

## Research Article

# Desferrioxamine Attenuates Doxorubicin-Induced Acute Cardiotoxicity through TGF- $\beta$ /Smad p53 Pathway in Rat Model

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Interaction of doxorubicin DOX with iron and the consequent generation of reactive oxygen species (ROS) is a major player in DOX-induced cardiomyopathy. Accordingly, this study has been initiated to investigate the preventive effect of the iron chelator, desferrioxamine (DFX), against DOX-induced acute cardiotoxicity in rats. Male Wistar albino rats were divided into four groups and were injected intraperitoneally (I.P.) with normal saline, a single dose of DOX (15 mg/kg), a single dose of DFX (250 mg/kg) and a combined treatment with DFX (250 mg/kg) 30 min prior to a single dose of DOX, (15 mg/kg). A single dose of DOX significantly increased mRNA expression of TGF- $\beta$ , Smad2, Smad4, CDKN2A and p53 and significantly decreased Samd7 and Mdm2 mRNA expression levels. Administration of DFX prior to DOX resulted in a complete reversal of DOX-induced alteration in cardiac enzymes and gene expression to normal levels. Data from this study suggest that (1) DOX induces its acute cardiotoxicity secondary to increasing genes expression of TGF- $\beta$ /Smad pathway. (2) DOX increases apoptosis through upregulation of CDKN2A and p53 and downregulation of Mdm2 gene expression. (3) The preventive effect of DFX against DOX-induced cardiotoxicity is mediated via the TGF- $\beta$ 1/Smad pathway.

## 1. Introduction

Doxorubicin (DOX) is a potent anthracycline chemotherapeutic agent used to treat a wide variety of human malignancies [1]. However, its clinical use is limited by a significant DOX-induced cardiotoxicity which can progress to heart failure [2–4]. The mechanism by which DOX-induced cardiotoxicity has not been fully understood yet; however, several mechanisms have been proposed. The mechanisms include increased oxidative stress [5], alteration of myocardial energy metabolism [6], altered molecular signaling [7], programmed cell death [4] and iron-dependent oxidative damage to biological macromolecules [8]. One of the mechanism explained DOX-induced cardiotoxicity is the formation of free radical-mediated myocytes damage. Iron (Fe) is a critical biogenic element necessary as oxidation-reduction catalysis and bioenergetics in all living cells. However, this metal plays a crucial role in the formation of reactive oxygen

species (ROS) [9]. There are two main pathways by which Fe may promote ROS formation in DOX-exposed cells, one of them is the formation of DOX-Fe complexes. The iron chelator, desferrioxamine (DFX), is used in the treatment of iron overload status and prevents interaction of DOX with iron, thus preventing DOX-induced damage in cultured heart cells [10]. ROS have been proposed as contributing to the deterioration of cardiac function in patients with both ischemic and nonischemic cardiomyopathies [11].

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is formed mainly by cardiac myofibroblast and fibroblast and is contributed to cardiac fibrosis development, hypertrophy, and apoptosis [12]. Previous report showed that TGF- $\beta$ 1 gene expression is increased in the left ventricular myocardium of patients with idiopathic hypertrophic cardiomyopathy or dilated cardiomyopathy and in animals after myocardial infarction [13]. ROS have been shown to play important roles in the development of apoptosis under various pathological

conditions. One potential mechanism whereby ROS promote apoptosis is activating TGF- $\beta$  [14]. The recombinant human latent TGF- $\beta$  was activated in cell free system in the presence of asbestos and ascorbic acid and that such activation was reduced significantly by addition of superoxide dismutase, catalase, or DFX [15]. ROS activated TGF- $\beta$  through two different mechanisms: direct by oxidation of latency association protein (LAP) and indirectly through activation of matrix metalloproteinase (MMPs) such as MMP-2 and MMP-9, which in turn cleave LAP to release active TGF- $\beta$  [16]. The activation of latent TGF- $\beta$  by asbestos-ascorbate mediated generation of ROS apparently also resulted from oxidative modification in LAP, leading to a loss of its ability to bind to TGF- $\beta$ 1 [15]. On the other hand, ROS generated by ultraviolet light B have been shown to activate TGF- $\beta$  in keratinocytes through stimulating MMP-2 and MMP-9 [17]. The initiation of TGF- $\beta$ /Smad signaling pathway started by the formation of heteromeric receptor complexes of specific type I and II serine/threonine kinases [18, 19]. After binding of TGF- $\beta$  to receptor type II, the receptor of type I is phosphorelated leading to phosphorylation of Smad-2 and -3 then formed a complex with Smad4. This complex then entered the nucleus and binded to TGF- $\beta$  responsive gene promoter that regulates their expression [20]. In addition, the expression of TGF- $\beta$ 1 and Smad-2, -3, and -4 is upregulated during the chronic phase of myocardial infarct scar healing [21]. Furthermore, Smad proteins are mediators for the TGF- $\beta$ -induced apoptosis [22]. Therefore, this study has been initiated to investigate the preventive effect of the DFX against DOX-induced cardiotoxicity in rat through studying genes expression in the TGF- $\beta$ /Smad pathways.

## 2. Material and Methods

**2.1. Animals.** Adult male Wistar albino rats, weighing 230–250 gm, were obtained from Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet diet essentially and tap water. The protocol of this study has been approved by Research Ethics Committee of the College.

**2.2. Materials.** Doxorubicin (Sandoz Pharma, Germany) and desferrioxamine (NOVARTIS pharma, Switzerland) were obtained from king Khalid University Hospital drug store.

**2.3. Experimental Design.** To achieve the ultimate goal of this study, a total of 40 adult male Wistar albino rats aged 12 weeks, weighted 230–250 gm were divided randomly into 4 groups of 10 animals each. Animals in the control group were injected intraperitoneally (I.P.) with normal saline. Rats in DOX group were injected with a single dose of DOX (15 mg/kg, I.P.). Rats in DFX group were given a single dose of DFX (250 mg/kg, I.P.). Animals in DOX + DFX group were injected with DFX (250 mg/kg) 30 min prior to a single dose of DOX (15 mg/kg, I.P.). At 48 hours after the last injection, animals were anesthetized with ether, and blood samples

were obtained by heart puncture. Part of blood was collected in nonsilicone coated 4.5 mg EDTA tube for complete blood picture analysis. Sera were separated for measurement of glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH), creatine phosphokinase (CK), and creatine phosphokinase-MB (CK-MB). Heart was excised and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA extraction.

### 2.4. Methods

**2.4.1. Determination of Serum Cardiotoxicity Enzymatic Indices.** Quantitative determination of GOT, LDH, and CK levels was measured according to the previous methods [23–25], respectively. Serum activities of CK isoenzyme were determined according to the previous published method [26]. A complete blood picture was measured using full automatic blood cell counter Model PCE 210 N (ERMA INC, Japan).

**2.4.2. Detection of TGF- $\beta$ , Smad2, Smad4, Smad7, Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A), Murine Double Minute (Mdm2), and p53 Gene Expression Level by Real-Time PCR Using  $\Delta\Delta\text{Ct}$  Method in Heart Tissues**

**Total RNA Extraction.** Total RNA were extracted from frozen heart tissue by Trizol method according to the manufacturer's protocol as previously described [27]. RNA quantity was characterized using a UV spectrophotometer (NanoDrop 8000, thermo scientific, USA); the isolated RNA has an A 260/280 ratio of 1.9–2.1. The integrity of RNA was assessed by running an aliquot of the RNA samples on a denaturing agarose gel stained with ethidium bromide.

**cDNA Synthesis and Real-Time PCR Methods.** First-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA by reverse transcription with a superscript first-strand synthesis system kit (Invitrogen, CA, USA), according to the manufacturer's instructions. Real-time PCR was done according to our previous study [4]. The Rat primers and probes used for quantification of  $\beta$ -actin gene, as an internal control, TGF- $\beta$ 1, Smad2, Smad4, Smad7, CDKN2A, Mdm2, and p53 were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) and listed in Table 1.

**2.4.3. Statistical Analysis.** Differences between obtained values (mean  $\pm$  SD,  $n = 10$ ) were carried out by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparison Test. a  $P$ -value of 0.05 or less was taken as a criterion for a statistically significant difference.

## 3. Results

A significant effect of DOX treatment on blood parameter, and interaction between DOX and DFX were observed in Table 2. A single dose of DOX treatment resulted in a significant decrease in hemoglobin (Hb%) concentration by 25%,

TABLE 1: Primers and probes sequence for TGF- $\beta$ , Smad2, Smad4, Smad7, CDKN2A, Mdm2, and p53.

Gene	Forward primer	Reverse primer	Probe
TGF- $\beta$	5'-TGCCTGACGGTCAGGTCA-3'	5'-CAGGAAGGAAGGCTGGAG-3'	FAM-CACTATCGGCAATGAGCG-GTTCCG-TAMRA
Smad7	5'-CCCCATCACCTTAGTTCGACTCT-3'	5'-GACAGTCTGCAGTTGGTTTGA-3'	VIC-CCCCTCCTCCTTACTCCAGAT-ACCCGA-TAMRA
Smad4	5'-ACCAACTTCCCCAACATTCT-3'	5'-ACTATGGCTGCCTGCCAGAA-3'	FAM-TGGCTTCCACAAGTCAGCCGGC-TAMRA
Smad2	5'-CCATCCCCGAGAACAATACTT-3'	5'-TGGTGGTCGCTAGTTTCTCCAT-3'	VIC-AACCCCAGAGCAATATTCCAG-AAACCCC-TAMRA
CDKN2A	5'-GGGTCACCGACAGGCATAAC-3'	5'-AGTCTCGCGTTGCCAGAAGT-3'	FAM-TGCTCAAGCACGCCAGGTGC-TAMRA
Mdm2	5'-GGTTGTGGGCTGCAGAGAAG-3'	5'-TGCAAGGATCAAATGTGTGTCA-3'	HEX-TCAGCCGGTAAAGGCGCCCACT-TAMRA,
p53	5'-CACCATGAGCGTTGCTCTGAT-3'	5'-GATTTCTTCCACCCGATAA-3'	FAM-ACGGCCTGGCTCCTCCCCAAC-TAMRA
$\beta$ -actin	5'-TGCCTGACGGTCAGGTCA-3'	5'-CAGGAAGGAAGGCTGGAAG-3'	HEX-CACTATCGGCAATGAGCG-GTTCCG-TAMRA

TABLE 2: Effect of DOX, DFX, and their combination on blood picture.

Group	Hematological parameters				
	Hb%	RBCs $\times 10^6$	WBCs $\times 10^3$	HCT %	Platelets $\times 10^3$
DOX	11.9 $\pm$ 0.39*#	4.9 $\pm$ 0.30*#	3.9 $\pm$ 0.78*#	28.0 $\pm$ 1.6*#	599 $\pm$ 44
DFX	16.3 $\pm$ 0.16	6.7 $\pm$ 0.16	7.9 $\pm$ 0.68	39.3 $\pm$ 1.7	607 $\pm$ 13
DOX + DFX	12.8 $\pm$ 0.47	6.6 $\pm$ 0.56	5.9 $\pm$ 0.44	36.5 $\pm$ 2.4	631 $\pm$ 49
Control	16.0 $\pm$ 0.22	6.3 $\pm$ 0.13	7.1 $\pm$ 0.23	39.8 $\pm$ 1.5	560 $\pm$ 41

Effect of DOX, DFX, and their combination on the levels of blood parameter in rats. Data are presented as mean  $\pm$  SEM ( $n = 10$ ). \* and # indicate significant change from control and their combination, respectively, at  $P < 0.05$ .

in white blood cells count by 45%, in red blood cells (RBCs) by 21%, and in hematocrit (HCT%) by 28% compared to the control group. In the pretreated group with DFX, there was a complete reversal of the blood parameters that were decreased in the DOX group compared to the control level.

Cardiac toxicity induced by DOX was measured by the changes in enzymatic levels of GOT, LDH, CK, and CK-MB in serum. Table 3 showed the effect of DOX, DFX pretreatment and their combination on cardiac enzyme levels after 48 h of treatment. There was a significant increase in the GOT, LDH, and CK levels in the DOX group by 146%, 118%, and 343%, respectively, compared to the control group. There was a significant increase in CK-MB levels in DOX-treated group by 92% compared to the control group. Administration of desferrioxamine 30 minutes prior to DOX resulted in complete reversal of DOX-induced increase in cardiac enzyme to control levels.

The expression levels of TGF- $\beta$ 1, Smad2, Smad4, Smad7, CDKN2A, Mdm2, and p53 genes are studied in heart tissue, to investigate whether DOX treatment stimulates the TGF- $\beta$ 1 signaling cascade that leads to cardiac apoptosis. The effect of DOX and its combination with DFX on TGF-

TABLE 3: Effect of DOX and DFX on serum cardiac enzyme and isoenzyme levels in rats.

Group	Serum enzyme level			
	GOT U/L	LDH IU/L	CK U/L	CK-MB U/L
DOX	118.8 $\pm$ 6.4*#	541.9 $\pm$ 18*#	1312.0 $\pm$ 67*#	325.0 $\pm$ 104*#
DFX	48.5 $\pm$ 4.7	217.0 $\pm$ 3.0	298.7 $\pm$ 15	202.0 $\pm$ 13
DOX + DFX	80.4 $\pm$ 7.8	333.0 $\pm$ 17	442.2 $\pm$ 21	210.0 $\pm$ 64
Control	48.1 $\pm$ 5.5	249.3 $\pm$ 13	296.2 $\pm$ 26	169.0 $\pm$ 11

Effect of DOX, DFX, and their combination on the serum levels of cardiac enzyme and isoenzyme levels in rats. Data are presented as mean  $\pm$  SEM ( $n = 10$ ). \* and # indicate significant change from control and their combination, respectively, at  $P < 0.05$ .

$\beta$ 1 expression was shown in Figure 1. A single dose of DOX resulted in significant increase in the gene expression of TGF- $\beta$ 1 by 5.6-folds and decrease in Smad7 by 0.5-fold expressions, compared to the control group. On the other hand, pretreatment with DFX, 30 minutes before DOX treatment, induced a significant repair of the DOX-induced

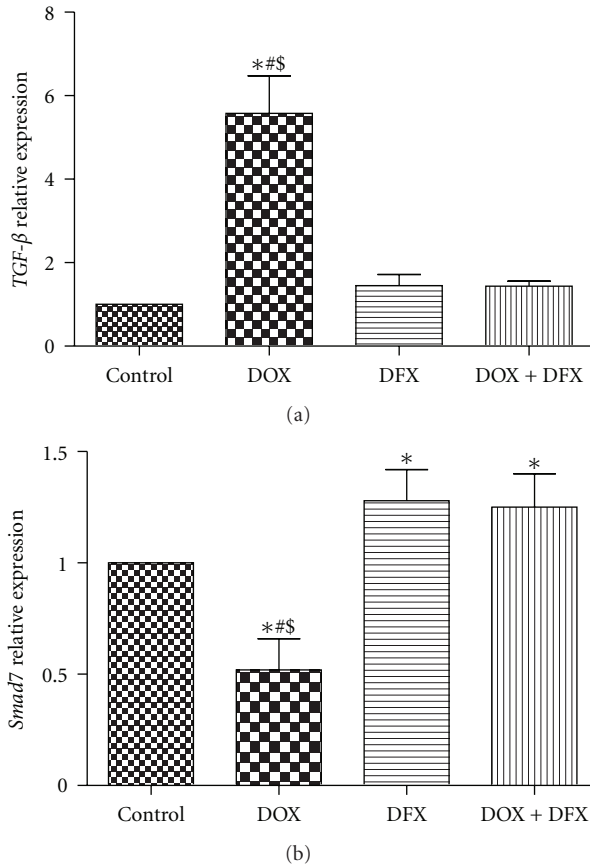


FIGURE 1: Effect of DOX, DFX, and their combination on the expression levels of TGF- $\beta$  (a) and Smad7 (b) in rat heart tissues. Data are presented as mean  $\pm$  SD ( $n = 10$ ). \*, # and \$ indicate significant change from control, DFX and DOX plus DFX, respectively, at  $P < 0.05$ .

alteration in the gene expression of TGF- $\beta$ 1 and Smad7 compared to the normal expression levels.

Figure 2 shows the effect of DOX, DFX, and their combination on the gene expression level of Smad2 (a) and Smad4 (b) in rat heart tissue. Treatment with a single dose of DOX was resulted in significant increases in the gene expression of Smad2 and Smad4 by 3.8- and 4-folds, respectively, compared to the control group. Interestingly, DFX pretreatment in combination with DOX resulted in a complete reversal change of Smad2 and 4 induced by DOX to the normal expression levels.

Figure 3 shows the effect of DOX, DFX, and their combination on the gene expression level of CDKN2A (a), Mdm2 (b), and p53 (c) in rat heart tissue. Treatment with DOX resulted in a significant decrease by 0.48-fold in Mdm2 and a significant increase by 5.2- and 3.7-folds in the gene expression of CDKN2 and p53, respectively, compared to the control group. Also, treatment with DFX 30 min prior to DOX resulted in complete reversal changes of Mdm2, CDKN2A, and p53 gene expression induced by DOX to the control levels.

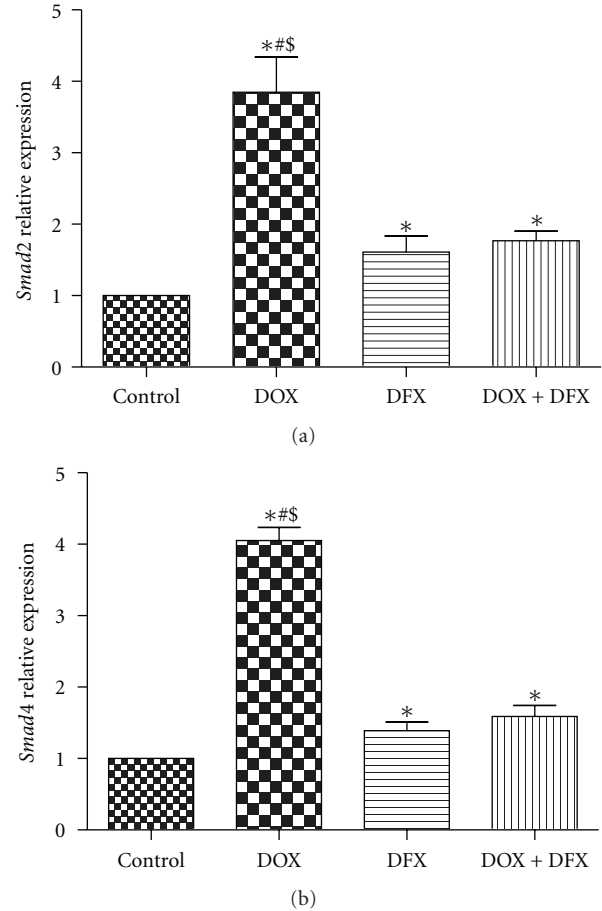


FIGURE 2: Effect of DOX, DFX, and their combination on the expression levels of Smad2 (a) and Smad4 (b) in rat heart tissues. Data are presented as mean  $\pm$  SD ( $n = 10$ ). \*, # and \$ indicate significant change from control, DFX and DOX plus DFX, respectively, at  $P < 0.05$ .

#### 4. Discussion

In the present study, the possible protective effect of iron chelator against doxorubicin-induced cardiotoxicity was investigated. It is well documented that doxorubicin may exert at least part of its cardiotoxicity by forming DOX-Fe complexes in heart resulting in ROS formation. Increase in serum LDH, CK, and GOT activity is well known diagnostic marker for myocardial function as these enzymes released from heart to the blood stream lead to increase their concentration in serum [28, 29]. In the current study, single dose of DOX (15 mg/kg) caused a significant increase in the serum level of GOT, LDH, and CK after 48 hours of treatment. The protective effect of DFX was clearly reflected in returning the cardiac enzymes and isoenzymes after combination treatment to their normal levels. Several reports found similar increases in the serum cardiac enzyme GOT, LDH, and CK levels following the induction of myocardial necrosis in rats [29, 30].

A single dose of DOX resulted in a significant decrease in RBCs and white blood cells (WBCs) counts, HCT% and Hb% concentration. The decrease in Hb% concentration

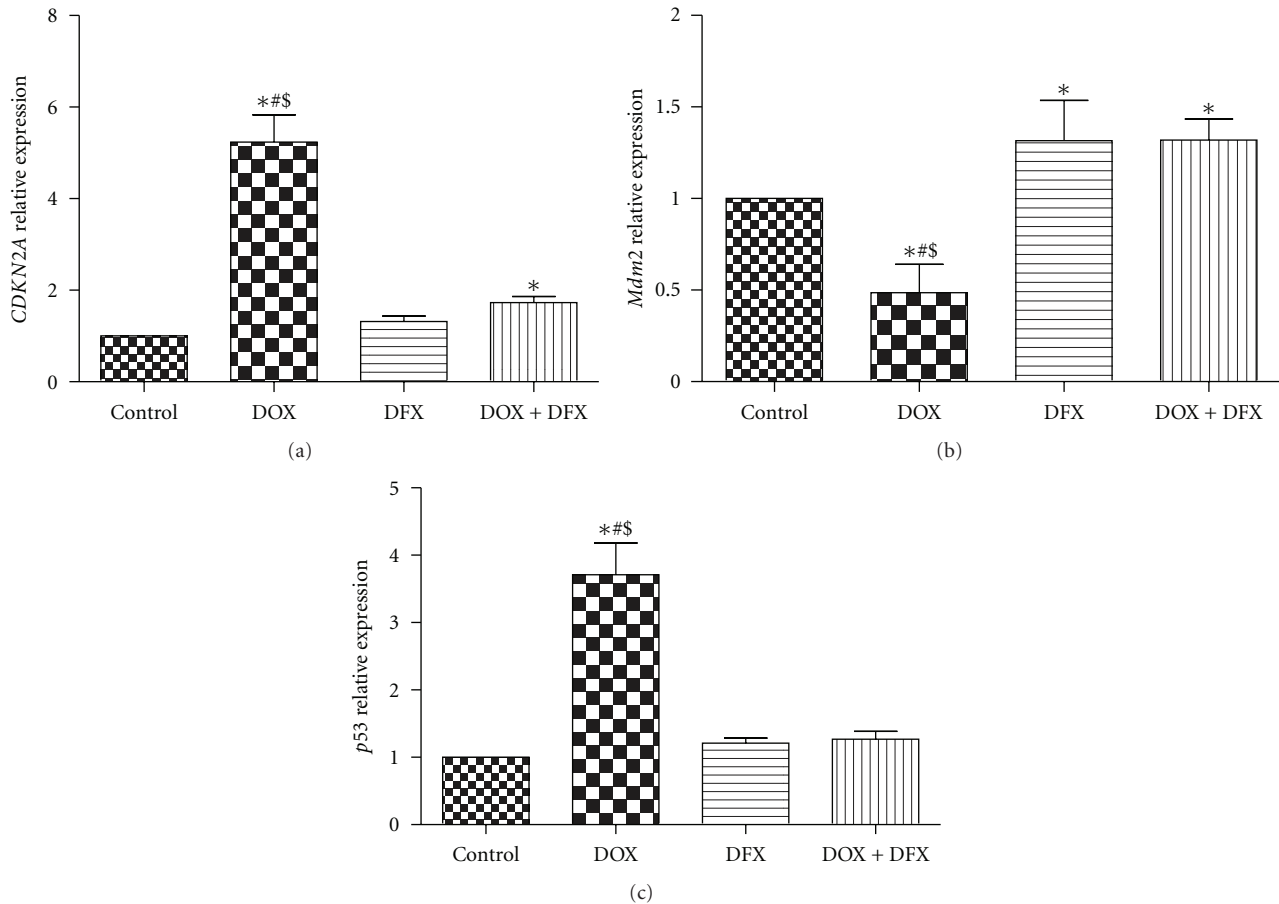


FIGURE 3: Effect of DOX, DFX, and their combination on the expression levels of CDKN2A (a), Mdm2 (b) and p53 (c) in rat heart tissues. Data are presented as mean  $\pm$  SD ( $n = 10$ ). \*, # and \$ indicate significant change from control, DFX and DOX plus DFX, respectively, at  $P < 0.05$ .

may be associated with decrease in the total content of body iron as a result of forming DOX-iron complex. Similarly, Piura and Rabinovich showed that treatment of patients with advanced/recurrent uterine sarcoma with a combination of DOX and ifosfamide had hematological toxicity represented in leukopenia in (80%), neutropenia in (80%), thrombocytopenia in (20%), and anemia in (20%) of the patients [31].

Oxidative stress is an important contributor to pathological remodeling, in the failing heart and plays critical role in cell growth, stress responses myocardial remodeling and programmed cell death [32]. The iron-mediated oxidative stress is thought to be responsible for DOX cardiotoxicity, but it is unclear whether it is also required for drug anti-tumor activity or not. Desferrioxamine acts as an antioxidant through its ability to decrease the amount of free iron available for the ROS production through the formation of DOX-iron complex [33]. The present study demonstrates that the pretreatment of DFX can prevent the cardiotoxicity induced by doxorubicin.

TGF- $\beta$ 1 gene downregulation leads to suppression of myocardial fibrosis and apoptosis [34]. In the present study, treated rats with DOX not only express high levels of cardiac TGF- $\beta$ 1, Smad2, and Smad4 but also exhibit downregulation

in Smad7 gene expression. These findings suggest the possible involvement of TGF- $\beta$ 1, Smad2, Smad4, and Smad7 genes in the regulation of cardiotoxicity process.

One of the most commonly mutated genes in human tumors is p53, tumor suppressor gene that controls both cell cycle arrest and apoptosis in response to DNA damage [35]. The p53 gene regulates the cell cycle by inhibiting the combination of cyclins with cyclin-dependent kinases. In the present study, we have explored the mechanism of CDKN2A and p53 genes as mediators in cardiotoxicity and their roles in apoptotic induction. The cell proliferation is controlled via a network of extracellular and intracellular cyclin-dependent kinases (CDKs) signaling pathways. Al-Khalaf and his colleagues showed that CDK inhibitor acts as apoptosis modulator through controlling the expression of transcription regulators [36]. Our data demonstrate that DOX increased apoptosis through upregulating CDKN2A and p53 gene expression.

Mdm2 oncogene is essential to p53 regulation which binds to and thereby inhibits p53. Therefore, Mdm2 gene acts as an inhibitor for p53 function or, as p53 promoter degradation by proteasome [37] and in this way, p53 levels are kept low in normal cells. The participation of Mdm2 in response

to DNA damage has been investigated in another study [38]. Léveillard and his colleagues investigated the importance of p53 control in Mdm2 knockout mice in which mice died early during development but are rescued from death by additional deletion of p53 gene [39]. In the current study, there was downregulation of Mdm2 gene expression levels in single dose DOX-treated group. Our results showed a decrease in Mdm2 gene expression level which triggers the apoptotic process by increasing the p53 gene expression. This clarifies the role of Mdm2 in cardiotoxicity and also shed new light on the clinical significance of TGF- $\beta$ /Smad p53 pathway. On the other hand, the DFX treatment in combination with DOX leads to downregulation of TGF- $\beta$ , Smad2 and Smad4 genes expression as well as upregulation in Smad7 expression that was associated with a significant lowering in cardiac apoptosis.

In conclusion data from this study suggest that (1) DOX induces its acute cardiotoxicity secondary to the increase in specific genes expression in TGF- $\beta$ /Smad pathway. (2) DOX increases apoptosis through upregulation of CDKN2A and p53 and downregulation of Mdm2 gene expression. (3) The preventive effect of DFX against DOX-induced cardiotoxicity is mediated via TGF- $\beta$ 1/Smad pathway.

## Conflict of Interests

All authors declare that there are no conflict of interests.

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