Pentoxifylline Prevents Adriamycin-Induced Myocardial Fibrosis and Apoptosis in Rats

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SUMMARY

Adriamycin (ADR) is a potent antineoplastic agent, but long-term treatment is limited by its cumulative, life-threatening cardiomyopathy. Recently, a few reports have shown that pentoxifylline (PTX) might produce cardioprotection in cardiac dysfunction. Here, we investigated the protective effects of PTX on ADR-induced cardiomyopathy in rats. Male rats were randomly assigned either to saline, ADR (adriamycin, 5 mg/kg/week), or A (adriamycin, 5 mg/kg/week) + PTX (pentoxifylline, 50 mg/kg/day) groups. After 3 weeks, these animals were sacrificed and the heart tissue was harvested for histological analysis and assessment of hepatocyte growth factor (HGF) and caspase-3 expression. Histopathological findings showed that PTX can alleviate myocardial damage caused by ADR. Cardiac fibrosis was significantly suppressed in the A+PTX group compared to that in the ADR group. The HGF gene expression was decreased significantly in the ADR group compared with the control group, but was increased in the A+PTX group. Caspase-3 was up-regulated in the ADR group, and down-regulated in the A+PTX group. These results show that treatment with PTX exerts a protective effect against ADR-induced myocardial fibrosis via regulation of HGF and caspase-3 gene expression. PTX may thus represent a useful new clinical tool for the treatment of ADR-induced cardiomyopathy. (Int Heart J 2015; 56: 651-655)

Key words: Antineoplastic, Cardiomyopathy

Adriamycin (ADR), an anthracycline drug, has been established as an effective agent against various kinds of solid tumors and hematologic malignancies.¹⁾ However, its clinical use is limited by its dose-dependent cardiotoxicity and irreversible cardiomyopathy which result in dilated cardiomyopathy (DCMP) with congestive heart failure.²⁾ The mechanism of ADR-induced DCMP is thought to be the up-regulation of reactive free oxygen radicals which can make the heart vulnerable to injury through lowering the activities of protective enzymes such as topoisomerase II and ghrelin.³⁻⁵⁾ The fibrotic mediators play an important role in the pathogenesis of DCMP induced by ADR. Hepatocyte growth factor (HGF), a well-known multifunctional cytokine, was shown to be involved in cardiac fibrotic processes and played an important role in anti-fibrosis during the course of DCMP.⁶⁻⁸⁾ These reports also showed that phosphodiesterases (PDEs) remarkably express in cardiomyocyte apoptosis, and can induce cAMP and cGMP-dependent apoptosis in cardiomyopathy.⁶⁻⁸⁾ In this pathway, caspases are crucial mediators. As critical participants in the orchestration of apoptosis, caspases cleave the target proteins to execute cell death. Among them, caspase-3 is the

most important one. It can target structural substrates including nuclear laminins, focal adhesion sites, and cell-cell adherence junctions, and catalyze the specific cleavage of many key cellular proteins.⁹⁻¹⁵⁾

Pentoxifylline (PTX) is a methylxanthine derivative and a nonspecific PDE inhibitor which has been used for decades. In recent years, many studies have showed that PTX can decrease the level of inflammatory mediators through blockade of the expression of inflammatory genes, and thereby reduce the generation of inflammatory factor.¹⁶⁻¹⁸⁾ The present study was designed to show the expression of HGF and caspase-3 in this ADR-induced cardiomyopathy after the administration of PTX in order to explore the mechanism of PTX acting on DCMP.

METHODS

Animals and experimental protocol: Thirty male Sprague-Dawley rats (6 weeks old, 200~220 g) were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China) and housed individually in cages at constant tempera-

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ture ($22 \pm 1^\circ\text{C}$) and humidity (60%). Animal and experiment protocols were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Sun Yat-sen University Institutional Animal Care and Use Committee. All of the rats were randomly divided into 3 equal groups ($n = 10$ per group). In the control group, animals were treated with saline. In the ADR group, animals were injected with ADR at a dose of 5 mg/kg/week iv; In the A+PTX group, animals were also injected with ADR at a dose of 5 mg/kg/week iv and received PTX at a dosage of 50 mg/kg/day by gavage through a gastric needle. On day 21, all animals were sacrificed by cervical dislocation under ether anesthesia. The hearts were removed and subjected to further analysis.

Pathological evaluation of the heart tissue: Heart tissues were immediately fixed in 4% paraformaldehyde (PFA) for 24 hours and embedded in paraffin. Subsequently, heart tissues were sectioned at a thickness of 5 μm and stained with Masson's-trichrome. Ten microscopic fields of blinded sections were randomly selected and analyzed using a color image analyzer (BX-51/Image-Pro plus, Olympus, Tokyo). To evaluate fibrotic changes of the heart, tissue sections were stained with Masson-trichrome. The fibrotic area of the total area was determined by computer assisted analysis in each tissue section. At least 10 fields at 200-fold magnification were captured and assessed in all the samples. The fibrosis extent and myocyte diameter in these photomicrographs was quantified by a blinded observer using the Image J program from NIH Image Software (National Institutes of Health, Bethesda, MD, USA). All data were analysed in a blind fashion by two independent investigators and then averaged.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA from the heart tissue was extracted using an RNeasy mini kit (Qiagen Ltd, UK) according to the manufacturer's protocol. Reverse transcription reactions were performed using murine leukemia virus reverse transcriptase and oligo-dT primers (Fermentas, Ltu). Specific primer sets for HGF and GAPDH used for RT-PCR analysis were: HGF forward, 5'-ACCAAGGAAGACCCATTACTGAAGA; HGF reverse, 5'-TTCCAAGGCTGGCATTTGATGC; GAPDH forward, 5'-ACCACAGT CCATGCCATCAC; GAPDH reverse, 5'-TCCACCACCCTGTTGCTGTA.

Western blot analysis: Separated heart tissues were powdered in liquid nitrogen before protein was isolated and suspended in radioimmunoprecipitation assay lysis buffer (Sigma Chemical Co., St Louis, USA). The homogenates were centrifuged at $10,000 \times g$ for 5 minutes at 4°C . The protein in supernatant was determined using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Next, 20 μg of each protein was administered separately to SDS-PAGE and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in TBST (0.1% Tween20, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4) for 1 hour, the membranes were incubated with polyclonal primary antibody HGF and caspase-3 (1: 100, both, Cell Signaling Technology, USA) overnight at 4°C . After washing in TBST 3 times, the membranes were incubated with HRP-conjugated secondary antibody (1:3000, Vector, Burlingame, USA) for 2 hours at room temperature. Signals were visualized using an enhanced chemiluminescence reagent (Amersham Bioscience, Uppsala, Sweden).

Apoptosis assay: Apoptotic nuclei were detected using termi-

nal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) staining kits (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Images were taken in 10 random fields (magnification X400) for each animal. Nuclei were counterstained with hematoxylin (blue). The numbers of TUNEL-positive cells and total cardiomyocyte nuclei were counted, and the apoptotic index of cardiomyocytes was expressed as the percentage of TUNEL-positive cells to total cells. The results are expressed as the percentages of apoptotic cell numbers in each field.

Statistical analysis: All data are expressed as the mean $\pm$ SEM. Statistical differences between different groups were analyzed using one-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* comparison. Values of $P < 0.05$ were considered significant.

RESULTS

Histological analysis of the heart after ADR treatment: Fibrosis has been suggested to be involved in cardiac stiffness and dysfunction in ADR-induced cardiotoxicity.¹⁹⁾ To explore the cardiac pathological changes of fibrosis, we performed Masson's trichrome staining after ADR administration in each animal group. Cardiomyopathy induced by ADR treatment was exacerbated in rats. There were no deaths and no signs of overt toxicity during the study. The histochemical features of myocardium in each group were examined in the fibrotic areas observed through microscopy. In the ADR group, the histological results showed that ADR injection gave rise to myocardial damage, such as myofibrillar loss, contraction band necrosis, and fibrosis after tissue apoptosis. However, these phenomena were alleviated when treated with PTX (Supplemental Figure 1A). The area of fibrosis seen on Masson's trichrome staining significantly increased in the ADR group compared with the A+PTX group ($21.08\% \pm 2.28\%$ versus $10.27\% \pm 3.28\%$, $P < 0.05$) (Supplemental Figure 1B).

PTX effect on HGF gene expression after ADR induced cardiomyopathy: HGF is an anti-fibrosis cytokine. The expression of HGF mRNA was significantly decreased in the ADR group compared with the control group ($P < 0.05$). Moreover, the HGF mRNA expression was increased 2- to 3-fold in the A+PTX group compared to the ADR group ($P < 0.01$) (Figure 1A and B). Similarly, the expression of HGF protein was also assessed and found to be decreased in the ADR group and in 40% of control specimens, but increased after PTX treatment (Figure 1C and D).

PTX administration inhibits myocardial apoptosis after ADR induced cardiomyopathy: HGF prohibits apoptotic signals via inhibition of caspase-3 activity.²⁰⁾ In this research, the protein expression of caspase-3 was also evaluated by Western blot analysis in each group. As shown in Figure 2, caspase-3 was found to be up-regulated in the ADR group ($P < 0.01$). After treatment with PTX, the expression of caspase-3 protein was significantly down-regulated ($P < 0.05$). We also performed TUNEL staining to assess the effect of PTX on ADR-treated cardiomyocyte apoptosis (Supplemental Figure 2). Compared with the control group, the percentage of apoptotic cells was increased in the cardiomyocytes of the ADR group (Figure 3). Remarkably, the rats treated with PTX showed a marked decrease in apoptotic cell death compared to the ADR treated rats

Figure 1. Expression of HGF in each group at day 21. **A:** The PCR original figure. The ADR group showed decreased HGF mRNA expression compared with the control group, and the A+PTX group showed higher HGF mRNA expression than the ADR group. **B:** The histogram of relative intensity derived from A. The relative value of HGF mRNA was also higher in the A+PTX group. **C:** The level of HGF protein decreased in the ADR group but increased in the A+PTX group. **D:** The histogram of relative intensity derived from C. The relative value of HGF protein was also higher in the A+PTX group than in the ADR group. Values are presented as the mean $\pm$ SEM. ** $P < 0.01$, $n = 10$ per group.

Figure 2. Outcome of Western blot and relative intensity analysis of caspase-3 protein expression in the myocardium of each group at day 21. **A:** The caspase-3 expression was higher in the ADR group, but lower in the A+PTX group. **B:** The histogram of relative intensity further demonstrated that the concentration of caspase-3 protein increased in the ADR group but significantly decreased in the A+PTX group. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$, $n = 10$ per group.

Figure 3. The apoptotic index of cardiomyocytes of the rats in each group. Quantitative analysis showed that the apoptotic index of cardiomyocytes was much higher in the ADR group than the other two groups. Values are presented as the mean $\pm$ SEM. ** $P < 0.01$, $n = 10$ per group.

($P < 0.01$) (Figure 3).

DISCUSSION

The results of the present study have demonstrated that PTX has a protective effect on injury to the heart in ADR-treated rats. ADR treatment was significantly associated with increased myocardial fibrosis and apoptosis. However, PTX treatment could regulate HGF gene expression and decrease cardiac fibrosis and down regulate caspase-3 mediated myo-

cardial apoptosis.

Adriamycin is one of the most effective and useful antineoplastic agents for the treatment of various malignancies. However, its practical therapeutic use is sometimes limited by the frequent induction of acute cardiotoxicity.^{21,22)} These cellular mechanisms, including myocardial fibrosis, apoptosis, and altered energy metabolism, have been proposed to account for the cardiomyopathy caused by ADR.^{19,23,24)}

Fibrosis has been suggested to be involved in cardiac stiffness and dysfunction, which are caused when increased collagen synthesized by the fibroblasts invades and replaces necrotic or apoptotic myocytes.¹⁹⁾ In this study, we observed that ADR-induced fibrosis was prevented by PTX, as presented by the attenuation of collagen deposition. PTX, a phosphodiesterase inhibitor, has a clinical application as an inhibitor of platelet aggregation. Several studies imply that its cellular effects also include anti-fibrotic effects in a variety of mesenchymal cells.²⁵⁻²⁸⁾ Furthermore, PTX administration has been shown to inhibit mitogenesis and collagen synthesis in renal fibroblast cultures.^{27,28)} Here, our study found an increased expression of myocardial HGF mRNA and protein occurred after the administration of PTX. HGF is a well-known anti-fibrosis factor in the development of heart disease. In the protection process, HGF can significantly reduce the generation of extracellular matrix, increase the production of matrix metalloproteinase-1 and urokinase plasminogen, as well as decrease TGF- β concentration.^{8,29,30)} TGF- β , which is a pathogenic factor that stimulates fibrosis through the accumulation of extracellular matrix, is up-regulated in cardiomyopathy. Moreover, PTX could improve liver regeneration via down-regulation of TGF- β 1 gene expression.³¹⁾ Thus, PTX may have improved the cardiac function by up-regulated HGF, which functions through further suppressing the expression of TGF- β . The exact mechanisms remain unclear and need further study.

ADR exposure causes activation of mitochondrial apoptosis in cardiomyocytes.³²⁾ The caspase cascade induction is considered one of the mechanisms of intensification of apoptosis in cardiomyocytes, which is manifested in increased activity of the caspase-3 effector caspase in the heart.³³⁾ Here, we also confirmed that ADR significantly induces myocardium apoptosis and increases caspase-3 activity. Compared with ADR-treated rats, PTX-treated rats underwent significantly less histologic change of myocardial tissue, and had a smaller apoptotic index, including lower TUNEL positive cells and down regulated caspase-3 expression. A number of previous studies have also demonstrated that HGF exerted an anti-apoptotic effect through the proapoptotic caspase-3 signaling pathway in various cells.^{34,35)} It has been reported that PTX can significantly decrease caspase-3 activities and reduce apoptosis to a significant extent in rat pups with hypoxic-ischemic encephalopathy.³⁶⁾ Collectively, our data suggest that PTX prevents the activation of myocardial apoptosis induced by ADR, and the anti-apoptotic effect of PTX is probably mediated through cellular signaling of the HGF pathways.

According to the results of our research, PTX treated rats exhibited significantly attenuated cardiac fibrotic changes from macro (histopathology) and micro (cytokine) aspects. Other histopathological improvements also occurred, including significant decreases in necrosis, interstitial fibrosis, and apoptosis. Therefore, PTX might reduce DCMP occurrence, which is an end-stage cardiomyopathic heart disease.

In conclusion, PTX might be effective in the treatment of ADR-induced cardiomyopathy. Our research implies that PTX can play an important role in relieving the cardiotoxicity due to a high cumulative dose of ADR. Furthermore, the cardio-protective effect of this drug may occur through blocking the caspase3-dependent apoptotic pathway and may create factors that play anti-apoptotic roles. Moreover, we found that PTX treatment significantly increased HGF, the anti-fibrotic cytokine, which plays an important role in reversing the process of heart disease. In summary, our results suggest that cardio-protection of ADR-induced cardiomyopathy can be improved by treatment with PTX.

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SUPPLEMENTAL FILES

Supplemental Figure 1, 2

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