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Original Research Communication

Apoptosis in Adriamycin Cardiomyopathy and Its Modulation by Probucol

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ABSTRACT

The dose-dependent cardiomyopathy and heart failure due to adriamycin have been shown to be due to increased oxidative stress and loss of myocytes. We examined the incidence of myocardial apoptosis as well as changes in the expression of apoptotic regulatory gene products in an established animal model of adriamycin cardiomyopathy. Rats were treated with adriamycin (cumulative dose, 15 mg/kg), and the hearts were examined for apoptosis as well as expression of Bax, caspase 3, and Bcl-2 at 0, 4, 10, 16, and 21 days after the treatment. A significant increase in the incidence of apoptosis was seen at 4 days, followed by a decline at 10 and 16 days of posttreatment. At 21 days, the number of apoptotic cells increased again and included cells of the conducting system. Expression of Bax corresponded to these biphasic changes, whereas the converse was true for the expression of Bcl-2. The latter peaked at 10 days followed by a decline at 16 and 21 days. The Bax/Bcl-2 ratio also correlated with the incidence of apoptosis. Expression of caspase 3 correlated with increased apoptosis, but only at early time points. Probucol (cumulative dose, 120 mg/kg), a known antioxidant as well as promoter of endogenous antioxidants, significantly reduced the incidence of apoptosis as well as expression of Bax. Adriamycin-induced hemodynamic changes were also prevented by probucol. These data suggest that adriamycin-induced apoptosis is mediated by oxidative stress and may play a role in the development of heart failure. Antioxid. Redox Signal. 3, 135–145.

INTRODUCTION

ADRIAMYCIN (DOXORUBICIN) is a highly active antineoplastic drug used for the treatment of a variety of human malignancies. However, the usefulness of adriamycin is limited by the fact that repetitive administration of the drug causes cardiomyopathy and heart failure (19, 35, 37). Although adriamycin-induced cell injury is multifactorial (10, 29, 36, 39, 40, 46), increased oxidative stress has been shown to play a major role in mediating the chronic cardiotoxic side effects of adriamycin (25, 38). Probucol, a lipid-lowering drug with antioxidant properties, has been shown to prevent adriamycin-induced cardiomyopathy as well as associated subcellular changes (41).

Apoptosis, or programmed cell death, is a highly conserved evolutionary event that is crucial for normal development and cell homeostasis. However, recent data suggest that an inappropriate or untimely loss or ventricular myocytes may play a major role in the pathogenesis of different cardiac disease states (26, 28, 33, 34). Whether the cardiomyocyte death associated with adriamycin-induced cardiomyopathy *in vivo* is a result of apoptosis remains to be determined. Earlier studies have

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reported the occurrence of apoptosis, due to adriamycin administration in kidney, intestine, and hair follicles as well as various cell lines (7, 49). In heart, adriamycin-induced apoptosis was reported to be limited to interstitial dendritic cells and macrophages, but not to myocytes (49). However, morphological features of hearts from patients and animals with adriamycin-induced cardiomyopathy suggest that myocyte cell death may be mediated at least in part through an apoptotic process (38). Furthermore, adriamycin-induced myocyte cell death in cell culture has been demonstrated to be mediated by a combination of apoptosis and necrosis (18).

Using an established model of adriamycin-induced cardiomyopathy and heart failure in adult rats, we examined whether the myocyte loss associated with adriamycin-induced cardiomyopathy is a result of an increase in the incidence of apoptosis. Expression of apoptosis regulatory gene products such as Bax, caspase 3, and Bcl-2 was also examined. As probucol is known to prevent adriamycin-induced cardiomyopathic changes, its effects on adriamycin-induced apoptosis in ventricular myocytes were also examined.

MATERIALS AND METHODS

Animal model

Male Sprague–Dawley rats (body weight, 250 ± 10 g) were maintained on normal rat chow. Rats were divided into four groups: control (CONT), adriamycin-treated (ADR), probucol-treated (PROB), and probucol + adriamycin-treated (PROB + ADR). Adriamycin (doxorubicin hydrochloride) was administered intraperitoneally in six equal injections (each containing 2.5 mg/kg of body weight) to animals in the ADR and PROB + ADR groups over a period of 2 weeks for a total cumulative dose of 15 mg/kg of body weight as described earlier (43). Probucol (cumulative dose, 120 mg/kg of body weight) was administered intraperitoneally to the PROB and PROB + ADR groups in 12 equal injections (each treatment containing 10 mg/kg) over a period of 4 weeks, 2 weeks before adriamycin administration and 2 weeks alternating with adriamycin injections. Animals in the CONT group were injected with the vehicle only (lactose, 75 mg/kg in saline) on the same regimen as ADR. All studies were approved by the Central Animal Care Committee of the University of Manitoba.

Hemodynamic studies

At the end of the 3-week posttreatment period, animals from all of the groups were assessed hemodynamically by a procedure described before (44). In brief, animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.). A miniature pressure transducer (Millar Micro-Tip) was inserted into the left ventricle via the right carotid artery. Left ventricle systolic pressure (LVSP), left ventricle end-diastolic pressure (LVEDP), aortic systolic pressure (ASP), and aortic diastolic pressure (ADP) were recorded.

Apoptosis and immunohistochemistry

After the last injection, rats were killed by decapitation on days 0, 4, 10, 16, and 21 for the ADR group and on day 21 for the CONT, PROB, and PROB + ADR groups. Hearts were removed and immediately fixed in buffered formalin. The hearts were cut into blocks, embedded in paraffin, and cut into tissue sections of 5 μm for the study of apoptosis and immunohistochemical staining for Bax, caspase 3, and Bcl-2. In each group and each time point, three or four hearts were sectioned, stained, and analyzed.

Apoptosis by conventional and confocal microscopy. Tissue sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol (100%, 90%, 70%, 50%, 30%, and phosphate-buffered saline). Tissue sections were treated with proteinase K (25 μg/ml in 10 m*M* Tris-HCl) for 30 min at 30°C, and washed twice in phosphate-buffered saline. Slides were then incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature to inactivate endogenous peroxidase activity. Tissue sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, and processed for terminal deoxynucleotidyltrans-

ferase-mediated nick end-labeling (TUNEL) assay.

In brief, sections were incubated for 1 h at 37°C in terminal deoxynucleotide transferase buffer containing 140 m*M* sodium cacodylate, 1 m*M* cobalt chloride, 30 m*M* Tris-HCl, pH 7.2, 50 units of terminal deoxynucleotide transferase, and 1 nmol of fluorescein isothiocyanate-conjugated dUTP or rabbit anti-fluorescein isothiocyanate conjugated to peroxide using diaminobenzoic acid as substrate (Boehringer Mannheim). To differentiate cardiac myocytes from noncardiac cells, sections were double-immunostained for the presence of sarcomeric myosin with anti-sarcomeric myosin antibody MF20 (provided by D. Bader, Vanderbilt University) followed by 10 μg/ml rhodamine-conjugated anti-mouse IgG. Following the terminal nucleotide transferase reaction, sections were washed three times in phosphate-buffered saline and mounted on glass slides. Tissue sections were visualized under bright field and confocal microscopy using an Olympus IX 70 Research microscope equipped with an Olympus Fluoview Laser Scanning Module.

Quantitative analysis of apoptotic myocytes was performed on four to six individual tissue sections, using four to six adjacent fields from the epicardial, myocardial, and endocardial regions. The percentage of positive myocyte cells was calculated counting positive nuclei per 500 myocyte nuclei counted. Cardiomyocytes were also distinguished morphologically from nonmyocytes using phase-contrast microscopy as well as by counterstaining of adjacent tissue sections with hematoxylin. This approach is similar to one adopted by others for quantitation of apoptosis in human hearts (22). For statistical analysis of the data, group means were compared by one-way analysis of variance.

Immunohistochemical staining for the detection of Bax, caspase 3, and Bcl-2. Immunoperoxidase staining was performed by using methods described previously with some modifications (23). In brief, tissue sections were deparaffinized, and endogenous peroxidase activity was inactivated as described above. Antigen unmasking was done by saponin treatment (5–10 μg/ml in distilled water) for 30 min at room temperature. Following washing, tissue sections were blocked with 1% normal goat serum to prevent nonspecific binding. Sections were incubated with rabbit antibodies directed toward rat Bax, caspase 3, and Bcl-2 followed by goat anti-rabbit antibody conjugated to horseradish peroxidase (1:500 dilution) in a humidified chamber at 37°C. The antibody against the active subunit for caspase 3 was obtained from Santa Cruz. Sections were washed and incubated with diaminobenzidine substrate for detection of Bax, caspase 3, and Bcl-2 expression by light microscopy. A reddish brown appearance in the cytoplasm of cardiomyocyte counted as a positive myocyte. Quantitative analysis of myocytes was performed by counting the number of positive cells as described above.

RESULTS

General observations and hemodynamics

Animals in the ADR group began to show enlargement of the abdomen 1 week after the last treatment. At 21 days, all animals in the ADR group had a significant amount of peri-

Table 1. Effects of Probucol on Adriamycin-Induced Hemodynamic Changes

Hemodynamic parameters	*CONT*	*ADR*	*PROB*	*PROB + ADR*
LVEDP	4.2 ± 1.5	29.5 ± 3.6*	2.9 ± 1.4	6.1 ± 1.8
LVSP	111.5 ± 3.2	83.2 ± 4.1*	108.4 ± 5.3	109.3 ± 4.2
ADP	70.2 ± 4.1	68.4 ± 3.1	71.6 ± 4.4	69.2 ± 3.1
ASP	106.9 ± 6.1	79.1 ± 5.2*	107.6 ± 3.6	103.5 ± 5.2

Data are means ± SEM of six or seven animals.
*$p < 0.05$ compared with all other groups.

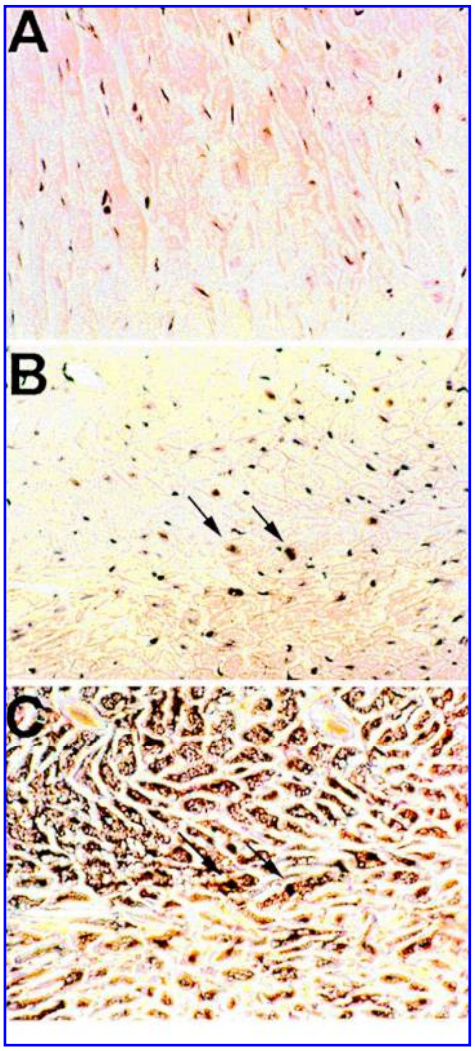


FIG. 1. (**A**) Control heart with TUNEL and hematoxylin staining. (**B**) Adriamycin, 4 days following treatment, TUNEL and hematoxylin staining. (**C**) Adriamycin, 4 days following treatment, TUNEL staining and phase contrast. Vacuolization of the cytoplasm, typical of adriamycin cardiomyopathy, can be seen in C. Arrows point to apoptotic nuclei.

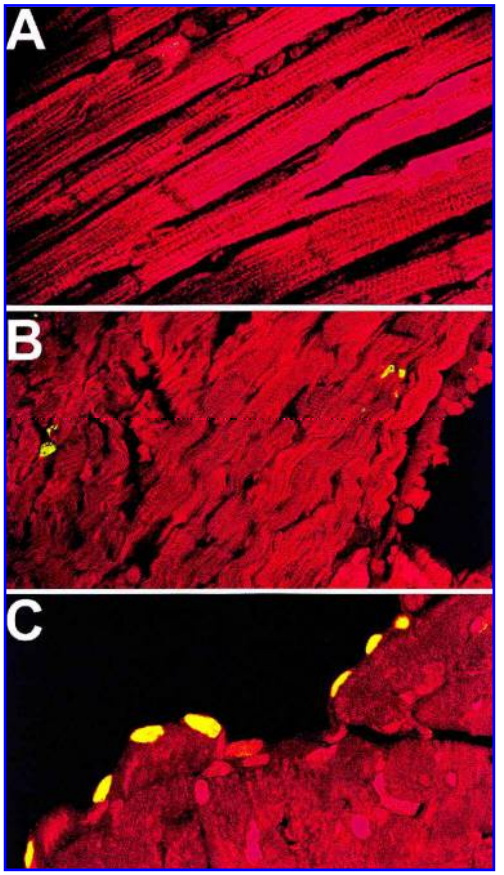
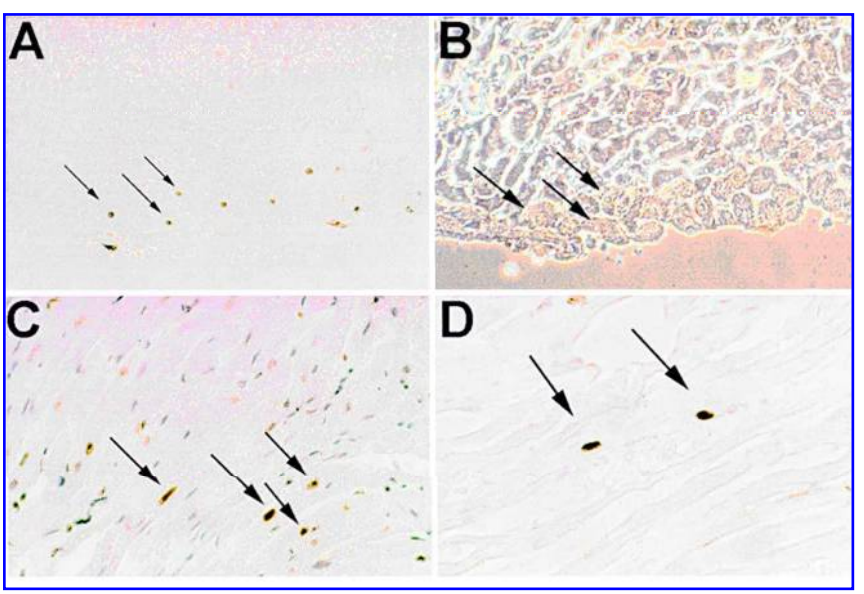


FIG. 3. Adriamycin group, 21 days following treatment. Confocal micrographs after tissue sections were stained for TUNEL (green) and sarcomeric myosin (red) are shown: (**A**) control; (**B**) myocardial region; and (**C**) subendocardial localization of apoptotic nuclei.

FIG. 2. TUNEL staining in 21-day posttreatment group. (**A**) Adriamycin alone; subendocardial localization of apoptotic nuclei. (**B**) Adriamycin alone; same general area in phase contrast. Purkinje cell layer in the subendocardial region as well as vacuolization in most of the cardiomyocytes is also apparent. (**C**) Adriamycin alone; TUNEL with hematoxylin. (**D**) Adriamycin plus probucol; TUNEL staining. Arrows point to apoptotic nuclei.



toneal fluid. By this time, ADR animals showed dyspnea and appeared weaker and slow. All these signs and ascites were absent in animals in the PROB + ADR, PROB, and CONT groups. Mortality in the ADR group at 21 days was 40%, and there was no death seen in any of the other three groups. At 21 days, whereas LVEDP of the animals in the ADR group was significantly elevated, LVSP and ASP were significantly depressed (Table 1). These parameters in PROB + ADR group animals were no different from those in the CONT and PROB groups (Table 1).

Analysis of apoptosis

Adriamycin treatment resulted in vacuolization in the cytoplasm and myofibrillar drop out, and the extent of these morphological changes increased with the posttreatment duration. Analysis of the hearts from the CONT group displayed an infrequent number of apoptotic events (0.08%) as indicated by TUNEL assay (Fig. 1A). In contrast, however, the hearts from the ADR group exhibited a significantly higher number of apoptotic myocytes at 4 days (Fig. 1B and C). The distribution of apoptotic cells in these hearts at 4 days was uniform throughout the myocardium (Fig. 1B and C). At 10- and 16-day posttreatment durations, the incidence of apoptosis was less than that observed at 4 days. However, at 21 days, the frequency of apoptotic cells increased (Fig. 2A–C). A similar distribution of the apoptotic cardiomyocytes in the myocardial as well as endocardial regions was confirmed by confocal microscopy using double staining (Fig. 3). Interestingly, at 21 days, some of the involved myocytes were found localized in the subendocardial region (Figs. 2A and B and 3C). Morphological assessment revealed that these myocytes were oblong, joined end to end, and manifested the appearance of Purkinje cell fiber. In addition, under phase contrast, cardiomyocytes in the ADR group showed vacuolization typical of this drug (Figs. 1C and 2B).

Quantitative analysis of different tissue sections from 0-, 4-, 10-, 16-, and 21-day posttreatment ADR groups was done, and a biphasic response in the frequency of apoptosis was apparent (Fig. 4A). At 4 days following treatment, ~6% of cardiomyocytes displayed evidence of apoptosis, which was decreased to ~1.3% at 10 days. The DNA fragmented nuclei observed in ADR group hearts rose to <3% and >5% at 16 and 21 days, respectively (Fig. 4A). Hearts from PROB + ADR group animals were analyzed at 21-day posttreatment duration, and these data are shown in Fig. 2D and 4B. There was a significant decrease in the number of apoptotic nuclei in the PROB + ADR group compared with the ADR group at 21 days (Fig. 4B).

Expression of Bax, Bcl-2, and caspase in myocytes

The immunohistochemical detection of Bax, Bcl-2, and caspase 3 in the adriamycin cardiomyopathy rat model was performed at 0, 4, 10, 16, and 21 days. Positive cardiomyocytes with the appearance of reddish brown color in the cytoplasm were counted. Figure 5 illus-

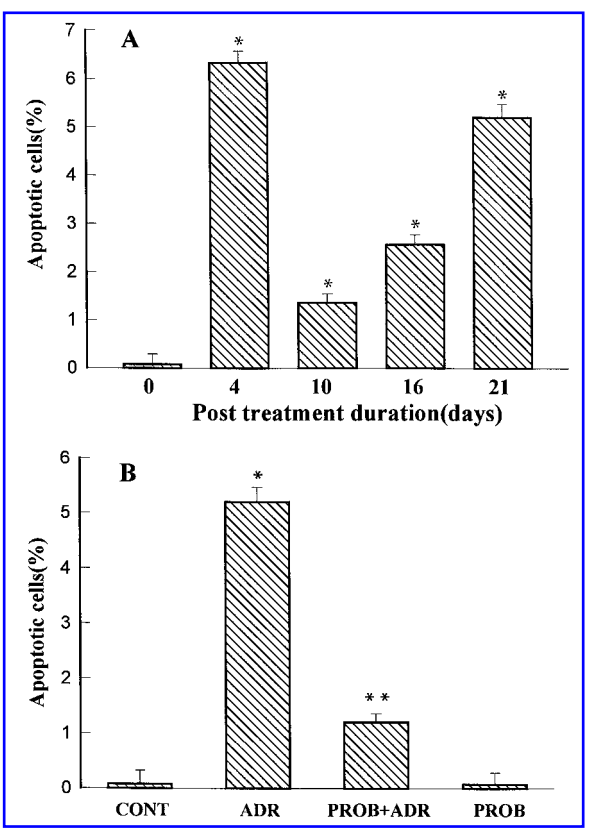


FIG. 4. (**A**) Percentage of apoptotic cardiomyocytes in the ADR group hearts at different time intervals (0, 4, 10, 16, and 21 days in the ADR group) after the last injection of adriamycin. (**B**) Percentage of apoptotic myocytes at 21 days following treatment. *, **Significantly different ($p < 0.05$) from the other three groups.

trates that Bax labeling, which was found rarely in control myocytes (Fig. 5A), was more frequently distributed in the cytoplasm of the ADR group (Fig. 5B). However, the expression for Bax was significantly reduced in the PROB + ADR group at 21 days (Fig. 5C). Quantitative analysis of the number of myocytes displaying positive staining for Bax revealed a maximum reaction that peaked at 4 days for 9.2% followed by a sharp decline to 4.4% at 10 days with a gradual rise to 5.8% and 6.2%, respectively, at 16 and 21 days following adriamycin treatment (Fig. 6). At 21-day posttreatment duration, the number of cells staining positive for Bax was only ~2% in the

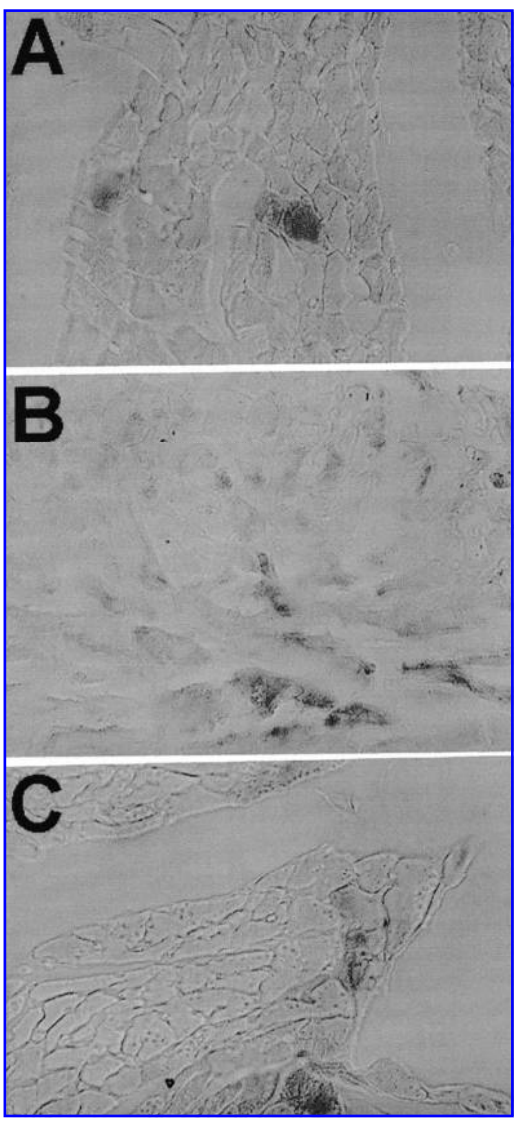


FIG. 5. Bax staining. (**A**) Control. (**B**) 21 days in the ADR group. (**C**) 21 days in the PROB + ADR group.

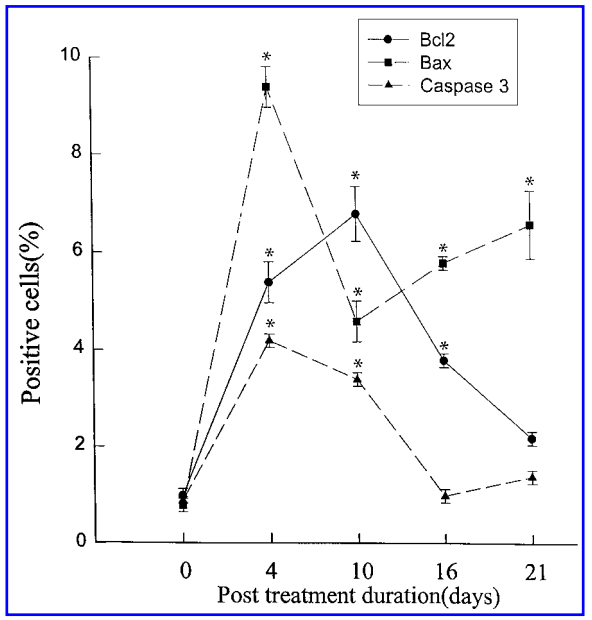


FIG. 6. Percentage expression of Bcl-2-, Bax-, and caspase-positive myocytes at different posttreatment durations in the ADR group hearts. *Significantly different ($p < 0.05$) from 0 time point value.

ADR + PROB group, which was significantly less than that in the ADR group (Fig. 7).

The distribution pattern for Bcl-2 proteins was also characteristic of posttreatment durations with adriamycin, and its expression in relation to Bax and caspase 3 is shown (Fig. 6). In the CONT hearts, <1% of the myocytes were

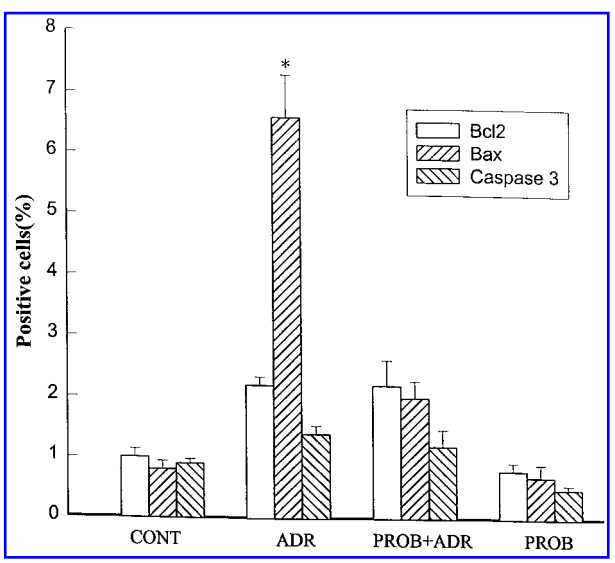


FIG. 7. Effects of probucol on adriamycin-induced changes in the expression of Bax, Bcl-2, and caspase at 21 days. *Significantly different ($p < 0.05$) from all other groups.

positively stained for Bcl-2 (Fig. 8A). The reaction in ADR hearts peaked at 10 days (Fig. 8B) followed by a decline at 16 and 21 days after treatment (Fig. 8C). The expression of Bcl-2 at 21 days in the ADR group was not different from that in the PROB + ADR group (Fig. 7).

A slight immunoreactive staining for caspase 3 protein was detected in <1% of the myocytes in the CONT group (Fig. 9A). In the ADR group heart, the maximum staining for caspase 3 occurred at 4 days after treatment (Fig. 9B) followed by a decline at 10, 16, and 21 days after treatment (Fig. 6). Some positive immunoreactive cells were observed at 21 days in the ADR group, but the reactivity was not different from that in the PROB + ADR group (Fig. 7).

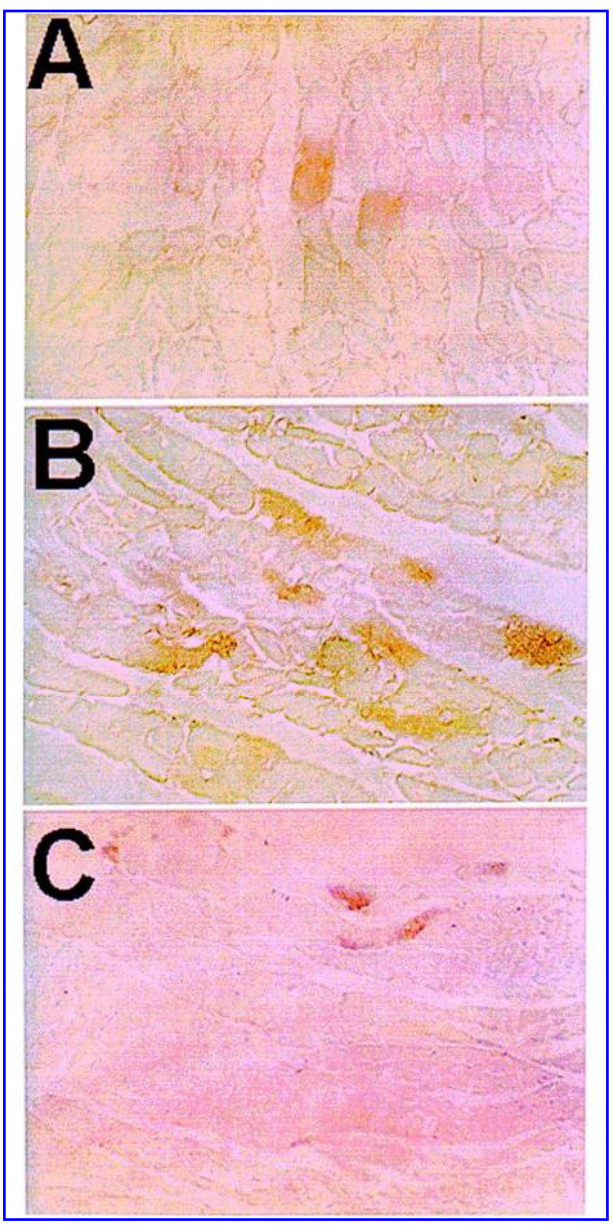


FIG. 8. Bcl-2 staining. (**A**) Control. (**B**) 10 days with adriamycin. (**C**) 21 days with adriamycin.

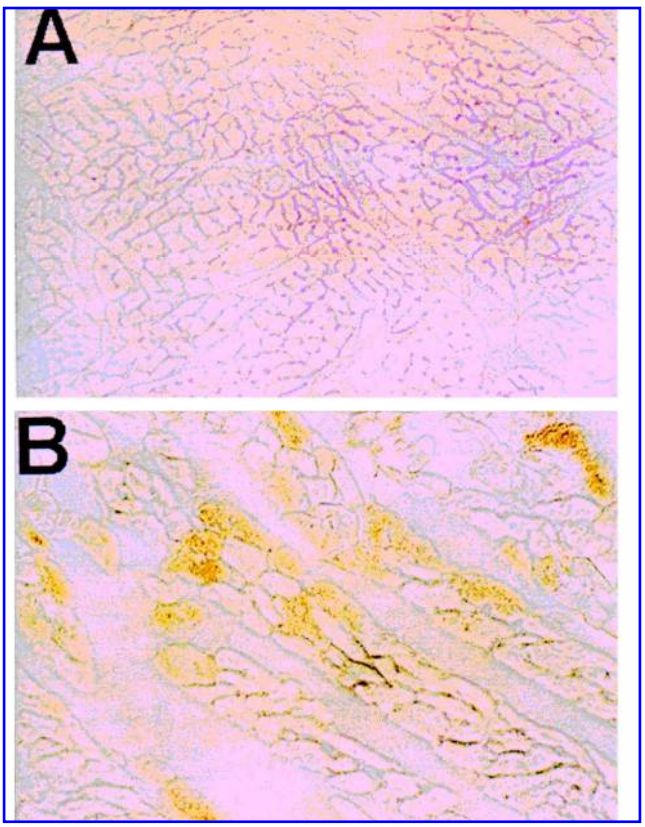


FIG. 9. Caspase 3 staining. (**A**) Control. (**B**) 4 days with adriamycin.

The ratio of Bax/Bcl-2 in ADR group hearts showed a biphasic response and correlated with the extent of apoptosis seen in these hearts (Fig. 10).

DISCUSSION

The study of adriamycin-induced cardiomyopathy and heart failure by using rat as an animal model was first reported in 1973 (8). The treatment dose and schedule used in the present study was established in our laboratory (48) and has been used by other groups (4, 24). These rats at 3 weeks after the last injection seem to mimic not only myocardial structural changes, but also the functional refractoriness of adriamycin-induced cardiomyopathy observed in chronic stages in humans (19, 46).

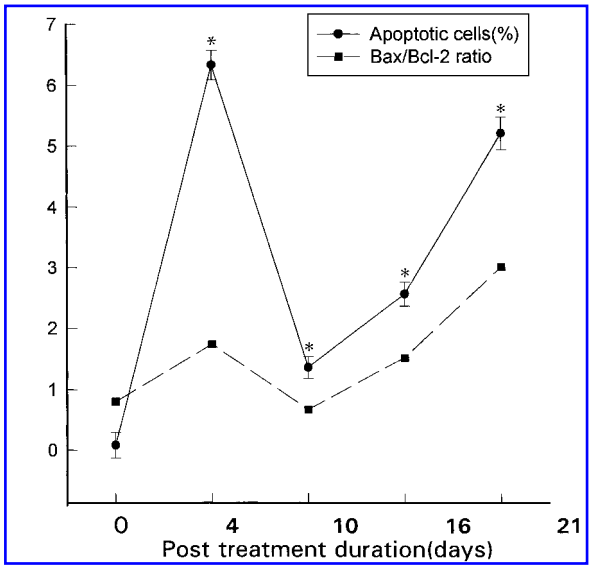


FIG. 10. Percentage of apoptotic myocytes and Bax/Bcl-2 ratio at different posttreatment durations in the ADR group. *Significantly different ($p < 0.05$) from the 0 time point.

Thus, the induction of cardiomyopathy in this model is highly reproducible and is time-related. Heart failure in the present study was confirmed by the presence of ascites, dyspnea, a rise in LVEDP, and depression of LVSP and ASP. It has also been established that the myocardial cell damage is characterized by the dilatation of the sarcoplasmic reticulum, loss of myofibrils, and ultimately emptying and loss of the myocytes (38, 41). These changes, typical for adriamycin cardiomyopathy, were also observed in the present study.

It is important to note that morphological features of adriamycin-induced cardiomyopathy suggest that the process may be a regulated event occurring in an orderly fashion. In a previous study on isolated adult rat cardiomyocytes, we reported that adriamycin causes apoptosis as evidenced by histochemical studies as well as DNA laddering (18). Apoptotic cell death in isolated myocytes has also been reported by others (13, 47). In isolated myocytes, both TUNEL assay and Hoechst 33258 staining revealed a significantly increased number of apoptotic nuclei and nucleosomal fragmentation upon adriamycin treatment (18). DNA laddering was also observed on agarose gel electrophoresis in the cells treated with adriamycin, whereas it was undetectable in control cells (13, 18, 47). The present *in vivo* study not only supports the previous findings, but also demonstrates, for the first time, that adriamycin-induced cardiomyopathy involves myocyte cell death through an apoptotic process. The extent of apoptosis in adriamycin cardiomyopathy seems to vary with the posttreatment duration. The first peak of apoptotic myocytes was seen at 4 days, and distribution of these cells was fairly uniform. The second peak of apoptotic myocytes at 21 days following treatment appears to be due to a higher incidence of cell death in the subendocardial cells. Judging from the location and morphology of these myocytes in phase contrast, hematoxylin staining, and confocal microscopy, it is likely that these may have been conducting cells.

The high incidence of apoptosis (5–6%) seen in the ADR group in this study cannot be conducive for life. Indeed, dilation of the heart, thinning of the ventricular wall, and high mortality are seen in patients (19, 37) as well as experimental animals (42, 44). In the present study, the mortality in the ADR group at 3 weeks of posttreatment was at 40%. Focal myocardial necroses have been reported in some patients who died in cardiogenic shock within 4 weeks of receiving their first or second course of adriamycin (5). However, in more chronic lesions after several cycles of anthracyclines, an inflammatory reaction is usually absent (12). The present animal model represents more of a chronic stage, which also does not show any inflammatory infiltrations (42). Although in the present study we focused on apoptosis, the occurrence of focal necrosis cannot be ruled out. However, in a recent report, apoptosis has been reported as the predominant form of cell death due to adriamycin as compared with necrosis (16).

Expression of Bax and caspase 3 has been reported to promote apoptotic cell death in various cell systems (14, 27, 31, 47). Induction of apoptosis by these pro-apoptotic factors in myoctes under different disease conditions has also been reported (2, 23, 31). In the present study, an increased incidence of apoptosis also correlated with an increased expression of Bax in the hearts of rats with adriamycin-induced cardiomyopathy. The level of Bax protein ex-

pression was maximal at 4 and 21 days following the last adriamycin treatment. Maximal activation of caspase 3 in these cardiomyopathic hearts was seen at 4 days. These findings corroborate and are consistent with previous studies on H9C2 cardiac muscle cells following doxorubicin treatment in which Bax and caspase 3 activation was seen (47). Furthermore, a higher expression of Bax and caspase 3 was associated with positive *in situ* nick-end labeling of DNA at these time points. A correlation between the frequency of TUNEL-positive nuclei and expression of Bax at 4 and 21 days and of caspase 3 at 4 days provides strong evidence to support the occurrence of apoptotic myocyte cell death in adriamycin cardiomyopathy. A lack of correlation between TUNEL positivity and caspase 3 expression at 21 days may suggest a greater role for Bax at these later time points.

Several recent studies have shown that Bcl-2 offers protection against drug-induced apoptosis in endothelial cells, lymphocytes, ventricular myocytes, and cultured vascular smooth muscle cells (17, 20, 23, 32). In the present study, the increased Bcl-2 protein expression in adriamycin cardiomyopathic rat hearts correlated with a reduced incidence of apoptosis, and the converse was true at 4 and 21 days when apoptosis was found to be maximal. Furthermore, the Bax/Bcl-2 ratio at different time points also correlated with apoptosis in adriamycin cardiomyopathy.

The role of oxygen free radicals in the pathogenesis of the cardiomyopathy has been supported by direct as well as indirect evidence (3, 21, 38). Increased levels of oxygen species due to adriamycin have been detected directly by electron spin resonance (1, 9, 15, 45), indirectly by an increase in tissue malondialdehyde, which is a breakdown product of lipid peroxidation (30, 38), and lower endogenous antioxidant enzyme activities (10, 21, 38). Furthermore, free radical scavengers, and drugs with antioxidant properties such as *N*-acetylcysteine, α-tocopherol, and probucol, reduce anthracycline-induced cardiotoxicity (25, 30, 44). Although the exact mechanism of the cytoprotective action of probucol remains to be elucidated, the drug is known to decrease myocardial oxidative stress by acting as an antioxidant as well as by promoting endogenous antioxidant enzyme activities (44). Increased oxidative stress has been suggested to be involved in apoptosis (6). In the present study, the adriamycin-induced apoptosis was modulated in animals treated with probucol, suggesting that the apoptosis, an event underlying adriamycin cardiomyopathy, may be mediated by the increased oxidative stress. A simplified scheme encompassing the proposed adriamycin-induced increase in oxidative stress, apoptotic regulatory proteins, apoptosis, and heart failure as well as the protective effects of probucol is shown in Fig. 11.

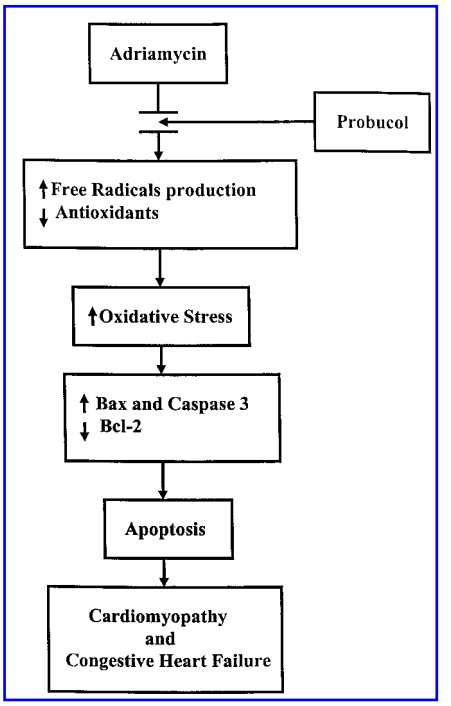


FIG. 11. Schematic representation of adriamycin-induced cardiomyopathy and congestive heart failure being mediated by an increase in oxidative stress and apoptosis, as well as the beneficial effects of probucol in mitigating these changes.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ADP, aortic diastolic pressure; ADR, adriamycin (doxorubicin)-treated; ASP, aortic systolic pressure; CONT, control; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; PROB, probucol-treated; PROB + ADR, probucol + adriamycin-treated; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end-labeling.

REFERENCES

1. Alegria AE, Samuni A, Mitchell JB, Riesz P, and Russo A. Free radicals induced by adriamycin-sensitive and adriamycin-resistant cells: a spin-trapping study. *Biochemistry* 28: 8653–8658, 1989.
2. Anversa P, and Kajstura J. Myocyte cell death in the diseased heart. *Circ Res* 82: 1231–1233, 1998.
3. Bachur NR, Gee MV, and Friedman RD. Nuclear catalyzed antibiotic free radical formation. *Cancer Res* 42: 1078–1081, 1982.
4. Beanlands RS, Shaikh NA, Wen WH, Dawood F, Ugnat AM, McLaughlin PR, Carere R, and Liu PP. Alterations in fatty acid metabolism in adriamycin cardiomyopathy. *J Mol Cell Cardiol* 26: 109–119, 1994.
5. Bristow MR, Thompson PD, Martin RP, Mason JW, Billingham ME, and Harrison DC. Early anthracycline cardiotoxicity. *Am J Med* 65: 823–832, 1978.
6. Buttke TM, and Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* 15: 7–10, 1994.
7. Cece R, Cazzaniga S, Morelli D, Sfondrini L, Bignotto M, Menard S, Colnaghi MI, and Balsari A. Apoptosis of hair follicle cells during doxorubicin-induced alopecia in rats. *Lab Invest* 75: 601–609, 1996.
8. Chalcroft SC, Gavin JB, and Herdson PB. Fine structural changes in rat myocardium induced by daunorubicin. *Pathology* 5: 99–105, 1973.
9. Costa L, Malatesta V, Morazzoni F, Scotti R, Monti E, and Paracchini L. Direct detection of paramagnetic species in adriamycin perfused rat hearts. *Biochem Biophys Res Commun* 153: 275–280, 1988.
10. Doroshow JH. Effect of anthracycline antibiotics on oxygen radical formation in rat heart. *Cancer Res* 43: 460–472, 1983.
11. Doroshow JH, Akman S, Chu FF, and Esworthy S. Role of the glutathione—glutathione peroxidase cycle in the cytotoxicity of the anticancer quinones. *Pharmacol Ther* 47: 359–370, 1990.
12. Ferrans VJ. Anthracycline cardiotoxicity. *Adv Exp Med Biol* 161: 519–532, 1983.
13. Ito H, Shimojo T, Fujisaki H, Tamamori M, Ishiyama S, Adachi S, Abe S, Marumo F, and Hiroe M. Thermal preconditioning protects rat cardiac muscle cells from doxorubicin-induced apoptosis. *Life Sci* 64: 755–761, 1999.
14. Kajstura J, Liu Y, Baldini A, Li B, Olivetti G, Leri A, and Anversa P. Coronary artery constriction in rats: necrotic and apoptotic myocyte death. *Am J Cardiol* 82: 30K–41K, 1998.
15. Kalyanaraman B, Perez-Reyes E, and Mason RP. Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. *Biochim Biophys Acta* 630: 119–130, 1980.
16. Khaw BA, Rammohan R, Narula J, and Pak K-Y. Te-99m glucaric acid targets the nucleoproteins of acutely necrotic myocardium but cannot target myocardial cell death due to apoptosis. *Circulation* 100: Suppl. 1, 310, 1999.
17. Kirshenbaum LA, and de Moissac D. The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation* 96: 1580–1585, 1997.
18. Kumar D, Kirshenbaum L, Li T, Danelisen I, and Singal P. Apoptosis in isolated adult cardiomyocytes exposed to adriamycin. *Ann NY Acad Sci* 874: 156–168, 1999.
19. Lefrak EA, Pitha J, Rosenheim S, and Gottlieb JA. A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* 32: 302–314, 1973.
20. Leszczynski D, Zhao Y, Luokkamaki M, and Foegh ML. Apoptosis of vascular smooth muscle cells. Protein kinase C and oncoprotein Bcl-2 are involved in regulation of apoptosis in non-transformed rat vascular smooth muscle cells. *Am J Pathol* 145: 1265–1270, 1994.
21. Li T, and Singal PK. Adriamycin-induced early changes in myocardial antioxidant enzymes and their modulation by probucol. *Circulation* 102: 2105–2110, 2000.
22. Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, and Fontaine G. Evidence of apoptosis in arrhythmogenic right ventricular dysplasia. *N Engl J Med* 335: 1190–1196, 1996.
23. Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, and Fujiwara H. Expression of Bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. *Circulation* 94: 1506–1512, 1996.
24. Morishima I, Matsui H, Mukawa H, Hayashi K, Toki Y, Okumura K, Ito T, and Hayakawa T. Melatonin, a pineal hormone with antioxidant property, protects against adriamycin cardiomyopathy in rats. *Life Sci* 63: 511–521, 1998.
25. Myers CE, McGuire WP, Liss RH, Ifrim i, Grotzinger K, and Young RC. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science* 197: 165–167, 1977.
26. Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, and Khaw BA. Apoptosis in myocytes in end-stage heart failure. *N Engl J Med* 335: 1182–1189, 1996.
27. Nicholson DW, Ali A, Thornberry NA, Vaillancourt

JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, and Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376: 37–43, 1995.
28. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S, Reed JC, and Anversa P. Apoptosis in the failing human heart. *N Engl J Med* 336: 1131–1141, 1997.
29. Olson HM, Young DM, Prieur DJ, LeRoy AF, and Reagan RL. Electrolyte and morphologic alterations of myocardium in adriamycin-treated rabbits. *Am J Pathol* 77: 439–454, 1974.
30. Olson RD, Boerth RC, Gerber JG, and Nies AS. Mechanism of adriamycin cardiotoxicity: evidence for oxidative stress. *Life Sci* 29: 1393–1401, 1981.
31. Oltvai ZN, Milliman CL, and Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74: 609–619, 1993.
32. Otsuki Y, Misaki O, Sugimoto O, Ito Y, Tsujimoto Y, and Akao Y. Cyclic bcl-2 gene expression in human uterine endometrium during menstrual cycle. *Lancet* 344: 28–29, 1994.
33. Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, and Voipio-Pulkki LM. Apoptosis in human acute myocardial infarction. *Circulation* 95: 320–323, 1997.
34. Sharov VG, Sabbah HN, Shimoyama H, Goussev AV, Lesch M, and Goldstein S. Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am J Pathol* 148: 141–149, 1996.
35. Singal PK, Deally CM, and Weinberg LE. Subcellular effects of adriamycin in the heart: a concise review. *J Mol Cell Cardiol* 19: 817–828, 1987.
36. Singal PK, Forbes MS, and Sperelakis N. Occurrence of intramitochondrial Ca^{2+} granules in a hypertrophied heart exposed to adriamycin. *Can J Physiol Pharmacol* 62: 1239–1244, 1984.
37. Singal PK, and Iliskovic N. Doxorubicin-induced cardiomyopathy. *N Engl J Med* 339: 900–905, 1998.
38. Singal PK, Iliskovic N, Li T, and Kumar D. Adriamycin cardiomyopathy: pathophysiology and prevention. *FASEB J* 11: 931–936, 1997.
39. Singal PK, and Panagia V. Direct effects of adriamycin on the rat heart sarcolemma. *Res Commun Chem Pathol Pharmacol* 43: 67–77, 1984.
40. Singal PK, and Pierce GN. Adriamycin stimulates low-affinity Ca^{2+} binding and lipid peroxidation but depresses myocardial function. *Am J Physiol* 250: H419–H425, 1986.
41. Singal PK, Siveski-Iliskovic N, Hill M, Thomas TP, and Li T. Combination therapy with probucol prevents adriamycin-induced cardiomyopathy. *J Mol Cell Cardiol* 27: 1055–1063, 1995.
42. Singal PK, Tong L, and Wheeler G. Progress and problems in adriamycin-induced cardiomyopathy. In: *Cardiac Dilatation,* edited by Jacob R, Seipel L, and Zucker IH. New York: Gustav Fischer Verlag, 1990, pp. 89–96.
43. Siveski-Iliskovic N, Hill M, Chow DA, and Singal PK. Probucol protects against adriamycin cardiomyopathy without interfering with its antitumor effect. *Circulation* 91: 10–15, 1995.
44. Siveski-Iliskovic N, Kaul N, and Singal PK. Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. *Circulation* 89: 2829–2835, 1994.
45. Thornalley PJ, and Dodd NJ. Free radical production from normal and adriamycin-treated rat cardiac sarcosomes. *Biochem Pharmacol* 34: 669–674, 1985.
46. Tong J, Ganguly PK, and Singal PK. Myocardial adrenergic changes at two stages of heart failure due to adriamycin treatment in rats. *Am J Physiol* 260: H909–H916, 1991.
47. Wang L, Ma W, Markovich R, Chen JW, and Wang PH. Regulation of cardiomyocyte apoptotic signaling by insulin-like growth factor I. *Circ Res* 83: 516–522, 1998.
48. Weinberg LE, and Singal PK. Refractory heart failure and age-related differences in adriamycin-induced myocardial changes in rats. *Can J Physiol Pharmacol* 65: 1957–1965, 1987.
49. Zhang J, Clark JRJ, Herman EH, and Ferrans VJ. Doxorubicin-induced apoptosis in spontaneously hypertensive rats: differential effects in heart, kidney and intestine, and inhibition of ICRF-187. *J Mol Cell Cardiol* 28: 1931–1943, 1996.

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2. Azhar Rashikh, Shibli Jameel Ahmad, Krishna Kolappa Pillai, Kanchan Kohli, Abul Kalam Najmi. 2012. Aliskiren attenuates myocardial apoptosis and oxidative stress in chronic murine model of cardiomyopathy. *Biomedicine & Pharmacotherapy* . [CrossRef]
3. Yi Lisa Lyu, Leroy F. LiuDoxorubicin Cardiotoxicity Revisited 351-369. [CrossRef]
4. Ana Ludke, Anita K. Sharma, Ashim K. Bagchi, Pawan K. Singal. 2011. Subcellular basis of vitamin C protection against doxorubicin-induced changes in rat cardiomyocytes. *Molecular and Cellular Biochemistry* . [CrossRef]
5. Sankarganesh Arunachalam, Sun Young Kim, Sun Hwa Lee, Young Hee Lee, Min Sun Kim, Bong Sik Yun, Ho Keun Yi, Pyoung Han Hwang. 2011. Davallialactone protects against adriamycin-induced cardiotoxicity in vitro and in vivo. *Journal of Natural Medicines* . [CrossRef]
6. Li Jing, Yingliang Wu, Jing Wu, Jun Zhao, Daiying Zuo, Shuangqing Peng. 2011. Peroxiredoxins are involved in metallothionein protection from doxorubicin cardiotoxicity. *European Journal of Pharmacology* **659**:2-3, 224-232. [CrossRef]
7. Jonathan R. Walker, Anita Sharma, Matthew Lytwyn, Sheena Bohonis, James Thliveris, Pawan K. Singal, Davinder S. Jassal. 2011. The Cardioprotective Role of Probucol Against Anthracycline and Trastuzumab-Mediated Cardiotoxicity. *Journal of the American Society of Echocardiography* **24**:6, 699-705. [CrossRef]
8. C. Zhang, Y. Feng, S. Qu, X. Wei, H. Zhu, Q. Luo, M. Liu, G. Chen, X. Xiao. 2011. Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in mice through SIRT1-mediated deacetylation of p53. *Cardiovascular Research* **90**:3, 538-545. [CrossRef]
9. António Ascensão, Paulo J. Oliveira, José Magalhães. 2011. Exercise as a beneficial adjunct therapy during Doxorubicin treatment—Role of mitochondria in cardioprotection. *International Journal of Cardiology* . [CrossRef]
10. Jin Zheng, Hui Ching Michelle Lee, Mohamad Mukmin bin Sattar, Yu Huang, Jin-Song Bian. 2011. Cardioprotective effects of epigallocatechin-3-gallate against doxorubicin-induced cardiomyocyte injury. *European Journal of Pharmacology* **652**:1-3, 82-88. [CrossRef]
11. Viorel G. Florea, Jay N. CohnDisease Prevention in Heart Failure 610-625. [CrossRef]
12. Chunmei Li, Jingwei Tian, Guisheng Li, Wanglin Jiang, Yanli Xing, Jian Hou, Haibo Zhu, Hong Xu, Guanbo Zhang, Zhifeng Liu. 2010. Asperosaponin VI protects cardiac myocytes from hypoxia-induced apoptosis via activation of the PI3K/Akt and CREB pathways. *European Journal of Pharmacology* **649**:1-3, 100-107. [CrossRef]
13. Shamjeet Singh, Sanjiv Dhingra, Dan D. Ramdath, Sudesh Vasdev, Vicki Gill, Pawan K. Singal. 2010. Risk Factors Preceding Type 2 Diabetes and Cardiomyopathy. *Journal of Cardiovascular Translational Research* **3**:5, 580-596. [CrossRef]
14. Fang Wang, Bo Yang, G. Hui Ling, Can Yao, Y. Sheng Jiang. 2010. Methylguanidine cytotoxicity on HK-2 cells and protective effect of antioxidants against MG-induced apoptosis in renal proximal tubular cells in vitro. *Renal Failure* **32**:8, 978-985. [CrossRef]
15. Y. Zhang, L. Wei, D. Sun, F. Cao, H. Gao, L. Zhao, J. Du, Y. Li, H. Wang. 2010. Tanshinone IIA pretreatment protects myocardium against ischaemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway in diabetic rats. *Diabetes, Obesity and Metabolism* **12**:4, 316-322. [CrossRef]

16. Karen Y. Wonders, David S. Hydock, Stephanie Greufe, Carole M. Schneider, Reid Hayward. 2009. Endurance exercise training preserves cardiac function in rats receiving doxorubicin and the HER-2 inhibitor GW2974. *Cancer Chemotherapy and Pharmacology* **64**:6, 1105-1113. [CrossRef]
17. Wenyuan Zhao, Dawn Zhao, Ran Yan, Yao Sun. 2009. Cardiac oxidative stress and remodeling following infarction: role of NADPH oxidase. *Cardiovascular Pathology* **18**:3, 156-166. [CrossRef]
18. Jinping Zhang, Anheng Liu, Rongrong Hou, Juan Zhang, Xin Jia, Weifeng Jiang, Jianzong Chen. 2009. Salidroside protects cardiomyocyte against hypoxia-induced death: A HIF-1#-activated and VEGF-mediated pathway. *European Journal of Pharmacology* **607**:1-3, 6-14. [CrossRef]
19. F. J. F. Broeyer, S. Osanto, H. J. Ritsema van Eck, A. Q. M. J. Steijn, B. E. P. B. Ballieux, R. C. Schoemaker, A. F. Cohen, J. Burggraaf. 2008. Evaluation of biomarkers for cardiotoxicity of anthracyclin-based chemotherapy. *Journal of Cancer Research and Clinical Oncology* **134**:9, 961-968. [CrossRef]
20. Sanjay Popat, Ian E Smith. 2008. Therapy Insight: anthracyclines and trastuzumab—the optimal management of cardiotoxic side effects. *Nature Clinical Practice Oncology* . [CrossRef]
21. Jae-Ok Shin, Eun-Seon Ju, Hyun-Mi Song, Soo-Hyeon Yun, Byung-Kwan Lim, Jin-Ho Choi, Duk-Kyung Kim, Eun-Seok Jeon. 2008. Effects of Cardiotrophin-1 on Adriamycin-Induced Apoptosis in H9c2 Cardiomyoblasts. *Korean Circulation Journal* **38**:5, 264. [CrossRef]
22. Ciobotaru E., Militaru M., Braslasu C.M., Braslasu E.D., Soare T., Joita S., Dinescu G.. 2008. Gravimetric and morphometric assessments in Wistar rats with experimental diabetes mellitus type 1 and cardiac failure. *Acta veterinaria* **58**:5-6, 583-592. [CrossRef]
23. Yao Sun. 2007. Oxidative Stress and Cardiac Repair/Remodeling Following Infarction. *The American Journal of the Medical Sciences* **334**:3, 197-205. [CrossRef]
24. Partha Mukhopadhyay, Sándor Bátkai, Mohanraj Rajesh, Nora Czifra, Judith Harvey-White, György Haskó, Zsuzsanna Zsengeller, Norma P. Gerard, Lucas Liaudet, George Kunos, Pál Pacher. 2007. Pharmacological Inhibition of CB1 Cannabinoid Receptor Protects Against Doxorubicin-Induced Cardiotoxicity. *Journal of the American College of Cardiology* **50**:6, 528-536. [CrossRef]
25. J FU, H HUANG, J LIU, R PI, J CHEN, P LIU. 2007. Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis. *European Journal of Pharmacology* **568**:1-3, 213-221. [CrossRef]
26. Billy Chen, Xuyang Peng, Laura Pentassuglia, Chee Chew Lim, Douglas B. Sawyer. 2007. Molecular and cellular mechanisms of anthracycline cardiotoxicity. *Cardiovascular Toxicology* **7**:2, 114-121. [CrossRef]
27. Delia Outomuro, Daniel R. Grana, Francisco Azzato, José Milei. 2007. Adriamycin-induced myocardial toxicity: New solutions for an old problem?. *International Journal of Cardiology* **117**:1, 6-15. [CrossRef]
28. Bo Su, Yun-Bo Yang, Qin-Hui Tuo, Bing-Yang Zhu, Xiao-Yong Lei, Weidong Yin, Duan-Fang Liao. 2007. Anti-apoptotic Effects of Probucol are Associated with Downregulation of Daxx Expression in THP-1 Macrophage. *Cardiovascular Drugs and Therapy* **21**:1, 37-45. [CrossRef]
29. Dinender K. Singla, Kuljeet Kaur, Anita K. Sharma, Sanjiv Dhingra, Pawan K. Singal. 2007. Probucol promotes endogenous antioxidant reserve and confers protection against reperfusion injury. *Canadian Journal of Physiology and Pharmacology* **85**:3-4, 439-443. [CrossRef]
30. Gregory D. Lewis, Marc J. Semigran. 2006. The emerging role for type 5 phosphodiesterase inhibition in heart failure. *Current Heart Failure Reports* **3**:3, 123-128. [CrossRef]
31. Mahmood Khan, Saradhadevi Varadharaj, Jagdish C Shobha, Madireddi U Naidu, Narasimham L Parinandi, Vijay Kumar Kutala, Periannan Kuppusamy. 2006. C-Phycocyanin Ameliorates Doxorubicin-Induced Oxidative Stress and Apoptosis in Adult Rat Cardiomyocytes. *Journal of Cardiovascular Pharmacology* **47**:1, 9-20. [CrossRef]

32. Jyh-Sheng You, Hui-Feng Huang, Ying-Ling Chang, Ying-Shiung Lee. 2006. Sheng-Mai-San Reduces Adriamycin-Induced Cardiomyopathy in Rats. *The American Journal of Chinese Medicine* **34**:02, 295-305. [CrossRef]
33. Jyh-Sheng You, Hui-Feng Huang, Ying-Ling Chang. 2005. Panax ginseng reduces adriamycin-induced heart failure in rats. *Phytotherapy Research* **19**:12, 1018-1022. [CrossRef]
34. Irmela Jeremias, Karsten Stahnke, Klaus -Michael Debatin. 2005. CD95/Apo-1/Fas: independent cell death induced by doxorubicin in normal cultured cardiomyocytes. *Cancer Immunology, Immunotherapy* **54**:7, 655-662. [CrossRef]
35. Yukitaka Shizukuda, Satoaki Matoba, Omar Y. Mian, Tammy Nguyen, Paul M. Hwang. 2005. Targeted disruption of p53 attenuates doxorubicin-induced cardiac toxicity in mice. *Molecular and Cellular Biochemistry* **273**:1-2, 25-32. [CrossRef]
36. J MARINGARCIA, M GOLDENTHAL. 2004. Mitochondria play a critical role in cardioprotection. *Journal of Cardiac Failure* **10**:1, 55-66. [CrossRef]
37. F. Mwale, J. Antoniou, S. Héon, N. Servant, C. Wang, G. M. Kirby, C. N. Demers, L. E. Chalifour. 2004. Gender-Dependent Reductions in Vertebrae Length, Bone Mineral Density and Content by Doxorubicin Are Not Reduced by Dexrazoxane in Young Rats: Effect on Growth Plate and Intervertebral Discs. *Calcified Tissue International* . [CrossRef]
38. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]