DXR + RD and DXR + PD groups
**$P < 0.05$ vs all other groups
***$P < 0.05$ vs SA + RD group

Concentration of PD in plasma

The average PD plasma concentrations in the SA + PD and DXR + PD groups were 168 ± 31 ng/ml and 255 ± 69 ng/ml, respectively. There was no significant ($P > 0.05$) difference in the plasma concentrations of PD between these two groups. Plasma from four control rats in the SA + RD group was also analyzed for PD, but none was detected.

Biochemical changes in heart and kidney

The weight, hydroxyproline content and lipid peroxidation levels of the heart and lipid peroxidation level of plasma are summarized in Table 2. There were no differences in any of the measured parameters of the heart between the two control (SA + RD and SA + PD) groups. DXR treatment caused significant reductions in heart weight, and in the level of lipid peroxidation of the heart in both the DXR + RD and DXR + PD groups as compared to the SA + RD and SA + PD control groups. However, DXR treatment increased the level of lipid peroxidation in the plasma and the hydroxyproline content of the heart in the DXR + RD group as compared to other groups. The treatment with PD caused significant reductions in DXR-induced increases in plasma lipid peroxidation and hydroxyproline content in the DXR + PD group (Table 2). Similar to the heart, DXR treatment decreased the right kidney weight significantly in the DXR + RD and DXR + PD groups as compared to the control SA + RD and SA + PD groups (Table 3). However, DXR treatment alone (DXR + RD group) caused significant increases in lipid peroxidation and hydroxyproline content of the kidney and increased the protein content of the urine as compared to other groups. Treatment with PD abrogated the DXR-induced increases in these biochemical measurements in the DXR + PD group. Although PD treatment caused a significant reduction in the lipid peroxidation of kidney in the DXR + PD group as compared to the DXR + RD group, the level of lipid peroxidation in this group was still significantly higher than in the SA + RD group (Table 3).

Pathological findings in heart and kidney

The animals treated with DXR alone manifested microscopic changes in the heart that consisted of disorganization of myofibrils and associated vacuolization of the myofibers. These changes were randomly distributed in the left and right ventricular wall and the intraventricular septum. The degree of vacuolization in individual myofibers ranged from a single small diameter vacuole to small clusters of several vacuoles of the same size (Fig. 3). While cytoplasmic vacuolization of myofibers was occasionally observed in the SA + RD group, its frequency was significantly less ($P < 0.05$) than in the DXR + RD group. Treatment with PD caused a significant reduction ($P < 0.05$) in the frequency of the vacuolization of myofibers in the DXR + PD group as compared to the DXR + RD group. There was no difference in the myofiber vacuolization between the two control (SA + RD and SA + PD) groups. The average scores of the cardiac lesions in the DXR + RD and DXR + PD groups were 2.2 ± 0.4 and 1.0 ± 0.3, respectively.

Two striking morphological alterations were seen in the kidneys of DXR-treated rats in the DXR + RD group. One was tubular regeneration confined in the cortical region only, and the other was tubular dilation with protein casts found in both the cortical and medullary regions (Fig. 4). The epithelial cells lining the regenerating tubules had pale-staining cytoplasm compared to the surrounding unaffected tubules, and prominent vesicular nuclei. Occasionally these tubules had thickening of the tubular basement membrane and contained protein casts. In a few focal areas, the tubules were surrounded by interstitial fibrosis. A mitotic figure was seen in one of the lining epithelial cells, which was consistent with the regenerative response. A few mononuclear cells were also seen dispersed around these cells, but this was not a consistent finding. Although these changes were occasionally seen in the SA + RD group, they were most severe in the DXR + RD group and markedly decreased in the DXR + PD group. The scores for the degree of severity of the lesions in the kidney were 2.0 ± 0.4 and 0.8 ± 0.2 for rats in the DXR + RD and DXR + PD groups, respectively.

The presence of protein casts in the renal tubules was most commonly seen in the DXR + RD group (Fig. 4). These casts were red and hyalinized in appearance with H&E staining and often caused dilation of the lumen of the renal tubules. Although the protein casts were most prominent in the cortex, these casts were also found in the medulla. The tubular epithelial cells adjacent to the casts ranged from flattened normal to pale ones with a regenerative appearance as described above. There was no evidence of tubular necrosis associated with these casts or in any other part of the kidney. Protein casts were not observed in any of the kidney sections examined from control animals. The DXR + PD group had significantly reduced tubular protein casts as compared to the DXR + RD group. The average scores for the protein casts in the kidney in the DXR + RD and DXR + PD groups were 2.0 ± 0.4 and 0.6 ± 0.2, respectively.

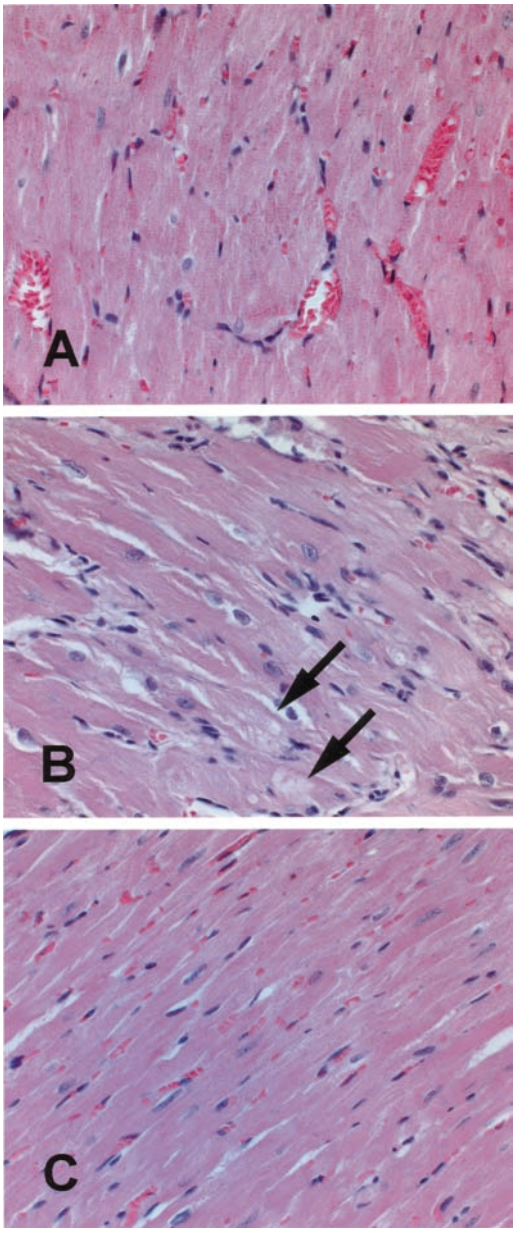

Fig. 3A–C Effects of PD on DXR-induced histopathological changes of the heart. Representative photomicrographs of longitudinal sections through left and right ventricles stained with hematoxylin and eosin, ×500. **A** Heart from a control rat in the SA + RD group showing normal myofibrils. **B** Heart from a rat in the DXR + RD group showing disorganization of myofibrils associated with vacuolization of myofibers (*arrows*). **C** Heart from a rat in the DXR + PD group showing near normal myofibrils. See the legend to Fig. 1 for experimental details and explanations of the abbreviations

Discussion

Rats receiving a cumulative dose of DXR of 16.25 mg/kg in seven injections over a period of 12 days

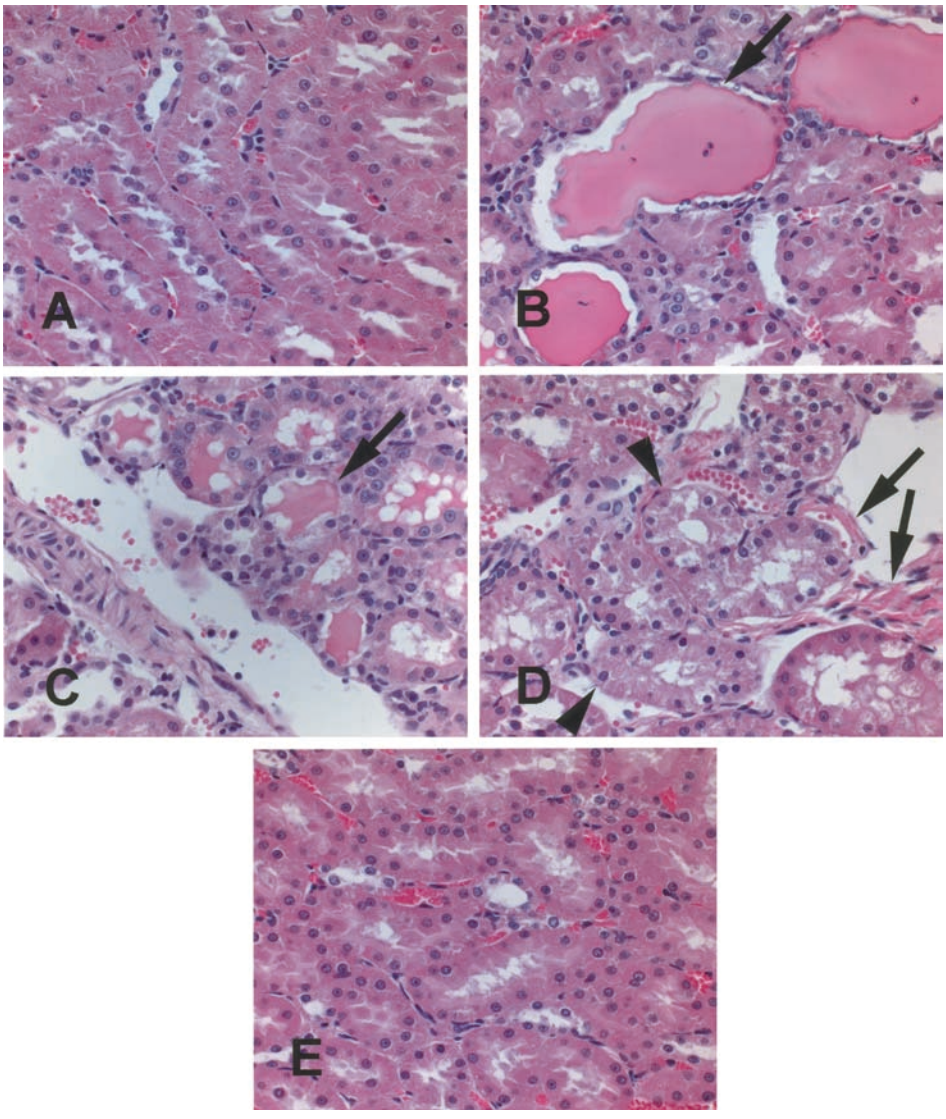

Fig. 4A–E Effects of PD on DXR-induced histopathological changes in the left kidney. Representative photomicrographs of kidney sections stained with hematoxylin and eosin ×500. **A** Kidney from a control rat in the SA + RD group showing normal structure of the renal cortical tubules. **B, C** Kidney from a rat in the DXR + RD group showing a range of tubular dilation with protein casts (*arrows*). **D** Regeneration of renal epithelial cells lining the tubules (*arrowheads*) in the cortex and interstitial fibrosis (*arrows*). **E** Kidney from a rat in the DXR + PD group showing a normal cortical region. See the legend to Fig. 1 for experimental details and explanations of the abbreviations

manifested systemic toxicity at 25 days after the last injection, characterized by significant reductions in daily food intake and body weight with or without PD as compared to the control groups. Similarly, DXR-treated rats in both the DXR + RD and DXR + PD groups had significantly decreased heart and kidney weights as compared to rats in the SA + RD and SA + PD control groups. This reflects the toxic effects of DXR on the heart and kidney; these effects were not minimized by PD treatment in the DXR + PD group. It is somewhat puzzling that DXR treatment caused significant reductions in lipid peroxidation in hearts from the DXR + RD and DXR + PD groups as compared to controls. This finding is similar to the finding in our previous study in which the formation of lipid peroxides in the heart remained unchanged during 14 and 19 weeks of the study period, even though the cumulative dose of DXR was 26 mg/kg given over a period of 13 weeks [42]. Other investigators also have not found any change in lipid peroxidation of the heart in response to DXR treatment [15]. The lack of any change in the MDAE content of the heart, as found in the present study as well as in previous studies, suggests that lipid peroxidation plays little role in DXR-induced cardiac toxicity.

The elevations of the MDAE levels, as an index of lipid peroxidation found in plasma, liver and kidney, as found in the previous study [42], may be a consequence of DXR-induced peroxidation of lipids accumulated as a result of nephrosis caused by this drug [5, 6, 31, 39]. This may explain why plasma and kidney contained higher levels of MDAE in the DXR + RD group than in any other group. Although renal toxicity is not generally considered an important consequence of DXR therapy in humans, renal lesions have been reported in cancer patients receiving DXR therapy [9]. Nevertheless, it is difficult to explain the finding in the present study of significantly decreased MDAE content of the hearts of the DXR + RD and DXR + PD groups as compared to those of the SA + RD and SA + PD control groups. The increase in hydroxyproline content of the hearts of the DXR + RD group suggests the accumulation of collagen as a result of DXR-induced inflammatory changes in the

heart. Its abrogation by PD treatment in the DXR + PD group was not unexpected since PD is known to suppress the deposition of collagen in a variety of organs including the heart in response to different toxic agents [25, 28, 37, 46]. The moderate degree of biochemical changes in the hearts of the DXR + RD group agrees with the minimal histopathological changes and the lack of any changes in cardiac and pulmonary physiological parameters observed in this group. This could be attributed to administration of a suboptimal cumulative dose of DXR to induce an overt cardiac toxicity.

On the contrary, DXR was found to cause significant increases in the lipid peroxidation and hydroxyproline content of the kidney and in lipid peroxidation of plasma and the protein content of the urine of the DXR + RD group. Treatment with PD minimized these increases in the DXR + PD group. These findings suggest that the kidney in rats is more vulnerable to DXR-induced toxicity than the heart. This may be partly due to exposure of the kidney to a higher concentration of DXR than the heart because DXR and its metabolites are partly excreted by the kidneys. The biochemical indices of kidney toxicity such as increased lipid peroxidation and hydroxyproline content and increased protein in the urine in response to DXR treatment in the DXR + RD group paralleled the histopathological changes of the kidney. We have previously reported degenerative changes in the cardiac muscle and renal tubular necrosis with intertubular protein cast formation and mild to moderate thickening of the mesangial matrix in the glomerular tufts of the kidney [42]. However, the cumulative dose of DXR in that study was 26 mg/kg administered over a period of 13 weeks as compared to a cumulative dose of 16.25 mg/kg administered over a period of 12 days in the present study. This suggests that the degenerative changes induced by DXR are dependent on the cumulative dose, duration of treatment and the time of evaluation after the cessation of treatment. It is not surprising that degenerative changes in the cardiac muscle have also been reported in rats in response to treatment with high doses of DXR [13, 42].

The mechanism involved in the DXR-induced oxidative damage to the tissues appears to involve the generation of ROS which initiate free-radical-mediated chain reactions, resulting in the oxidation of unsaturated fatty acids of the membrane into lipid peroxides [10, 19]. This may explain the higher levels of MDAE, an index of lipid peroxidation, and hydroxyproline content, an index of collagen accumulation, in the kidneys of rats in the DX + RD group than of rats in the control groups. The results of the present study are at variance with previously reported results indicating an increase in lipid peroxidation of the heart, even though the DXR doses and treatment regimens in the two studies were almost similar [40].This discrepancy may be attributed to the sources of DXR and strains of rats used in the two studies.

It is remarkable that dietary intake of PD minimized DXR-induced increased fibrosis in the heart, the mild degenerative changes of cardiac muscle and all the biochemical and histopathological indices of kidney toxicity, including increased protein in urine, in the DXR + PD group. In addition, PD treatment in this group also caused significant reductions in ascites and its protein content by 64% and 68%, respectively, as compared to the levels in the DXR + RD group. Furthermore, PD treatment had survival benefits since there was a 12.5% cumulative mortality in the DXR + RD group as compared to zero mortality in the DXR + PD group. These beneficial effects of PD against DXR-induced cardiac and renal toxicity were obtained when the plasma concentrations of PD averaged 255 ± 69 ng/ml on the day the animals were killed. Whether a lower concentration of PD would provide the same level of beneficial effects against DXR-induced cardiac and renal toxicity needs to be investigated.

The beneficial effects of PD against DXR-induced cardiac and renal toxicity as found in this study are similar to its beneficial effects against bleomycin-induced lung toxicity in hamsters and mice [25, 28], and vanadate-induced renal toxicity in rats [1]. PD treatment has also been shown to reverse renal and cardiac fibrosis in streptozotocin-diabetic rats [37] and dimethylnitrosamine-induced hepatic fibrosis in rats [46]. The biochemical mechanism involved in the minimization by PD treatment of the oxidative damage to different organs in response to a number of chemicals may reside in its ability to directly scavenge ROS including O_2^-, H_2O_2, and ·OH as demonstrated both in vitro and in vivo [17, 38].

It is documented that the chemicals capable of generating ROS activate nuclear transcription factor-kB (NF-kB). This allows its translocation into the nucleus where it binds to the promoter regions of inducible nitric oxide synthase (iNOS) and of inflammatory cytokine genes containing the NF-kB motif, and stimulates their synthesis [3, 22, 27]. An excess production of nitric oxide [21, 43] and some cytokines such as TNF-α have been implicated in the tissue injury and dysfunction in a number of organs [47]. Strategies that block an excess production of nitric oxide [18, 21] and TNF-α [11] or neutralize TNF-α by neutralizing antibody [23] have been shown to protect against their deleterious effects. According to the prevailing theory, NF-kB is an oxidant-sensitive transcription factor [45], and it is activated in some cell lines in response to elevated levels of ROS [4, 44]. Since DXR is known to cause oxidative damage by generating ROS in tissues, it is conceivable that it activates NF-kB and thus stimulates the production of an excess amount of nitric oxide and TNF-α, which are directly responsible for heart and kidney toxicity.

This theory would explain the protective effect of aminoguanidine, a specific inhibitor of iNOS against DXR-induced cardiac toxicity [40]. This would also explain the protective effects of PD against cardiac and renal toxicity, because PD is known to be antioxidant by scavenging ROS [17, 38], and compounds

having antioxidant effects are known to block ROS-induced activation of NF-kB, thus minimizing the tissue damage in response to oxidants [20, 32]. The protective effect of PD against DXR-induced cardiac and renal toxicity as found in the present study is similar to the inhibitory effects of other antioxidants, including 5,6,7,8-tetrahydroneopterin, propolis, and alpha-lipoic acid, on Adriamycin-induced cardiotoxicity [2, 24, 30]. Regardless of the mechanisms, it appears that PD is an effective drug in preventing the DXR-induced renal injury at the biochemical, functional and morphological levels and mild cardiac injury as seen in this study.

Although it is difficult to extrapolate rat data to humans, it is possible that oral ingestion of PD in patients on DXR chemotherapy for the management of cancers may provide a survival benefit, improve their food intake and protect their vital organs including heart, kidney and liver against DXR-induced toxicity and organ dysfunction.

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References

1. Al-Bayati MA, Xie Y, Mohr FC, Margolin SB, Giri SN (2002) Effect of pirfenidone against vanadate-induced kidney fibrosis in rats. Biochem Pharmacol 64:517
2. AL-Majed AA, Gado AM, AL-Shabanah OA, Mansour MA (2002) Alpha-lipoic acid ameliorates myocardial toxicity induced by doxorubicin. Pharmacol Res 46:499
3. Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141
4. Barchowsky A, Dudek EJ, Treadwell MD, Wetterhan KE (1996) Arsenic induces oxidant stress and NF-kB activation in cultured aortic endothelial cells. Free Radic Biol Med 18:321
5. Bertani T, Remuzzi G, Rocchi G, Delaini F, Sacchi G, Falchetti M, Donnati MB (1984) Steroids and adriamycin nephrosis. Appl Pathol 2:32
6. Bizzi A, Ceriani L, Gerundino M, Spina A, Tacconi MT, Venorani E (1983) Adriamycin causes hyperlipidemia as a consequence of nephrotoxicity. Toxicol Lett 18:29
7. Blum RH (1975) An overview of studies with Adriamycin (NSC) in the United States. Cancer Chemotherapy Rep 6:247
8. Bristow MR (1982) Toxic cardiomyopathy due to doxorubicin. Hosp Pract 17:101
9. Burke JF, Laucius F, Brodovsky HS, Soriano RZ (1977) Doxorubicin hydrochloride-associated renal failure. Arch Intern Med 137:385
10. Bus JS, Gibson JE (1979) Lipid peroxidation and its role in toxicology. In: Hodgson E, Bend JR, Philpot RM (eds) Reviews in biochemical toxicology. Elsevier North Holland, New York, p 125
11. Cain WC, Stuart RW, Lefkowitz DL, Starnes JD, Margolin SB, Lefkowitz SS (1998) Inhibition of tumor necrosis factor and subsequent endotoxin shock by pirfenidone. Int J Immunol 20:685
12. Chabner BA, Ryan DP, Paz-Ares L, Carbonero RG, Calabresi P (2001) Antineoplastic agents. In: Hardman JG, Limbird LE, Gilman AG (eds) Goodman and Gilman's the pharmacological basis of therapeutics, 10th edn. McGraw-Hill, New York, p 1389
13. Chopra S, Pillai KK, Husain SZ, Giri DK (1995) Propolis protects against doxorubicin-induced myocardiopathy in rats. Exp Mol Pathol 62:190
14. DeAtley SM, Aksenov MY, Aksenova MV, Jordan B, Carney JM, Butterfield DA (1999) Adriamycin-induced changes of creatine kinase activity in vivo and in cardiomyocyte culture. Toxicology 134:51
15. Facchinetti T, Delaini F, Salmona M, Donati MB, Feuerstein S, Wendel A (1983) The influence of selenium intake on chronic adriamycin toxicity and lipid peroxidation in rats. Toxicol Lett 15:128
16. Ferrans VJ (1978) Overview of cardiac pathology in relation to anthracycline cardiotoxicity. Cancer Treat Rep 62:955
17. Giri SN, Leonard S, Shi X, Margolin SB, Vallyathan V (1999) Effects of pirfenidone on the generation of reactive oxygen species in vitro. J Environ Pathol Toxicol Oncol 18:169
18. Giri SN, Biring I, Nguyen T, Wang Q, Hyde DM (2002) Abrogation of bleomycin-induced lung fibrosis by nitric oxide synthase inhibitor, aminoguanidine in mice. Nitric Oxide 7:109
19. Goodman J, Hockstein P (1977) Generation of free radicals and lipid peroxidation by redox cycle of adriamycin and daunorubicin. Biochem Biophys Res Commun 77:797
20. Gurujeyalakshmi G, Wang Y, Giri SN (2000) Taurine and niacin block lung injury and fibrosis by down-regulating bleomycin-induced activation of transcription nuclear factor-kB in mice. J Pharmacol Exp Ther 293:82
21. Gurujayalakshmi G, Wang Y, Giri SN (2002) Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. Nitric Oxide 4:399
22. Henkel T, Mechleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA (1993) Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 365:182
23. Hinshaw L, Olson P, Kuo G (1989) Efficacy of post-treatment with anti-TNF monoclonal antibody in preventing the pathophysiology and lethality of sepsis in the baboon. Circ Shock 27:362
24. Iliskovic N, Hasinoff BB, Malisza KL, Li T, Danelisen I, Singal PK (1999) Mechanisms of beneficial effects of probucol in adriamycin cardiomyopathy. Mol Cell Biochem 196:43
25. Iyer SN, Wild JS, Schiedt MJ, Hyde DM, Margolin SB, Giri SN (1995) Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. J Lab Clin Med 125:779
26. Iyer SN, Hyde DM, Giri SN (2000) Anti-inflammatory effect of pirfenidone in the bleomycin–hamster model of lung inflammation. Inflammation 24:477
27. Janssen Y, Barchowsky A, Treadwell M, Driscoll KE, Mossman BT (1995) Asbestos induces nuclear factor B (NF-kappa B) DNA binding activity and NF-kappa B dependent gene expression in tracheal epithelial cells. Proc Natl Acad Sci U S A 92:8458
28. Kehrer JP, Margolin SB (1997) Pirfenidone diminishes cyclophosphamide-induced lung fibrosis in mice. Toxicol Lett 90:125
29. Kitada M, Horie T, Awazu S (1994) Chemiluminescence associated with doxorubicin-induced lipid peroxidation in rat heart mitochondria. Biochem Pharmacol 48:93
30. Kojima S, Icho T, Hayashi M, Kajiwara Y, Kitabatake K, Kubota K (1993) Inhibitory effect of 5,6,7,8-tetrahydroneopterin on adriamycin-induced cardiotoxicity. J Pharmacol Exp Ther 266:699
31. Kunitoma M, Yamaguchi Y, Matsushima K, Futagawa Y, Bando Y (1985) Hyperlipidemic effects of adriamycin in rats. Jpn J Pharmacol 39:323
32. Leff JA, Wilke CP, Hybertson BM, Shanley CJ, Repine JE (1993) Postinsult treatment with N-acetylcysteine decreases IL-1 induced neutrophil influx and lung leak in rats. Am J Physiol 265:L501
33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265

34. Mansoor JK, Hyde DM, Schelegle ES (1997) Pulmonary vagal reflexes breathing pattern are not altered in elastase-induced emphysema in rats. Exp Lung Res 23:441
35. McManus JFA, Mowry RW (1960) Staining methods, histologic and histochemical. Paul B. Hoeber, Medical Division of Harper and Brothers, New York, p 223
36. Minotti G, Ronci R, Salvatorelli E, Menna P, Cairo G (2001) Doxorubicin irreversibly inactivates iron regulatory protein 1 and 2 in cardiomyocytes: evidence from distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. Cancer Res 61:8422
37. Miric G, Dallemagne C, Endre Z, Margolin SB, Taylor SM, Brown L (2001) Reversal of cardiac and renal fibrosis by pirfenidone and spironolactone in streptozotocin-diabetic rats. Br J Pharmacol 133:687
38. Misra HP, Rabideau C (2000) Pirfenidone inhibits NADPH-dependent microsomal lipid peroxidation and scavenges hydroxyl radicals. Mol Cell Biochem 204:119
39. Montilla P, Tunez I, Munoz MC, Lopez A, Soria JV (1997) Hyperlipidemic nephropathy induced by adriamycin: effect of melatonin administration. Nephron 76:345
40. Mostafa AM, Nagi MN, Al Rikabi AC, Al-Shabanah OA, El-Kashef HA (1999) Protective effect of aminoguanidine against cardiovascular toxicity of chronic doxorubicin treatment in rats. Res Commun Mol Pathol Pharmacol 106:193
41. Ohkawa H, Ohismi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Biochemistry 95:351
42. Robison TW, Giri SN, Wilson DW (1989) Effects of chronic administration of doxorubicin on myocardial creatine phosphokinase and antioxidant defenses and levels of lipid peroxidation in tissues and plasma of rats. J Biochem Toxicol 4:87
43. Salch D, Barnes PJ, Giad A (1997) Increased production of potent oxidant peroxy nitrite in the lungs of patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 155:1763
44. Schreck R, Albermann K, Baurel PA (1992) Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (review). Free Radic Res Commun 17:221
45. Sun Y, Oberley LW (1966) Redox regulation of transcriptional activators. Free Radic Biol Med 21:335
46. Tada S, Nakamuta M, Enjoji M (2001) Pirfenidone inhibits dimethylnitrosamine-induced hepatic fibrosis in rats. Exp Pharmacol Physiol 133:687
47. Tracy KJ, Beutler B, Lowry SF, et al (1986) Shock and tissue injury induced by recombinant human cachectin. Science 234:470
48. Woessner JF Jr (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 93:440
49. Zima T, Tesar V, Stipek S, Crkovska J, Poledne R, Teminova J, Platenik J, Rychlik I, Merta M, Nemecek K (1997) The influence of cyclosporin on lipid peroxidation and superoxide dismutase in adriamycin nephropathy in rats. Nephron 75:464