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All-trans-retinoic acid ameliorates doxorubicin-induced cardiotoxicity: in vivo potential involvement of oxidative stress, inflammation, and apoptosis via caspase-3 and p53 down-expression

Asmaa F. Khafaga[1] · Yasser S. El-Sayed[2]

Abstract The present study aimed to investigate the potential protective effect of all-trans-retinoic acid (ATRA, a natural derivative of vitamin A) against doxorubicin (DOX)-induced in vivo cardiac toxicity and its underlying mechanisms. Forty male albino rats were allocated into control, ATRA (0.5 mg/kg bwt, intraperitoneally daily), DOX (2.5 mg/kg bwt, intraperitoneally twice weekly for 3 weeks), and DOX + ATRA groups. Serum lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase-cardiac type (CK-MB), troponin I, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) were measured. In addition, cardiac glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), and malondialdehyde (MDA) were determined. Cardiac tissues were examined for histopathologic changes and immunoexpression of pro-apoptotic caspase 3 and tumor-suppressor p53 proteins. DOX caused severe myocardial damage; degenerative and necrotic changes and worsened cardiac function biomarkers; and elevated serum LDH, CK, CK-MB, and troponin I. In addition, DOX inhibited cardiac antioxidative enzymes (GSH, GSH-Px, SOD, CAT) activities and enhanced MDA level. DOX increased serum proinflammatory cytokines (TNF-α, IL-6) and area percent of caspase 3 and p53 immunoexpression in heart tissues.

Pretreatment with ATRA maintained cardiac function biomarkers, and reduced proinflammatory cytokines, lipid peroxidation, and immunoexpression of caspase 3 and p53. Moreover, ATRA improved cardiac histoarchitecture, as well as the activities of antioxidative enzymes. Collectively, ATRA can counteract DOX-induced cardiomyopathy through antioxidative and anti-inflammatory properties, besides suppression of the activation of the mitochondrial apoptotic pathway.

Keywords All-trans-retinoic acid · Oxidative stress · Cytokines · Apoptosis · Immunohistochemistry · Cardiotoxicity

✉ Yasser S. El-Sayed
elsayed-ys@vetmed.dmu.edu.eg; yasser_tf@yahoo.com

Asmaa F. Khafaga
soma_path2008@yahoo.com

[1] Department of Pathology, Faculty of Veterinary Medicine, Alexandria University, Edfina 22758, Egypt

[2] Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Egypt

Introduction

Doxorubicin (DOX) is a broad-spectrum anti-neoplastic agent with a potent chemotherapeutic efficacy against various malignancies and tumorigenesis. It is widely used in the treatment of several tumors, such as liver carcinoma, breast cancer, and non-Hodgkin's lymphoma (Jordan 2002; Weiss 1992). However, the cardiotoxicity is considered as the major adverse effect of DOX, which limits its clinical use (Barry et al. 2007; Gianni et al. 2008). Lipid peroxidation and subsequent production of free radicals are supposed to be integrated into DOX-induced cardiotoxicity (Simunek et al. 2009). In addition, the mitochondrial dysfunction, excessive production of proinflammatory cytokines (Pecoraro et al. 2016), and cardiac membrane injuries (Hiona et al. 2011) may be also implicated in the pathogenesis of cardiotoxicity. DOX cardiotoxicity has a poor prognosis for available treatment, and it is frequently fatal (Jordan 2002; Takemura and Fujiwara 2007). Accordingly, extensive researches on various cardioprotectant are being proposed to mitigate DOX-induced cardiotoxicity.

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The dietary vitamin A is normally metabolized in the cytoplasm to several isomeric forms of retinoic acid, of which all-trans-retinoic acid (ATRA) is the major biologically active form (Lee and Lee 2006). Previous investigations indicated that ATRA has antioxidant, anti-inflammatory, and anti-neoplastic properties (Rao et al. 2010). Furthermore, it has been suggested that ATRA plays a role in the cellular proliferation and tissue hemostasis (Canete et al. 2017; Doldo et al. 2015), and in retrieving the adverse effect in various conditions, such as uterine toxicity (Chatterjee and Chatterji 2011), hepatic ischemia and hepatitis (Nagy 2012; Rao et al. 2010), neurotoxicity (Cheng et al. 2013; Kim et al. 2013), and cardiotoxicity (Choudhary et al. 2008; Lou et al. 2013; Nizamutdinova et al. 2013). A few studies have been proposed to investigate the role of combined chemotherapy of DOX and ATRA in a single nano-system in the enhancement of anti-neoplastic activity (Sun et al. 2015; Zhang et al. 2015). However, only one study was conducted to evaluate the in vitro cardioprotective potential of ATRA against DOX cardiotoxicity in H9C2 cells and primary cardiomyocytes (Yang et al. 2016), since the in vivo protective effect of ATRA against DOX-associated cardiac injury remains unclear. This study was thereby designed to assess the potential protective effects of ATRA against in vivo DOX cardiotoxicity, in the light of cytopathic effects, oxidative stress, and apoptotic signaling pathways.

Materials and methods

Animals and experimental design

Forty male Wistar albino rats, 6–8 weeks old and 175–200 g body weight, were used for the experimental procedures. They were obtained from the closed bred colony, Pharos University, Alexandria, Egypt. Prior to experimentation, rats were housed in metal cages under controlled environmental conditions (24–27 °C temperature, 55% RH, and 12 h light/dark cycle) for 2 weeks. All animals fed on a standard laboratory diet and received water ad libitum during the experimentation.

After the acclimatization period, rats were assigned into four groups (n = 10/each). Group I (control) was intraperitoneal (ip) injected daily with olive oil (2.5 ml/kg bwt), the vehicle used for DOX. Group II (ATRA) was administered daily with ATRA (Sigma-Aldrich Chemical Co., St. Louis, USA) 0.5 mg/kg bwt ip, for three consecutive weeks, which asserted as the optimum effective dosage (Chatterjee and Chatterji 2011). Group III (DOX) received DOX (BMC United Pharmaceuticals Co., Cairo, Egypt) 2.5 mg/kg bwt ip, twice weekly to a cumulative dose 15 mg/kg bwt, as described earlier (Kumar et al. 2001). Group IV (ATRA + DOX) was administered ATRA, an hour before DOX administration in the similar regime of Groups II and III, respectively. Twenty-four hours of the last injection, all animals were anesthetized and weighed. The experimental protocol and dosage regimen used in this study are summarized in Fig. 1. Blood samples were then collected from the inner eye canthus into heparinized capillary tubes. Next, rats were immediately euthanized and grossly examined, and hearts were rapidly harvested, washed, and weighed. The relative heart weight (RHW) was calculated according to the following equation: RHW = (heart weight/body weight) × 100. Instantly after weighting, hearts were cut longitudinally with one half, which was thoroughly washed with ice-cold phosphate buffer saline (PBS) and kept at − 80 °C. A 10% cardiac tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) using a homogenizer with a Teflon pestle. The homogenate was centrifuged at 14,000×g for 15 min at 4 °C to remove cell debris. Aliquots of the supernatant were used for determination of lipid peroxidation and antioxidants biomarkers. The other split was collected in neutral buffered formalin 10% for cytopathological and immunocytochemical evaluations. The number of rats and their suffering were minimized.

Measurement of cardiac function biomarkers

Collected blood samples were immediately centrifuged for 10 min at 3000 rpm for serum separation and further colorimetric estimation of lactate dehydrogenase (LDH, EC 1.1.1.27) (Buhl and Jackson 1978), creatine kinase (CK, EC 2.7.3.2) (Hughes 1962), and creatine kinase-cardiac type isoenzyme (CK-MB) (Wu and Bowers 1982) activities following the providers' manual of commercial-supplied assay kits (BioAssay Systems, USA). Additionally, serum troponin I concentration was determined using enzyme-linked immunosorbent assay (ELISA) capture (Spectrum Diagnostics, Egypt) (Penttila et al. 1997).

Assessment of pro-inflammatory biomarkers

To test whether cardiac damage was associated with pro-inflammatory cytokine induction, we measured serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) using ELISA assay following the manufacturers' instruction (Abcam, USA). All assays were performed in duplicates, and cytokine concentrations (pg/ml) were determined at 450 nm using a computerized, automated microplate ELISA reader (Sorin Biomedica SpA., Italy).

Oxidative damage and antioxidant capacity assay

Lipid peroxidation in cardiac tissues was estimated in heart homogenates as malondialdehyde (MDA). The principle of

Fig. 1 Schematic summary of the study protocol. Allocated animals were intraperitoneally injected with olive oil (2.5 mg/kg bwt twice weekly), all-trans-retinoic acid (ATRA; 0.5 mg/kg bwt daily), doxorubicin (DOX; 2.5 mg/kg bwt twice weekly), or ATRA + DOX as in the previous dosage for 3 weeks. One day of the last injection, animals were anesthetized and weighed, and blood and heart samples were collected. *ATRA was given an hour before DOX administration
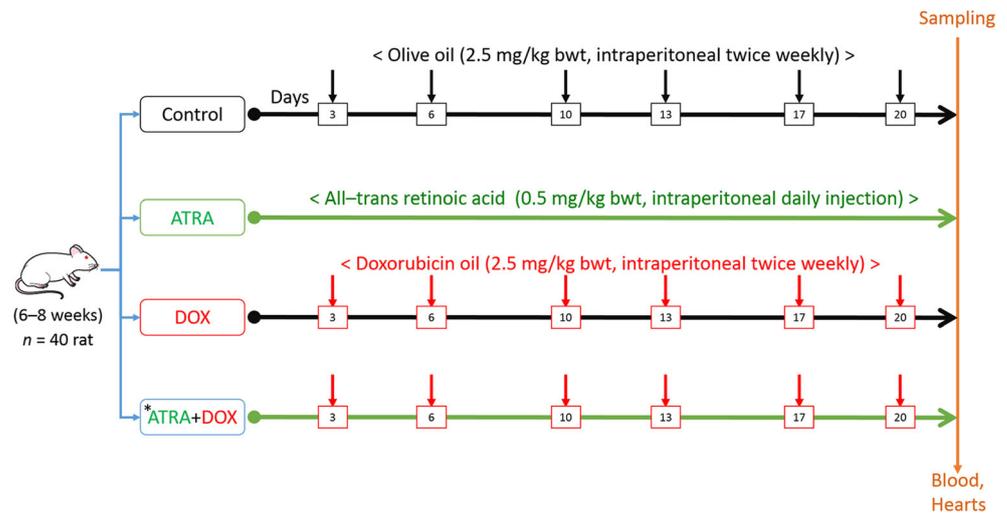
the colorimetric assay was based on the reaction between one molecule of MDA in homogenate with two molecules of thiobarbituric acid resulting in pink-colored complex, its absorbance measured at 532 nm (Mihara and Uchiyama 1978). The tissue content of reduced glutathione (GSH) was assayed by the colorimetric method using commercially supplied kits (Bio-Diagnostic, Egypt). The GSH assay was based on the reduction of 5,5′–dithiobis (2–nitrobenzoic acid) with GSH to a yellow-colored complex; its absorbance was read at 405 nm within 15 min (Adams et al. 1983). The superoxide dismutase (SOD, EC 1.15.1.1) activity was colorimetrically determined using commercially available kits (Bio-Diagnostic, Egypt). The assay depends on the ability of the SOD enzyme to inhibit the phenazine methosulphate-mediated reduction of the nitroblue tetrazolium dye (Sun et al. 1988). The activity of glutathione peroxidase (GSH-Px, EC 1.11.1.9) was evaluated according to the previously described methods (Paglia and Valentine 1967). Evaluation of catalase (CAT, EC 1.11.1.6) activity was performed on the basis of the decomposition rate of H_2O_2 at 240 nm (Aebi 1984). Protein was determined using Bradford's reagent.

Histopathologic examination and semi-quantitative scoring

Collected cardiac tissue specimens of control and treated rats were immediately fixed in buffered formalin for 24 h then processed through the paraffin-embedding technique. Several sections of 5 μm thickness were deparaffinized with xylene, stained with hematoxylin and eosin (H&E), and finally examined under light microscopy. Additionally, the extent of cardiac tissue damage was then evaluated using a semi-quantitative scoring assay, in which five random fields were examined from each section. The severity of lesions was scored and graded according to the percentage of tissue involvement, as follows:

None (−): representing no involvement of the examined field
Mild (+): representing involvement of 0–25% of the examined field
Moderate (++): representing involvement of 25–50% of the examined field
Severe (+++): represented involvements of 50–100% of the examined field

Immunohistochemical assessment

Several 4-μm-thick sections were prepared from paraffin blocks, deparaffinized in xylene, and rehydrated in descending grades of ethanol. The sections were pretreated with 0.01 mol/L citrate-buffered saline (pH 6.0) for antigen retrieval, quenched with 0.3% (v/v) H_2O_2 in phosphate-buffered saline for endogenous peroxidase activity, and incubated for an hour with 10% (v/v) normal goat serum to block nonspecific binding of the immunological reagents. After that, the sections were incubated overnight at 4 °C with mouse monoclonal antibodies against caspase 3 and p53 (Dako Corporation, Life Trade, Egypt). Streptavidin-biotin complex and horseradish peroxidase were then applied, and immunohistochemical staining kits were used to visualize the reaction products. Sections were incubated for a minute in diaminobenzidine tetra-hydrochloride to develop the peroxidase labeling. Counterstaining was finally conducted using Mayer's hematoxylin solution. For quantitative analysis, area and area percentage of caspase 3 and p53 immunoexpression were measured in myocardial sections of control and treated groups using the ImageJ software (Wayne@codon. nih.gov, Rasband 1997–2016). Original immunohistochemical photographs were obtained from 10 random fields per section with total magnification 100 (magnification lens 10). Area of positive immunoreactivity was determined by a

standard measuring frame and then masked by red binary color to be measured.

Statistical analyses

All values in tables and figures were expressed as means ± standard error of means (SEM). Collected data were subjected to one-way analysis of variance (ANOVA) using the SAS® software followed by post hoc Duncan's multiple range test to detect differences between groups. Differences were statistically considered significant at $p < 0.05$.

Results

Body weight and relative heart weight

DOX-treated rats showed a significant drop ($p < 0.05$) in their body weight (155.9 ± 4.21 g) as compared to control rats (211.9 ± 2.49 g). However, rats treated with ATRA + DOX showed significant ($p < 0.05$) improvement in their body weights (178.9 ± 3.21 g) compared to DOX-treated rats. Noticeably, Duncan's multiple range post hoc test showed that there were no significant ($p > 0.05$) differences in rats' body weights between ATRA-treated (204.9 ± 3.12 g) and control (211.9 ± 2.49 g) groups. Generally, the relative heart weights showed no significant ($p > 0.05$) changes between treated and control rats (Table 1).

Serum cardiac biomarkers and pro-inflammatory cytokines

As shown in Table 1, DOX evoked cardiotoxicity as showed by the elevation of serum cardiac biomarkers. The LDH, CK, and CK-MP activities and troponin I level were significantly increased ($p < 0.05$) in DOX-treated rats (1673 ± 18.4, 793 ± 4.78, 547.0 ± 3.00, and 21.04 ± 1.12 U/L, respectively) compared to control values (590 ± 4.08, 206 ± 3.06,

182.4 ± 1.54, and 11.14 ± 0.17 U/L, respectively). Alternatively, these biomarkers were significantly reduced ($p < 0.05$) following pretreatment of DOX-treated rats with ATRA (1207 ± 5.33, 461 ± 3.32, 346.8 ± 3.38, and 17.04 ± 0.64 U/L, respectively) compared to DOX-treated rats, even though they were not identical to controls. Treatment with ATRA alone had no effect on the serum cardiac biomarkers compared to control.

Furthermore, the data presented in Table 1 showed the levels of pro-inflammatory cytokines; IL-6 and TNF-α were significantly ($p < 0.05$) increased in DOX-treated rats (91.87 ± 3.25 and 84.78 ± 5.96 pg/mL, respectively) compared to controls (32.13 ± 2.63 and 31.83 ± 2.83 pg/mL, respectively). Meanwhile, their values were significantly ($p < 0.05$) diminished following pretreatment with ATRA (64.95 ± 6.66 and 62.87 ± 7.40 pg/mL, respectively) compared to DOX-treated rats. Moreover, administration of ATRA alone did not produce any alterations in the assessed proinflammatory cytokines.

Cardiac oxidative parameters

To estimate the protective effect of ATRA against DOX-induced cardiac oxidative damage in the rat, we examined the lipid peroxidation and antioxidant biomarkers in the myocardium. Lipid peroxidation was estimated as MDA, which was significantly increased ($p < 0.05$) in DOX-treated rats (86.11 ± 0.71 μmol/g protein) compared to controls (35.29 ± 0.43 μmol/g protein). However, cardiac antioxidant cellular molecules (GSH, GSH-Px, CAT, and SOD) were significantly ($p < 0.05$) lowered in DOX-treated animals (6.92 ± 0.20 nmol/g protein, 3.89 ± 0.15, 14.31 ± 0.37, and 17.28 ± 0.31 U/mg protein, respectively) than that of controls (16.19 ± 0.23 nmol/g protein, 7.43 ± 0.19, 27.26 ± 0.28 and 32.40 ± 0.19 U/mg protein, respectively). Administration of ATRA shortly before DOX resulted in an improvement of the cardiac oxidative state. It significantly ($p < 0.05$) decreased lipid peroxidation biomarker (53.55 ± 0.55 μmol/g protein)

Table 1 Effect of doxorubicin, all-trans-retinoic acid, and their combination on the body weight, relative heart weight, serum cardiac function biomarkers, and proinflammatory cytokines of male Wistar albino rats

Groups (n = 10)	Body and relative heart weights		Cardiac function biomarkers				Proinflammatory cytokines	
	BW (g)	RHW (%)	LDH (U/L)	CK (U/L)	CK-MB (U/L)	Troponin I (U/L)	IL-6 (pg/ml)	TNF-α (pg/ml)
CTR	211.9 ± 2.49a	0.59 ± 0.03	590 ± 4.08c	206 ± 3.06c	182.4 ± 1.54c	11.14 ± 0.17c	32.13 ± 2.63c	31.83 ± 2.83c
ATRA	204.9 ± 3.12a	0.57 ± 0.02	587 ± 5.07c	202 ± 2.76c	176.7 ± 2.89c	11.68 ± 0.18c	31.71 ± 2.50c	32.09 ± 2.84c
DOX	155.9 ± 4.21c	0.52 ± 0.01	1673 ± 18.4a	793 ± 4.78a	547.0 ± 3.00a	21.04 ± 1.12a	91.87 ± 3.25a	84.78 ± 5.96a
ATRA + DOX	178.9 ± 3.21b	0.54 ± 0.05	1207 ± 5.33b	461 ± 3.32b	346.8 ± 3.38b	17.04 ± 0.64b	64.95 ± 6.66b	62.87 ± 7.40b

All values are expressed as mean ± SEM. Mean values of different letters (a, b, c) within the same column are significantly different ($p < 0.05$, ANOVA with Duncan's post hoc test for multiple comparisons)

CTR control, ATRA all-trans-retinoic acid, DOX doxorubicin, BW body weight, RHW relative heart weight, CK creatine kinase, CK-MB creatine kinase-cardiac type, IL-6 interleukin-6, TNF-α tumor necrosis factor-α

Table 2 Effect of doxorubicin, all-trans-retinoic acid, and their combination on cardiac lipid peroxidation and antioxidant biomarkers in male Wistar albino rats

Groups ($n = 10$)	MDA (μmol/g protein)	GSH (nmol/g protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)
CTR	35.29 ± 0.43c	16.19 ± 0.23a	7.43 ± 0.19a	27.26 ± 0.28a	32.40 ± 0.19a
ATRA	34.67 ± 0.30c	16.68 ± 0.22a	7.57 ± 0.21a	27.13 ± 0.32a	32.10 ± 0.32a
DOX	86.11 ± 0.71a	6.92 ± 0.20c	3.89 ± 0.15c	14.31 ± 0.37c	17.28 ± 0.31c
ATRA + DOX	53.55 ± 0.55b	10.26 ± 0.25b	4.81 ± 0.15b	18.91 ± 0.29b	22.82 ± 0.25b

All values are expressed as mean ± SEM. Mean values of different letters (a, b, c) within the same column are significantly different ($p < 0.05$, ANOVA with Duncan's post hoc test for multiple comparisons)

CTR control, *ATRA* all-trans-retinoic acid, *DOX* doxorubicin, *MDA* malondialdehyde, *GSH* reduced glutathione, *CAT* catalase, *SOD* superoxide dismutase, *GSH-Px* glutathione peroxidase

and increased antioxidant parameters (GSH 10.26 ± 0.25 nmol/g protein, GSH-Px 4.81 ± 0.15 U/mg protein, CAT 18.91 ± 0.29 U/mg protein, and SOD 22.82 ± 0.25 U/mg protein) compared to the DOX-treated group. Furthermore, ATRA injection alone did not produce any significant changes ($p > 0.05$) in oxidative parameters in relation to the control group (Table 2).

Histopathological observations and semiquantitative scoring

Myocardial tissues from the control and ATRA-treated rats showed normal histoarchitecture with a centrally located basophilic nucleus and normal striation of muscle fibers (Fig. 2a, b, Table 3). On the other hand, cardiomyocytes from the DOX-intoxicated rats revealed various degenerative changes: eosinophilia, fragmentation, hyalinization, and disorganization of myofibrillar arrays. Vacuolization of the sarcoplasm and formation of single large or multiple small vacuoles were evident in both muscle fibers and arteriolar wall (Fig. 2c, Table 3). Severe congestion and interfibrillar hemorrhage were also detected (Fig. 2d, Table 3). Additionally, numerous areas of interfibrillar edema (Fig. 2e, Table 3), coagulative necrosis, and myocytolysis were observed. Moreover, areas of myofibrillar loss were intensely infiltrated by various inflammatory cells including mononuclear cells and fibroblasts (Fig. 2f, Table 3). Co-treatment with ATRA resulted in an improvement of DOX-related myocardial damages; scattered patches of disorganized and fragmented cardiomyocytes with moderate vascular congestion were the commonly observed lesions (Fig. 2g, h, Table 3). The semiquantitative scoring of the reported lesions indicates a significant increase in myocardial injury in DOX-treated rats compared to control or ATRA-treated rats. Rats received ATRA shortly before DOX significantly prevented these myocardial damages as compared to DOX-intoxicated rats (Table 3).

Immunohistochemistry and histomorphometric findings

Compared to control (Figs. 3a and 4a) and ATRA-treated rats (Figs. 3b and 4b), the caspase 3 and p53 immunoexpression showed strong immunopositivity in DOX-treated groups (Figs. 3c and 4c), while in pretreatment with ATRA there is a mild to moderate immunopositivity of caspase 3 and p53 levels compared to DOX-treated rats (Figs. 3d and 4d), respectively. In addition, comparing to control and ATRA-treated rats, DOX-treated groups showed significant ($p < 0.001$) increment of the mean area percent of caspase 3 and p53 immunoreactivity, but co-treatment of DOX with ATRA resulted in a significant decline ($p < 0.001$) in their immunostaining area percent compared to DOX-treated rats. Treatment with ATRA alone had no significant ($p > 0.05$) effect on area percent of both caspase 3 and p53 immunostaining compared to control rats (Figs. 3e and 4e).

Discussion

To the best of our knowledge, this is the first work that reveals the in vivo cardioprotective role of ATRA against DOX-induced cardiotoxicity. Serum cardiac enzymes, such as CK, CK-MB, and LDH, as well as troponin I act as highly sensitive and specific biomarkers that evaluate the severity of myocardial damages. Commonly, DOX-treated rats exhibited significant increment in the activities of these cardiac function biomarkers (Chen et al. 2015; Kelleni et al. 2015; Sun et al. 2013, 2016; Yu et al. 2013), reflecting cardiomyocyte damages and loss of their membrane permeability and integrity (Bertinchant et al. 2003; Rohilla et al. 2012). In contrast, co-treatment with ATRA significantly improved the DOX-disturbed cardiac function, supporting its promising role as a cardioprotectant, and hence preservation of cardiac function. Oxidative stress is one of the major cellular reactions against toxic insults (Winczura et al. 2012). Previous research demonstrated that DOX-induced cardiotoxicity is associated with the generation of free radicals in cardiac tissues (Hrdina et al. 2000; Naidu

Fig. 2 Representative photomicrograph of myocardial tissue of rat following doxorubicin (DOX) and/or all-trans retinoic acid (ATRA) treatment (H&E, × 100). The light microscopic findings of myocardial tissue of control (**a**) and ATRA (0.5 mg/kg bw, ip daily)-administered (**b**) rats showed normal histological limits of myocardial tissues. The DOX (2.5 mg/kg bw, ip twice weekly)-administered rats showed severe cardiac injury with prominent vacuolization of sarcoplasm and arteriolar wall (arrows) (**c**), hyalinized myofibers with extensive inter-myofibrillar hemorrhage (asterisk) (**d**), interfibrillar edema (arrows) (**e**), and area of myocytolysis infiltrated with mononuclear inflammatory cells (yellow arrows) and fibroblasts (blue arrows) (**f**). ATRA + DOX-treated rats showed mild degeneration of myocardial tissues (arrows) (**g**) and least congestion of cardiac blood vessels (arrows) (**h**). *CTR* control, *ATRA* all-trans-retinoic acid, *DOX* doxorubicin
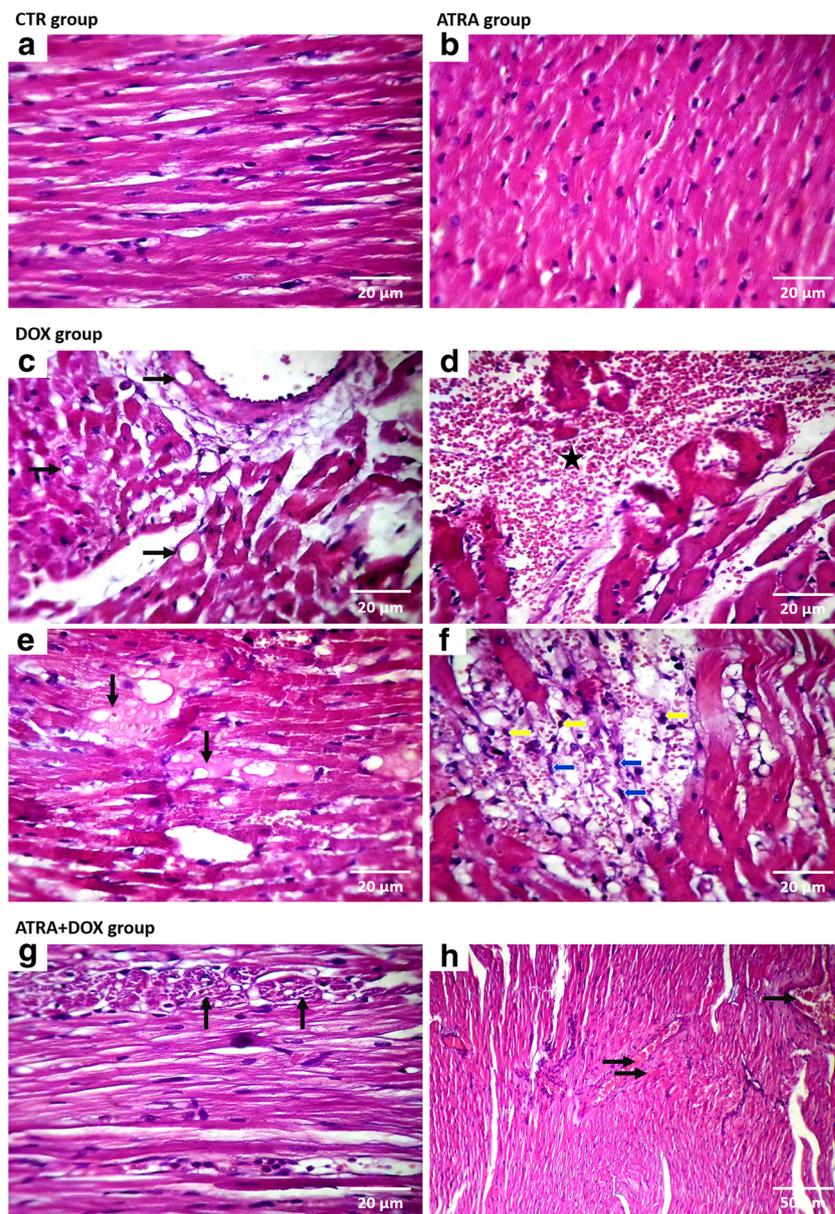
et al. 2002). Furthermore, DOX free radicals are non-enzymatically produced, and then react with iron; this DOX-iron complex is able to reduce oxygen to superoxide anion and hydrogen peroxide, which are involved in hydroxyl radicals formation (Malisza and Hasinoff 1995). In addition, MDA (the end product of lipid peroxidation process) is an indicator for free radicals-induced lipid breakdown (Abdel-Daim et al. 2017, 2016; El-Far et al. 2017; El-Sayed et al. 2015; Mohamed et al. 2016). In this work, a significant increment in MDA content and reduction of antioxidant enzymes activity involving GSH, GSH-Px, CAT, and SOD were recorded in DOX-intoxicated rats. Consistent with our findings, DOX-induced oxidative stress and depletion of antioxidant enzymes have been implicated in many tissues, such as the liver, kidneys (El-Moselhy and El-Sheikh 2014), brain (Pal et al. 2012),

and in particular, heart tissues (Chen et al. 2015; Elberry et al. 2010; Sterba et al. 2013; Sun et al. 2013, 2015; Yu et al. 2013). Remarkably, pretreatment with ATRA decreased MDA content and improved antioxidant enzyme activity in cardiac tissue compared to DOX-treated rats, thereby indicating the antioxidative potential of ATRA against DOX-induced oxidative stress. The eventual mechanism by which ATRA acts as an antioxidant may be mediated through scavenging free radicals-induced oxidative stress and preventing the peroxyl radical-dependent peroxidation (Marnett and Ji 1994).

Another theory of DOX-induced cardiotoxicity is an initiation of pro-inflammatory cytokines production (Abd El-Aziz et al. 2012; Pecoraro et al. 2016), which is associated with chronic heart failure (Hedayat et al. 2010), a leading cause of death worldwide. It is well documented that overexpression

Table 3 Incidence and severity of cardiac histopathological findings in the examined heart in control and after the various treatments in male Wistar albino rats

| Groups (n = 10)	Lesion severity	Myocardial degeneration	Myocardial necrosis	Interstitial inflammation	Interstitial fibrosis	Interstitial edema	Interfibrillar hemorrhage	Myocardial vaculation
CTR	None (−)	8 (80%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
	Mild (+)	2 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Moderate (++)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Severe (+++)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ATRA	None (−)	8 (80%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
	Mild (+)	2 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Moderate (++)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Severe (+++)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
DOX	None (−)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Mild (+)	0 (0%)	0 (0%)	0 (0%)	4 (10%)	0 (0%)	0 (0%)	4 (40%)
	Moderate (++)	2 (20%)	4 (40%)	6 (60%)	4 (40%)	4 (40%)	6 (60%)	4 (40%)
	Severe (+++)	8 (80%)	6 (60%)	4 (40%)	2 (20%)	6 (60%)	4 (40%)	2 (20%)
ATRA + DOX	None (−)	2 (20%)	2 (20%)	0 (0%)	6 (60%)	0 (0%)	2 (20%)	0 (0%)
	Mild (+)	4 (40%)	4 (40%)	8 (80%)	4 (40%)	8 (80%)	4 (40%)	6 (60%)
	Moderate (++)	4 (40%)	4 (40%)	2 (20%)	0 (0%)	2 (20%)	4 (40%)	4 (40%)
	Severe (+++)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Incidence is the number of rats with lesions per total examined. Severity of lesions was scored and graded by determining the percentage of tissue involvement. Lesion scoring: (−) absence of the lesion = 0%, (+) mild = 0–25%, (++) moderate = 25–50%, and (+++) severe = 50–100% of the examined tissue sections. *CTR*: control; *ATRA*: all-trans retinoic acid; *DOX*: doxorubicin

of IL-6 (Kanda and Takahashi 2004) and TNF-α (Feldman et al. 2000) is involved in the pathogenesis of cardiac failure. Similarly, DOX induced in vivo overexpression of pro-inflammatory cytokines, IL-6 and TNF-α; therefore, it elicits an inflammatory response in cardiac tissues, and further cardiotoxicity. Several reports have also demonstrated overexpression and release of inflammatory mediators including TNF-α and IL-6 in the serum of DOX-intoxicated rats (Abd El-Aziz et al. 2012; Sun et al. 2016) and plasma of heart failure patients (Gullestad et al. 2012). Accumulating evidence suggests a primary role for IL-6 and TNF-α in cardiovascular toxicity as reliable predictors of morbidity and mortality (Hedayat et al. 2010). Conversely, ATRA pretreatment inhibited pro-inflammatory mediators production, which might be mediated through reduction of ERK1/2 phosphorylation (Kirchmeyer et al. 2008), thereby improving its anti-inflammatory activity against DOX-induced cytokines release.

It has been reported that DOX-induced cardiotoxicity depends mainly upon the apoptotic death of cardiomyocytes (Kalay et al. 2006). The most direct cause of apoptotic death is the activation of caspases that have a major role in controlling apoptosis-associated chemical and cellular alterations (Lebda et al. 2017). DOX has the ability to activate caspase 3, the hallmark of the apoptotic

signaling pathway (Kalyanaraman et al. 2002). The strong immunopositivity and significant increment of immunostaining area of caspase 3 were apparent following DOX administration (Sun et al. 2013), indicating cardiomyocyte apoptosis. However, the reduced immunoexpression of caspase 3 follwoing co-treatment with ATRA was reflecting its anti-apoptotic effect against DOX-induced apoptotic cell death. Caspases were activated through two distinct pathways: the intrinsic (mitochondrial-dependent) apoptotic pathway and the extrinsic (death receptor-dependent) pathway (Pryor et al. 2006). The mitochondrial-dependent apoptotic pathway is the major pathway for DOX-induced myocardial apoptosis (Xiao et al. 2012). Furthermore, initiation of this intrinsic pathway has been directly dependent on activation of the p53 tumor-suppressor protein. p53 protein has a critical role in the regulation of cellular response to DNA damage and has the ability to interfere with the release of cytochrome c leading to subsequent initiation of cardiomyocytes apoptosis (Sun et al. 2013; Yoshida et al. 2009). Our findings revealed that DOX also induced overexpression with marked elevation in the immunostaining area of p53 tumor-suppressor protein. It has been reported that DOX evoked p53 overexpression in H9C2 cardiomyocytes (Sun et al. 2013), which contributes to the development of

Fig. 3 Representative photomicrograph of caspase 3 immunoreactivity in rat's myocardium following doxorubicin (DOX) and/or all-trans retinoic acid (ATRA) treatment. Cardiomyocytes from **a** control and **b** ATRA-treated rats showed negative immunoreactivity for caspase 3. **c** Cardiomyocytes from DOX-treated rats showed strong positive immunoreactivity for caspase 3 (arrows). **d** Cardiomyocytes from ATRA + DOX-treated rats showed mild to moderate immunoreactivity for caspase 3 (arrows). **e** Area percent of caspase 3 immunoexpression/field in control and treated groups; all values are expressed as mean ± SEM, $n = 10$. Mean value were significantly different from control (***$p \leq 0.001$), ATRA (###$p \leq 0.001$), DOX ($$$$p \leq 0.001$), one-way ANOVA with Duncan's post hoc test for multiple comparisons
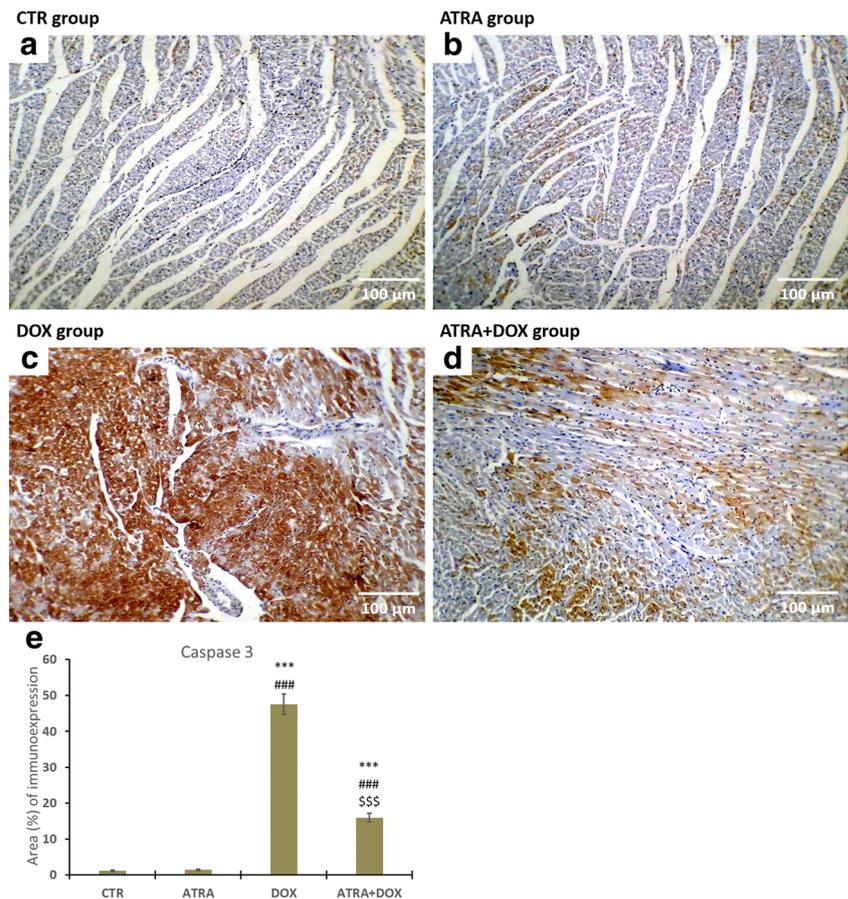
apoptosis (Liu et al. 2008). Interestingly, the protective intervention with ATRA shortly prior to DOX injection effectively reduced the area of p53 immunoexpression, signifying in vivo anti-apoptotic efficacy of ATRA. Recently, it has been demonstrated that ATRA reduced caspase 3 immunopositivity in both H9C2 cells and primary cardiomyocytes compared to DOX-treated cells (Yang et al. 2016). Others summarize the role of ATRA in activation of caspase 3 and p53 in tumor cell lines as a possible explanation for its tumor preventive activity (Gumireddy et al. 2003; Hormi-Carver et al. 2007; Mrass et al. 2004). As well, the normal cells were found more sensitive to DOX-induced apoptosis than the cancer cell lines (Bruynzeel et al. 2007). In this context, more than one mechanism can mediate apoptosis, which depends on the provided stimulus of cell death and the studied cell type (Bruynzeel et al. 2007). Moreover, the difference may be attributed to the diverse dose schedule of ATRA, and because we held our comparison with DOX, which is a potent activator for caspase 3 and p53 proteins. Altogether, ATRA pretreatment appears to protect against the diverse DOX-induced cell death pathways, because of its ability to scavenge ROS and then reducing the apoptotic myocardial cell death.

In the present study, the biochemical, oxidative, inflammatory, and immunohistochemical alterations in cardiac tissues of DOX-treated rats were also confirmed by histopathological examination. Toxopathologic analysis of DOX-treated rats revealed severe myocardial damages as reported in various DOX-related cardiotoxicity models (Abdel-Raheem and Abdel-Ghany 2009; Ashour et al. 2011; Sun et al. 2013). DOX caused severe cardiac lesions ranging from degenerative changes (hyalinization, vacuolization, and sarcoplasmic fragmentation) to myocardial necrosis, myocytolysis, intense inflammatory infiltration, interfibrillar edema, and circulatory disorders. Consequently, it appears that oxidative stress evoked by DOX plays an important role in promoting cardiomyocyte apoptosis and necrosis with subsequent cardiotoxicity (Minotti et al. 2004; Zhang et al. 2009) through various signaling pathways including activation of caspase 3 and p53. Interestingly, pretreatment with ATRA extensively ameliorated DOX-induced cardiomyopathy, suggesting antioxidative, anti-inflammatory, and anti-apoptotic potentials against DOX cardiotoxicity. Noteworthy, administration of ATRA shortly before DOX is a regime of choice that is successfully used in the treatment of acute promyelocytic leukemia. It has been shown that

Fig. 4 Representative photomicrograph of p53 immunoreactivity in rat's myocardium following doxorubicin (DOX) and/or all-trans retinoic acid (ATRA) treatment. Cardiomyocytes from **a** control and **b** ATRA-treated rats showed negative immunoreactivity for p53. **c** Cardiomyocytes from DOX-treated rats showed strong positive immunoreactivity for caspase 3 (arrows). **d** Cardiomyocytes from ATRA-treated rats showed mild to moderate immunoreactivity for p53 (arrows). **e** Area percent of p53 immunoexpression/field in control and treated groups; all values are expressed as mean ± SEM, $n = 10$. Mean value were significantly different from control (***$p \leq 0.001$), ATRA (###$p \leq 0.001$), DOX ($\$\$\$p \leq 0.001$), one-way ANOVA with Duncan's post hoc test for multiple comparisons
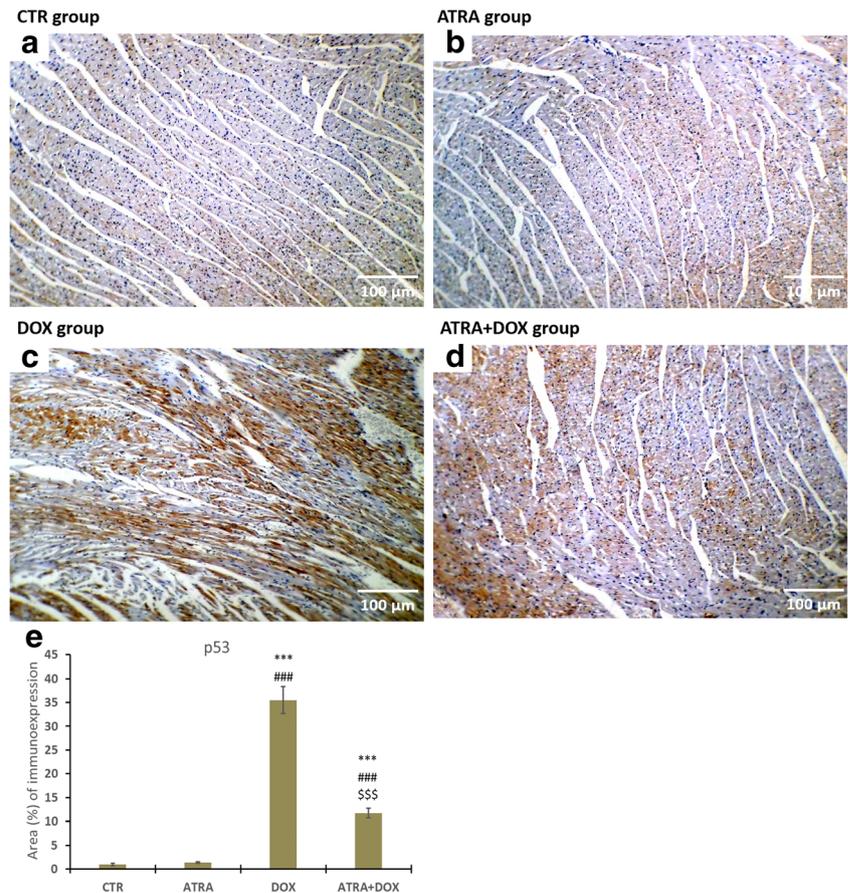
combination of both drugs improved the treatment outcome, and could increase the disease-free survival and overall survival compared to the DOX alone (Tallman et al. 1997). Moreover, recent studies have been concluded that the combined chemotherapy of ATRA and DOX in a single nano-system enhanced the antitumor effect of DOX (Sun et al. 2015; Zhang et al. 2015).

Conclusion

In summary, these results demonstrated that ATRA can be a novel candidate that could be used in combination with DOX as a cardioprotective agent against in vivo DOX-induced cardiomyopathy. The proposed mechanisms may be mediated via diminishing excess release of proinflammatory cytokines, amelioration of cardiac oxidative stress, and suppression of caspase 3 and p53 activation. Further research will be carried out to clarify whether ATRA modulates the in vivo cancer-killing ability of DOX.

Compliance with ethical standards The experimental approach was done in accordance with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and certified by the Committee of the Faculty of Veterinary Medicine, Alexandria University.

Conflict of interest The authors declare that they have no conflict of interest.

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