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Antiapototic Effect of Tauroursodeoxycholic Acid Protects against Acute Doxorubicin Induced Cardiomyopathy in Rats

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Abstract **Background and purpose:** Cardiotoxic carrdiomyopathy mediated by inflammation, oxidative stress lead to apoptosis with subsequently cell damage and heart failure. Tauroursodeoxycholic acid (TUDCA) is a bile acid that have anti-inflammatory and anti-oxidant effect, the current study aimed to examine the effect of administration of TUDCA on doxorubicin induced acute cardiomyopathy and explore the possible mechanism/s. **Methods**: Cardiomyopathy was induced in rats by single intraperitoneal (ip) injection of doxorubicin (DOX) (15 mg/kg). **Results:** doxorubicin administration exerted elevated levels of cereatine kinase-MB (CK-MB) and Lactate Dehydrogenase (LDH) in serum, in addition to myocardial TNF-α content, MDA levels, caspase-3, caspase-9 and caspase-12. However decrease in cardiac SOD and catalase content. Histopathological examination of myoendocardial section showed marked degeneration of cardiac muscle, distention of sarcoplasmic reticulum with marked accumulation of collagen fibers. Decrease immunoreactivity to antitroponin antibody. Echocardiography showed reduced LV fractional shortening, ejection fraction, and cardiac output. Treatment with TUDCA (250 mg/kg, ip) for 10 days significantly ameliorated histological changes and decreased the myocardium peroxidative damage in addition to decrease the cardiac markers for apoptosis. **Conclusion**: TUDCA can protect the heart from cardiotoxic effect of doxurubicin, The protective effects obtained by TUDCA is due to its inhibition of both endoplasmic stress and mitochondrial damage associated with chemotherapy treatment, with antioxidant and antiinflamatory properties. These results confirm the possible use of TUDCA to protect the heart during chemotherapy regimen.

Keywords: *TUDCA, cardiomyopathy, doxorubicin, rats*

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1. Introduction

Cardiotoxic cardiomyopathy is major health problems which threatens patients received chemotherapy for treatment of malignancy. Doxorubicin is an effective chemotherapeutic anticancer drug for various cancers [1,2], but its major adverse effect is cardiomyopathy. Doxorubicin cardiomyopathy has a poor prognosis that can lead to irreversible congestive heart failure [3,4]. The currently available cardiomyopathy treatment does not seem to improve prognosis. Thus preventive treatments have been studied.

Doxorubicin-induced cardiotoxicity by many mechanisms, including production of free radical that cause cardiac cell damage, moreover, induce mitochondrial apoptosis [5,6]. In addition, recent studies show that sarcoplasmic reticulum (SR)-associated functions have important roles in cardiac dysfunction [7,8]. SR stress is associated with cardiomyocyte apoptosis, these suggest that inhibition of SR stress has crucial roles in cardioprotection in model of reperfusion injury [9,10]. So, the search for an effective and safe drug against doxorubicin-induced heart failure is a critical issue in the recent years.

Tauroursodeoxycholic acid (TUDCA) is a bile acid produced in the body in a low concentration. TUDCA is synthesised by conjugation of ursodeoxycholic acid, which used clinically for treating amyotrophic and lateral sclerosis cholestatic liver diseases [11,12,13]. Study on TUDCA proved many beneficial and protective functions, in model of cardiac infarction, as it has been shown an anti-apoptotic effects and enhances cardiac function [14]. Moreover, treatment with TUDCA prevent hyperglycemia and improved insulin sensitivity in the liver, muscle, and adipose tissues and reduced fatty liver in diabetic and obese mice [15]. Another study showed an anti-inflammatory effect of TUDCA in induced poly cystic ovary in rat's model [16]. TUDCA reverse endoplasmic stress and its downstream pathway [17].

Interestingly, some studies identified the presence of G protein-coupled bile acid receptor 1 mRNA in mice and

human cardiomyocytes [18,19], furthermore, certain studies have shown that administration of TUDCA downregulate glycogen synthase kinase-3β and upregulate protein kinase B in mouse cardiomyocyte, and this associated with cardiac hypertrophy [20]. Moreover, in study on cardiomyocytes of neonates, they observed the ability of taurocholate to modify contractility through muscarinic M2 receptor [21].

In light of previous studies demonstrating the protective effect of TUDCA in different tissue, the aim of present study is to examine the possible protective effect of administration of TUDCA on doxorubicin induced acute cardiomyopathy and explore the possible mechanism/s.

2. Materials and Methods

2.1. Experimental Animals

This study was conducted in the scientific and medical research center (ZSMRC) in faculty of medicine, Zagazig University in the period from 1st of July 2017 to 8th of September 2017, this study was performed on 30 adult male albino rats weighing 180–200 g were obtained from the Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were housed in clean cages for 2 weeks for acclimatization in animal house, and kept under controlled temperature (25 ± 2°C) and 12 h light/dark. Rats were allowed free access to water and a standard chow diet. Experimental design and animal handling procedures were performed according to the guidelines of the animal ethics committee of the Faculty of medicine, Zagazig University (IACUC).

2.2. Experimental Design

30 rats were randomly divided into 3 equal groups (n = 10), group I (control group): rats received saline ip for 10 days. Group II: treated with DOX, rats received single i.p injection of doxorubicin hydrochloride (15 mg/kg) [23] (Sigma Aldrich), on the 6th day of the experiment. Group III: DOX + TUDCA treated rats, received a daily dose of_TUDCA (250 mg/kg/day, i.p.) [22] (Sigma Aldrich) for 10 days + DOX (15mg/kg; i.p. in saline) on the 6th day of the study.

2.3. Echocardiographic Measurement

On the last day, and after 12 hrs of fasting, the animals were anesthetized with i.p injection of urethane (1200 mg/kg) [24]. After induction of anesthesia, the chest of each rat was carefully shaved. The rats were positioned in the supine position with spread front legs, and an ultrasound gel was applied to the chest. Transthoracic echocardiography was performed using a GE ultrasonography and 7.5 MHz. transducer. The heart was first imaged in two-dimensional (2-D) mode in the parasternal long axis view. From this view, the M-mode line was put perpendicular to the interventricular septum and passed through the left ventricle (LV) structures, at the level of the chordae tendinea, just below the mitral valve, and M-mode images were obtained [25].

The parameters of left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD) and left ventricular mass (g) were measured. Functions were assessed by the following parameters: cardiac output, left ventricular fractional shortening (LVFS %) which was calculated from the M-mode using the following equation (LVFS %) = [(LVEDD - LVESD)/LVEDD] X 100. And left ventricular ejection fraction (EF %) which was calculated by the echocardiography machine according to the Teicholz formula. Each measurement was obtained by averaging results from three consecutive heart beats.

2.4. Sample Collection and Biochemical Assays

After echocardiographic measurement, the rats were decapatated and blood samples were collected. separatation of serum for Lactate Dehydrogenase (LDH) and cereatine kinase (CK-MB) assays. The heart was then rapidly isolated andcut in to two halves, one was prepared for histopathological examination and immunohistochemistry study, the other half was prepared in ice-cold saline as 10% homogenates for the determination of oxidative stress markers, TNF-α, caspase-3, caspase-9 and caspase-12 content.

2.5. Measurement of Serum Lactate Dehydrogenase and Cereatine Kinase-MB

It was enzymatically assayed using a commercially kits (sigma Aldrich).

Measurement of cardiac lipid peroxide, SOD and catalase:

Maleic dialdehyde (MDA) as indicator of lipid peroxidation, SOD and catalase according to the method described by [26], using a commercially kits (sigma Aldrich)

Measurement of Cardiac TNF-α Content:

Cardiac TNF-α content was assayed using Enzyme ELISA Linked Immunosorbent Assay using a microplate reader (Salzburg, Austria)

Determination of Cardiac caspase3, 9 and caspase 12 content:

Caspase-3 content, cleaved caspase-9 and cleaved caspase-12 were measured using sandwich enzyme-linked immune-sorbent assay technology using a rat ELISA kit (Biotech Co., China) according to previous method [27].

2.6. Histopathological examinations

Histological analysis using haematoxylin & eosin (H&E) stain:

One part of the heart was fixed in 10% buffered formalin, dehydrated in an ascending series of ethanol (70, 80, 96, and 100%) concentration, the samples were embedded in paraffin and cut into 5-µm thick slices. The sections were stained at room temperature with haematoxylin working solution and 1% eosin (H&E) for 2 min respectively [28].

Histological analysis with Mallory's trichrome stain:

The other part of cardiac tissue was stained with Mallory's trichrome stain [29] for identification of collagen fibers under a light microscope (magnification, ×400).

Immunohistochemistry method for Antitroponin I antibody:

Sections from all tissue samples were cut to 4-6 mm and processed for immunohistochemical examination by astreptavidin-biotin-peroxidase complex (ABC) method. Tissue sections were placed on 3-amino-propyltrieyhoxysilanecoated e slides, de waxed, and hydrated. Antigen retrieval (Anti cardiac troponin I antibody) (4c2) (ab10231) abcam UK was facilitated by heating in citrate buffer (pH 6.0) for 10 min in a microwave oven with a power of 800 watts. The slides were then dipped in freshly prepared absolute methanol containing hydrogen peroxide 3% vol/vol for 15 min to quench endogenous peroxidase activity. Tissue sections were treated with goat anti-cTnI (1:100; C-19: sc-8118) for 1 hour. Following washing with phosphate buffered solution, the slides were then incubated with anti-goat immunoglobulin G diluted at 1:300 in PBS at room temperature. For 30 min. then, sections were incubated with ABCg diluted at 1:300 in Tris-buffered solution at room temperature for 30 min. After washing with PBS, the slides were treated for 5 min at room temperature with 3, 39-diaminobenzidine tetrahydrochlorid eg (DAB) in PBS (0.5 mg DAB/ ml) containing hydrogen peroxide 30% vol/vol. At the end, sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted [30].

2.7. Analysis of Immunohistochemistry Study

The percentage of troponin immune reactivity was quantified using the public domain image- processing software "Image J 1.49v/Java 1.6.0_244 (National Institutes of Health, USA). The analyzer of image has been calibrated for measurements before use to automatically convert the image pixels into micrometer units.

2.8. Statistical Analysis

All data were presented as mean ± standard deviation of mean. Statistical analysis was performed by SPSS version 18 software. The intergroup variation was measured by one way analysis of variance (ANOVA) followed by LSD Post hoc test. The minimal level of significance was identified at $p<0.05$.

3. Results

3.1. Echocardiographic Data

Heart rate, LV systolic (LVSD) and diastolic diameters (LVDD) showed significant increase, however, cardiac output (CO), LV ejection fraction (LVEF) and LV fractional shortening (LVFS) were significantly lower in DOX group when compared to control, all these parameters are reversed significantly in DOX group treated with TUDCA, as shown in Table 1.

Effect of TUDCA on necrosis and apoptosis of cardiac tissue:

Cardiac apoptosis was evaluated by serum levels of CK-MB, LDH and cardiac caspase-3 levels, Serum total LDH, CK-MB and caspase-3 levels were increased significantly in DOX-treated rats as compared to control group ($p< 0.001$) (Table 2). On the other hand, TUDCA treatment significantly reduced the serum LDH, CK-MB and caspase-3 levels as compared to DOX group ($p < 0.001$).

Effect of TUDCA on inflammatory response and oxidative stress:

Inflammation was evaluated by measuring TNF-α and oxidative stress markers. The protective myocardial antioxidant activity of CAT, SOD in rats treated with DOX exhibited a significant decline ($p< 0.001$), This was associated by significant increase in myocardial MDA and TNF-α content in DOX treated group when compared with control group ($p< 0.001$). Treatment of DOX group by TUDCA significantly increased the myocardial levels of antioxidant enzymes with significantly decrease in MDA and TNF-α contents compared with their respective DOX-treated rats ($p< 0.001$) as shown in Table 2.

Effect on Sarcoplasmic reticulum (SR) mediated apoptosis:

Determined by the activation of caspase-12 (Table 2), it is one of cysteine protease families that play important roles in regulating pathological cell death. Caspase-12 was significantly elevated in DOX treated group when compared with control group ($p < 0.001$), treatment of DOX group by TUDCA significantly decreased the myocardial levels of caspase-12 ($p < 0.001$).

Table 1. Echocardiographic data in all studied groups: heart rate; LVEF, cardiac output. LVFS, LVSD, LVDD, values are mean ± standard deviation ($\overline{X}$ ± SD)

parameters	Groups (n=10)	Control Group (I)	DOX group (II)	TUDCA + DOX group (III)
HR (bpm)	$\overline{X}$ ± SD	310± 45	330 ± 40	316± 35
	P value of LSD		P <0.001[a]	P<0.001[b]
LVEF %	$\overline{X}$ ± SD	0.90± 0.02	0.85± 0.01	0.88± 0.02
	P value of LSD		P <0.001[a]	P<0.001[b]
CO (ml/min)	$\overline{X}$ ± SD	72.60± 2.43	56.7± 3.02	65.7± 2.9
	P value of LSD		P <0.001[a]	P<0.001[b]
LVFS%	$\overline{X}$ ± SD	0.58± 0.02	0.51± 0.01	0.56± 0.02
	P value of LSD		P <0.001[a]	P <0.001 [b]
LVESD (mm)	$\overline{X}$ ± SD	2.60± 0.15	5.1± 0.13	3.3± 0.33
	P value of LSD		P <0.001[a]	P<0.001[b]
LVEDD (mm)	$\overline{X}$ ± SD	6.50± 0.11	8.9 ± 0.67	6.9± 0.87
	P value of LSD		P <0.001[a]	P<0.001[b]

a= significant compared with group (I) significant (P<0.001); b= significant compared with group (II) (P<0.001).

Table 2. Serum LDH, CK-MB levels and cardiac content of TNF-α, MDA, CAT, SOD, caspase-3, caspase-9 and caspase-12 in all studied groups

Parameters / Groups		Group I	Group II	Group III
Serum LDH (IU/L)	$\bar{X}$ ± SD	780±50.6	1705±60.3	956±52.9
	P value of LSD		P < 0.001[a]	P < 0.001[b]
Serum CK-MB (IU/L)	$\bar{X}$ ± SD	641±25.4	960±29.4	794±23.3
	P value of LSD		P < 0.001[a]	P < 0.001[b]
CAT (u/ g protein)	$\bar{X}$ ± SD	130.33±3.47	49.3± 4.74	101.80± 3.77
	P value of LSD		P < 0.001[a]	P < 0.001[b]
SOD (U/ mg protein)	$\bar{X}$ ± SD	62.58±7.55	44.85±5.27	58.30±7.36
	P value of LSD		P < 0.001[a]	P < 0.001[b]
MDA (nmol/ g protein)	$\bar{X}$ ± SD	5.37± 1.26	10.26±1.93	6.01± 2.01
	P value of LSD		P < 0.001[a]	P < 0.001[b]
TNF-α (pg/mg protein)	$\bar{X}$ ± SD	28.01 ± 9.1	124.38 ± 8.24	60.51 ± 7.35
	P value of LSD		P < 0.001[a]	P < 0.001[b]
Caspase-3 content (ng/g protien)	$\bar{X}$ ± SD	7.6±4.56	50.6±3.26	20.5±4.53
	P value of LSD		P < 0.001[a]	P < 0.001[b]
Cleaved Caspase-9 content (arbitrary od/mg protien)	$\bar{X}$ ± SD	5000 ± 141.2	11000± 161.5	6000± 150.2
	P value of LSD		P < 0.001[a]	P < 0.001[b]
Cleaved Caspase-12 content (arbitrary od/mg protien)	$\bar{X}$ ± SD	21000±221	70000± 321	40000± 231
	P value of LSD		P < 0.001[a]	P < 0.001[b]

a= significant compared with group (I) significant (P<0.001); b= significant compared with group (II) (P<0.001).

Effect on mitochondrial mediated apoptosis:

Caspase-9 is important marker for this process, it was significantly higher in DOX treated group when compared with control group (p< 0.001), treatment of DOX group by TUDCA significantly increased the myocardial levels of caspase-9 when compared with DOX group (p< 0.001) as shown (Table 2).

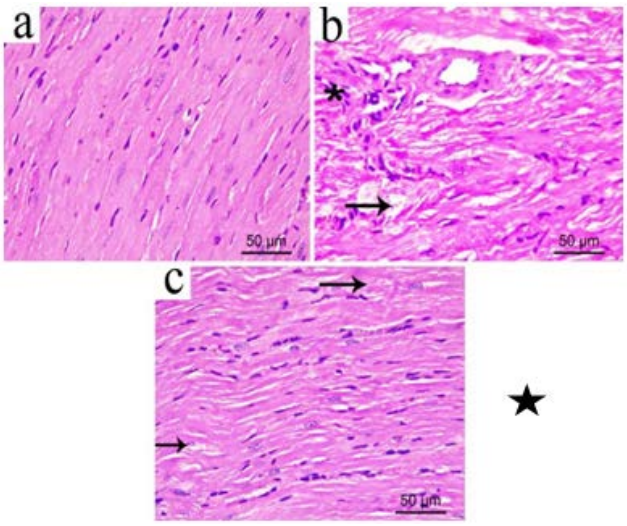

Figure 1. a. Section in myocardium of control rats showing normal muscle fibers with intact myocytes and no extracellular matrix changes. **b** section in myocardium of DOX group showing marked degeneration and myofibrillar disarrangement, vacuolization of the cytoplasm (arrow), Myocyte loss and inflammatory cell infiltration detected (stare). **c.** section in myocardium of DOX + TUDCA group showing mild degeneration of muscle fibers(arrow) (H&E x400)

3.2. Histopathological Study

In control rats, myocardial fibers were arranged regularly with clear striations. No observed degeneration or necrosis with normal intermuscular spaces. The myocardial biopsy in DOX group revealed characteristic diagnostic features of doxorubicin cardiomyopathy. There were loss of myofibrils, vacuolization of the cytoplasm and distention of sarcoplasmic reticulum (SR), (Figure 1 b) when compared to normal myocardium of control group (Figure 1a). On the other hand treatment with TUDCA markedly decreased these effects with mild degenerative changes (Figure 1 c).

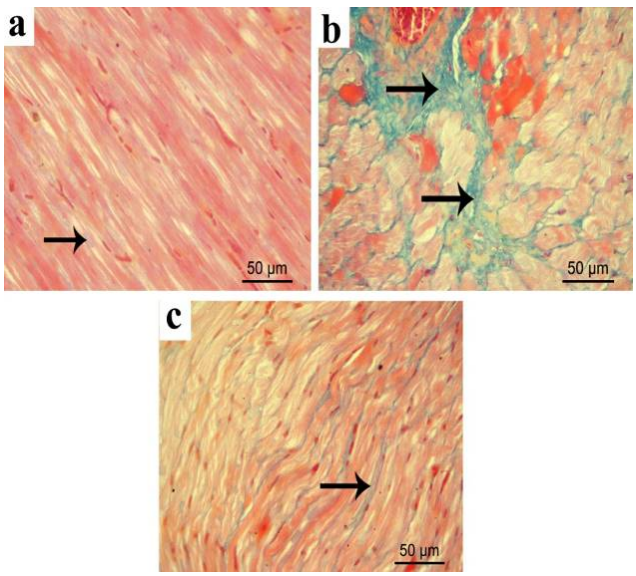

Figure 2. a. Section in myocardium of control rats showing minimal basophilic collagen fibers surrounding the cardiomyocyte bundles (arrow) b. section in myocardium of DOX group showing marked accumulation of collagen fibers (arrow).**c.** section in myocardium of DOX + TUDCA group showing mild accumulation of collagen fibers (arrow) (Mallory trichrome x 400)

Histological examination of Mallory's trichrome-stained rat myocardium sections of control group revealed minimal

basophilic collagen fibers surrounding the cardiomyocyte bundles (Figure 2 a). In contrast, in DOX group; the myocardium exhibited marked accumulation of collagen fibers (Figure 2 b) surrounding the bundles of cardiomyocytes. However, treatment with TUDCA exhibited mild accumulation of collagen fibers (Figure 2c) surrounding the bundles of cardiomyocytes. The results were confirmed by statistical analysis of the % area of collagen fibers in three studied groups (Figure 3).

Immunohistochemical expression in control group showed strong positive antitroponin I antibody (Figure 3 a) immunoreactivity in the sarcoplasm of the cardiomyocytes showing weak positive immunoreactivityin the sarcoplasm of the cardiomyocytes in the DOX group (Figure 3 b). In contrast, the DOX + TUDCA group revealed mild positive immunoreactivity in the sarcoplasm of the cardiomyocytes (Figure 3 c).

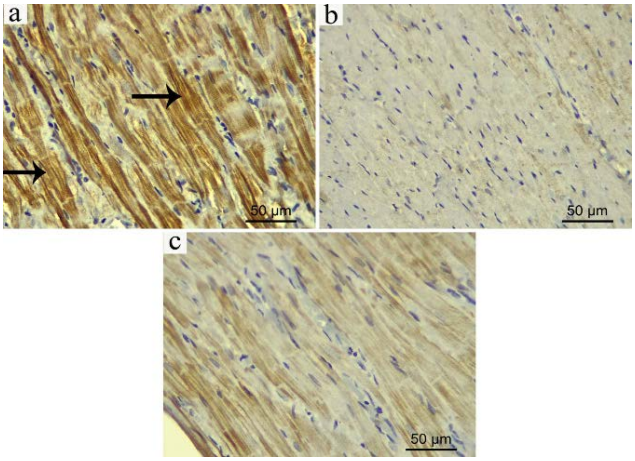

Figure 3. (a) Immunohistochemical reaction for antitroponin antibody in the control rats, it show strong positive immunoreaction in the sarcoplasmic reticulum of cardiomyocytes (arrows). **(b)** Weak positive reaction in the cardiomyocytes sarcoplasm of in DOX group is observed. **(c)** In DOX + TUDCA treated group, mild positive immunoreaction in the sarcoplasm of cardiomyocytes is noticed. (Immunohistochemistry x 400)

Table 3. Percentage area of collagen fibers and immunohistochemistry of antiroponin anitboy in all groups

	Control	Dox	DOX + TUDCA
% area of collagen fibers	7.38 ± 0.71	39.9 ± 0.88	21.87 ± 1.17
% area of antitroponin antibody expression	82.07 ± 0.91	33.76 ± 1.27	63.81 ± 2.03

4. Discussion

This current study is the first to determine the protective properties of TUDCA against cardiotoxic cardiomyopathy. In the current study, the administration of doxorubicin has cytotoxic effect on myocardium with significant necrosis and apoptosis of cardiac muscle confirmed by increase in serum CK-MB and LDH levels in addition, myocardial MDA, TNF-α and caspase 3 content with increase in collagen fibers content compared to control rats, this was associated with significant decrease in the myocardial antioxidant activity as confirmed by the reduction in myocardial CAT and SOD levels. All these changes were associated with myofibril degeneration, cytoplasmic vaculation and leukocytic infiltration. Moreover, increase in left ventricular systolic and diastolic diameters, this accompanied with disturbed cardiac function evidenced by reduced cardiac output, LV fractional shortening and ejection fraction with compensating increase in heart rate. These results are in line with previous studies investigating effect of acute doxorubicin toxicity in mice [31,32,33]. On the other hand, few studies observed no significant change in systolic function two days after doxorubicin treatment [34,35]. These difference in echo results are probably related to differences in doxorubicin dose and duration.

Also, the contractility of myocardium is decreased due to release of troponin which is confirmed by immunohistochemistry analysis which revealed strong positive immunoreactivity in control group compared with DOX group. On the other hand treatment with TUDCA showed increase of antitroponin antibody immunoreactivity. The above result consisted with Bertinchant et al. [36] who stated that among different markers of heart ischemia the troponin showed the greatest capability to detect myocardial damage confirmed by echocardiographic and histologic studies. Also Sabaheta et al. [37] who concluded that the troponine used as a good sensitive marker of myocardial necrosis with decrease cardiac contractility. As confirmed with our echocardiographic result.

In the current study, The high levels of myocardial MDA and TNF-α induced by doxorubicin and the low levels of myocardial SOD and catalase contents indicate a state of myocardial inflammation associated with oxidative stress which is in line with previous studies stated that oxidative stress promotes the transcription of TNF-α [38,39]. Our results confirm a previous results showed increase in cardiac oxidative stress with doxorubicin treatment [20,21,31]. The current results confirm the pivotal role of inflammation and oxidative stress in doxorubicin cardiomyopathy, Moreover, treatment of rats with TUDCA significantly reduced the serum LDH, CK-MB level which are classical biomarkers of cardiotoxicity [4] TUDCA administration significantly reduce MDA and TNF-α levels and significantly increase CAT and SOD levels compared with the DOX group, and this is consistent with the previous results showing that TUDCA exerts anti-oxidant and anti-inflammatory effect [11,12,39].

Multiple mechanisms suggesting the apoptotic effect of Doxorubicin in various malignancies by the generation of oxidative stress, arrest of cell cycle and autophagy [40] we measured cardiac content of Caspase-3 as an index for apoptosis, caspase-9 as index for mitochondrial role of apoptosis and caspase-12 as marker for endoplasmic reticulum pathway for apoptosis. In the current study, caspase-3, caspase- 12 and caspase-9 are elevated in DOX group.

We confirmed the previous data showing that doxorubicin induces apoptosis of cardiomyocytes and increased immuno-reactive caspase-3 expression [41-45]. Moreover, In our study doxorubicin increased both caspase-9 and caspase-12 content in cardiac tissue, in line with our results, yang et al. [46] observed elevated caspase-12 in a model of DOX induced cardiotoxicity using lower dose of doxurubocin (10 mg/kg), but in the

reverse of us they observed no increase in the activation of the mitochondrial pathway of apoptosis, this difference may be that they used lower dose of doxorubicin. This means that even with low pharmacological dose of doxurubicin it affected the SR-mediated pathway of apoptosis via activation of caspase-12, however we suggesting an additional role for mitochondrial pathway in DOX induced cardiomyopathy, Gewirtz [47] observed that the severity of injury in cardiac muscle correlates with mitochondrial free radical production.

Our results confirmed the antiapoptotic role of TUDCA, We found that TUDCA significantly reduced caspase-3, caspase-9 and caspase-12 activity in DOX group pretreated with TUDCA, Caspase 12 is essential for SR-induced apoptosis and important markers of SR stress. Our results are in line with many studies observed that TUDCA significantly decreased endoplasmic stress mediated apoptosis [48-52]. Regarding caspase-9, our results is in line with many studies on models of ischemia in hepatocytes, neurons [38,39,53]. It was previously found that, TUDCA inhibited ER stress and this was associated with inhibition of cytochrome c release and caspase 9 activation in the mitochondria [12,19,54-57].

5. Conclusion

In conclusion, our current study have confirmed the cardiotoxic effect of doxorubicin in rats. In addition a protective role for TUDCA in model of DOX induced cardiomyopathy which was attributed to antagonizing oxidative stress in addition to its direct antiapoptotic effect suggesting that TUDCA might be used as an adjuvant therapy to protect the heart during DOX therapy.

In our model, TUDCA prevent apoptosis by inhibition of ER-stress with its subsequent mitochondrial damage. These mechanisms of TUDCA action suggests important intervention methods for SR-stress induced cardiac diseases.

Declaration of Interest Statement

There is no conflict of interests regarding the publication of this research.

Author contributions

Eman R. Abozaid and Nadine A Raafat conceived the study; they were also responsible for the experimental procedure; statistical analyses; wrote the initial draft. Amal Al-Shahat Ibrahim was responsible for histopathology examination, interpretation and immunohistochemistry examination and analysis. All of the authors shared in interpretation of the data, took part in rewriting the article, revising it critically for intellectual content and have approved the final manuscript.

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