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Effect of caffeic acid on doxorubicin induced cardiotoxicity in rats

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Abstract

Doxorubicin (Dox) is one of the most potent broad-spectrum antitumor anthracycline antibiotics, its use is limited by the development of life-threatening cardiomyopathy. Doxorubicin generates free radicals and induces oxidative stress associated with cellular injury. Further, it has been shown that free radicals are involved in doxorubicin-induced toxicity. The goal of this study is to investigated the cardio-protective effects of caffeic acid on doxorubicin induced cardiotoxicity. The rats were randomized into three equal groups, sham group without treatment, doxorubicin treated group at a dose 3mg/kg IP every other two days and group treated with doxorubicin plus caffeic acid 40mg/day. Two weeks later LV function measurment were performed and blood samples were collected from the heart to measurment plasma levels of cardiac Troponin-I (cTn-I), oxidative stress parameter malondialdehyde (MDA) and high a sensitive c-reactive protein (hs-CRP). The hearts were excised for cardiac tissue cytokines (TNF-α, IL-1β, IL-10) measurement and microscopic examination. Rats in the Dox+caffeic acid group had improved LV function, reduced cytokine expression, decreased myocardial marker injury (cTn-I) and less MDA, hs-CRP levels in comparison with the Dox group. Pathological finding appeared nearly normal in the Dox+caffeic acid without fibrosis. The results of the present study reveal that caffeic acid has a promising cardioprotective effect against doxorubicin-induced cardiotoxicity.

Keywords: Doxorubicin, Cardiotoxicity, Inflammatory response, Left ventricular function,

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Introduction

Doxorubicin (DOX) is one of the most active anthracycline antibiotics that has been used for long time in the therapy of many types of human malignancies [1], [2] either alone or combination with other cytocidal agents [3]. Its clinical uses are limited by seriously high incidence of cardiotoxicity, acute effects can occur immediately after treatment and are characterized by transient arrhythmias, reversible hypotension and pericarditis [4] while, chronic cardiotoxicity can manifest years to decades after treatment. It is irreversible and dose

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dependent cardiotoxicity that is characterized by progressive left ventricular dysfunction and may lead to congestive heart failure [5]. Anthracyclines act by inhibiting DNA and RNA synthesis, blocking topoisomerase II to prevent DNA and RNA transcription and replication, and creating iron-mediated free radicals that damage DNA [6].

The pathogenesis of Doxinduced cardiotoxicity and heart failure is complex and may involve various signaling mechanisms including selective inhibition of cardiac muscle gene expression [7], disturbance of myocardial adrenergic signaling [8], cellular toxicity from metabolites of DOX [9], induction of cardiac cell apoptosis [10], production of reactive nitrogen species [11] and a number of DOX-induced biochemical changes have been identified that can damage cardiac reactive oxygen species [12].

Free radical damage to cardiac myocytes is thought to be the primary mechanism for anthracycline-induced cardiomyopathy [13] and also by induction of immunogenic reactions with the presence of antigen presenting cells in the heart [14]. The heart is particularly weak against the free radicals produced by DOX administration, as it contains less free radical detoxifying substances such as superoxide dismutase, glutathione and catalase than do other metabolic organs such as liver or kidney and its highly oxidative metabolism [15]. Additionally, DOX has a very high affinity by cardiolipin, a phospholipid that is present in mitochondrial membranes of heart, resulting in the accumulation of DOX inside cardiac cell [16].

TNF-□ and IL-1β is a potent pro-inflammatory cytokines, play an important role in the symptoms associated with anthracycline therapy. Bien et al. (2007) [17] showed a strong association between oxidative stress and cardiac inflammatory response including cytokine release after DOX treatment. ROS have been demonstrated to trigger the production and the release of TNFα via up-regulation of nuclear factor-kB (NF-KB) [18]. Thus, these reports indicated the pathophysiological relevance of TNF-α in DOX-induced cardiotoxicity.

Sauter et al. (2011) [19] found that doxorubicin induces increased blood levels of IL-1β when injected into mice. Interleukin IL-10 is an important immunoregulatory cytokine produced by many cell populations. Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes. IL-10 controls inflammatory processes by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, as well as antigen-presenting and costimulatory molecules in monocytes/macrophages, neutrophils, and T cells [20]. The troponins, a protein located in the contractile apparatus of myocytes, that are part of cardiac and skeletal muscle, troponins are sensitive and specific markers for myocardial injury. In patients with acute coronary syndromes the plasma levels of troponin correlate well with short-term, as well as long-term, mortality [21]. Adamcova et al. (2005) [22] demonstrated that cardiac troponins are the serum biomarkers of choice for monitoring potential drug-induced myocardial injury in both clinical and preclinical studies. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. Free radicals generate the lipid

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peroxidation process in an organism. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status in cancerous patients [23].

High-sensitivity C-reactive protein (hsCRP) is widely used to assess patient health [24] is a global inflammatory marker and a critical component of the immune system. Systemic inflammation is associated with increased rates of cardiovascular disease in adults and may also be involved with the mechanisms underlying anthracycline-related cardiotoxicity and pediatric cardiomyopathy [25]. As such, elevations in hsCRP may be a strong indicator of cardiac stress [26].

Caffeic acid (3,4-dihydroxycinnamic acid) is one of the natural phenolic compounds widely distributed in plant materials such as vegetables, fruits, coffee, and tea [27]. This substance as an antioxidant can scavenge a number of reactive species, including 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) peroxyl, and hydroxyl radicals [28] as well as superoxide anion, peroxynitrite, and mutagenic compounds such as nitrosamines [29]. Caffeic acid also inhibits 5-lipoxygenase (5-LOX) activity [30]. Others studies have been also reported to have antitumor activity, anti-inflammatory properties [31] and anti-HIV replication activity [32]. The fact that caffeic acid produced analgesic effect in the nociceptive model is indicative that it had both central and periferic antinociception and the mechanism of action could be partially related to lipoxygenase and/or cyclooxygenase of the arachidonic acid cascade and/or opioid receptors [33].

Materials and Methods

A 21 adult Sprague-Dawly rats were obtained from Animal Resource Center, the National Center for Drug Control and Researches The animals were apparently healthy and they were housed in the animal house of College of Medicine/University of Kufa, at temperature controlled environment (25±2°C) with ambient humidity. Lights were maintained on a 12 h light/dark cycle. The rats received standard chow diet with water. Rats in the study were maintained in accordance with the guidelines established by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Experimental Groups

After 2 weeks of acclimatization period, the animals were randomized into 3 groups (7 rats each) treated for 2 weeks and as following: Sham group; rats received no treatment; cardiotoxic group (DOX) was given DOX IP at adose 3mg/Kg every other 2 days plus normal saline as vehicle orally; Caffeic acid group (CA); DOX plus CA 40mg/Kg/day orally. Doxorubicin HCL vial ("Ebewe" pharma Ges. m.b.H. Nfg. KGA4866 Unterach, AUSTRIA) 50mg/25ml was diluted in saline and given IP at adose 3mg/Kg [34]. Caffeic acid (CA) 3,4dihydroxycinnamic acid was

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purchased from Sigma AldrichCompany (Batchno. 21909058). Saline 0.9% (as asolvent) according to body weight of animal was administered at adose 40mg/kg daily [35].

Preparation of Samples

At the end of the experiment, the body weight was recorded and the animals were anesthetized by ketamine at 80 mg/kg and xylazine at 8 mg/kg IP [36]. The thoracoabdomenal incision was performed and blood sample was collected directly from the heart by heart puncture. At the end of experiment, about 2.5 mls of blood were collected from the heart. The blood sample was placed in a tube containing disodium EDTA (22 mg/ml) as anticoagulant and mixed thoroughly then centrifuged at 3000 rpm for 15 min. Then it was used for determination plasma cTn-I according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Life Diagnostics. USA). The remaining blood was placed in a tube without anticoagulant and left for 30 minutes in room temperature and used to obtain serum via centrifugation at 3000 rpm for 10 minutes and then used for determination of MDA and hsCRP according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science Inc, China and Mybiosource, USA).

Tissue Preparation for TNF-α, IL-1β and IL-10Measurement

The basal side of the heart tissues was rinsed with ice cold saline to remove any red blood cells or clots, then homogenized with a high intensity ultrasonic liquid processor in 1:10 (w/v) phosphate buffered saline that contained 1% Triton X-100 and a protease inhibitor cocktail [37]. The homogenate was centrifuged for 20 min at 4°C. The supernatant was collected for determination of TNF-α, IL-1β and IL-10according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine. USA and RayBio, USA).

Tissue Sampling for Histopathology

The apical side of the heart was fixed in 10% formalin and processed by routine histological methods and embedded in paraffin blocks 5μm- thick horizontal sections were cut and stained with hematoxylin- eosin (H&E) for subsequent histological examination [38]. After fixation, evaluation of scores were performed by an investigator who was blinded to the experimental treatment groups. Scoring system (no abnormalities, mild, moderate, severe) was used to classify the heart tissue changes, myocardial necrosis, according to the severity of the damage [39]. Left ventricular function analysis hemodynamic analysis was planned for all animals by using Transonic® Animal Research Flow meters T106 (single channel) [40].

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Statistical Analysis

Statistical analysis was done by using SPSS (statistical package for social sciences) version 17. The data was expressed as mean± standard deviation (SD) unless otherwise stated. ANOVA was used for comparison in different groups and further exploration for the site of statistical significance between paired combinations of study groups was performed using LSD (least significance difference), Pearson correlation coefficient (r) for correlation between different variables. In all tests, $P<0.05$ was considered to be statistically significant.

Results Effect of Dox Treatment on Inflammatory Parameter

At the end of the experiment, the level of myocardial TNF-α, IL-1β, IL-10, serum hs-CRP significantly ($P<0.05$) increased in cardiotoxic group as compared with sham group. The levels of cardiac TNF-α, IL-1β and IL-10 and serum hs-CRP of CA treated group were significantly ($P<0.05$) lower than that of cardiotoxic group. The values of cardiac TNF-α, IL1β, IL-10 and serum hs-CRP are showed in table 1.

Table 1.
Cardiac TNF-α, IL-1β, IL-10(pg/ml) and hs-CRP(µg/ml) levels of the three experimental groups at the end of the experiment. The data expressed as mean ±SD (n = 7 in each group); $P < 0.05$

Markers	Sham	Cardiotoxic	CA	P value
	mean±SD	mean±SD	mean±SD	
TNF	10.114±1.607	92.457±15.824	20.857±3.602	<0.001
hs-CRP	0.808±0.073	2.634±0.264	0.847±0.151	<0.001
IL-10	121.2±16.861	141.8±14.930	205.1±33.216	<0.001
IL-1B	13.60±5.741	232.414±19.79	38.1429±1.615	<0.001

Table 2.
Plasma level of (cTn-I) (ng/ml) of the three experimental groups at the end of the experiment. The data expressed as mean ±SD (n = 7 in each group); $P<0.05$.

Group	CTnI(ng/ml)
Sham	0.0660±0.0145
DOX	0.4106±0.0929
CA treated	0.1661±0.0493

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Table 3.
Serum level of (MDA) (nmol/L) of the three experimental groups at the end of the experiment. The data expressed as mean ±SD (N = 7 in each group); *P*< 0.05

Group	MDA(nmol/L)
Sham	1.615±0.049
DOX	4.045±0.167
CA treated	2.924±0.140

Table 4.
EF % values of the three experimental groups at the end of the experiment. The data expressed as mean ±SD (N = 7 in each group); *P*< 0.05.

Group	EF%
Sham	60.31±5.594
DOX	26.162±1.938
CA treated	43.045±3.899

Table 5.
LV function of the three experimental groups at the end of the experiment. The data expressed as mean ±SD (n = 7 in each group); *P* < 0.05.

Groups	End-systolic Volume (uL)	End-diastolic Volume(uL)	End-systolic pressure (mmHg)	End-diastolic pressure (mmHg)	Cardiac Output (ml/min)
Sham	13.42 ± 0.25	35.25 ± 0.36	123.8 ± 0.47	28.99 ± 2.03	5.03 ± 0.57
Cardiotoxic (dox)	45.23 ± 0.42	63.77 ± 0.51	66.35 ± 3.40	15.40 ± 3.53	1.51 ± 0.43
CA	21.28 ± 0.60	41.40 ± 0.54	99.22 ± 1.04	19.84 ± 0.28	3.28 ± 0.17

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Effect of DOX treatment on plasma level of cardiac troponin I (cTn-I)

At the end of the experiment; the level plasma of (cTn-I) was significantly increased (P<0.05) in cardiotoxic group as compared with sham group. The plasma level of (cTn-I) of CA treated group was significantly (P<0.05) lower than that of cardiotoxic group. The values of plasma levels of (cTn-I) are showed in table 2.

Effect of DOX treatment on serum level of malondialdehyde (MDA)

At the end of the experiment, the level of serum MDA was significantly (P<0.05) increased in cardiotoxic group as compared with sham group. The serum MDA level in CA treated group was significantly (P<0.05) lower than that of cardiotoxic group. The values of serum levels of (MDA) are showed in table 3.

Effect of DOX treatment on left ventricular ejection fraction EF%

At the end of the experiment the value of EF% significantly (P<0.05) decreased in cardiotoxic group as compared with sham group. While for CA treated groups, there was significant (P<0.05) increase in EF% value as compared with cardiotoxic group but lower than sham group. The changes in EF% value are summarized in table 4.

Effect of Dox treatment on Left ventricular function

At the end of the experiment, the end systolic and diastolic (LVES, LVED) volume significantly (P<0.05) increased in induced untreated group as compared with sham group. while LVES, LVED volume was significantly (P<0.05) decreased in CA group as compared with cardiotoxic one. For cardiotoxic group the end systolic and diastolic pressure value significantly (P< 0.05) decreased at the end of the experiment as compared with sham group but in CA treated group there was significant increase as compared with cardiotoxic group. The cardiac output value (CO) for cardiotoxic group was significantly (P< 0.05) lower than sham group and for CA treated group was significantly (P<0.05) higher than cardiotoxic one, showed in table 5. In histological section, the heart of animals of sham group and cardiotoxic group, there is a morphological structure that corresponds to physiological appearance of the organ. Cardiomyocytes are regular, cylindrical, with no visible signs of degeneration or necrosis. Between cardiomyocytes is endomysium in normal quantities, with no signs of fibrosis, Fig 8.

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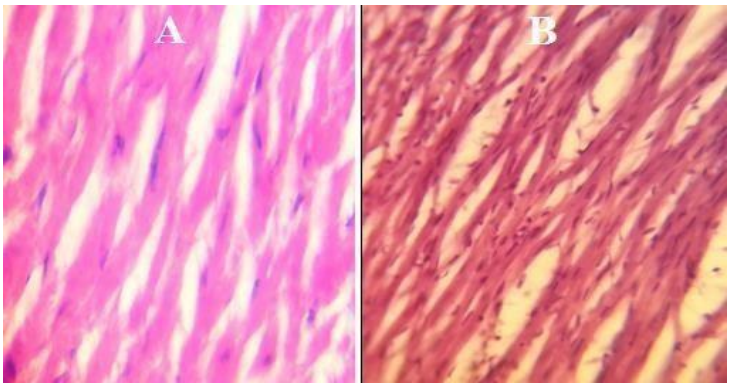

Figure 8.
Photomicrograph represent the histopathological changes in rats.

A. Section of normal rat myocardium shows the normal architecture.

B. Section of DOX treated rat myocardium shows the normal architecture. sections stained with H&E (X 40).

Discussion

Cardiac toxicity is a major dose-limiting factor for application of doxorubicin as a cancer chemotherapeutic agent. Although the precise biochemical mechanisms of Dox cardiotoxicity remain uncertain, it has been demonstrated that the cardiac toxicity associated with Dox administration is mediated, at least in part, by induction of oxidative stress [41] and cardiac cell apoptosis [42]. Redox signals play vital roles in the pathogenesis of acute and chronic stress conditions by mediating the expression patterns of proteins and genes associated with redox balance [43]. Under normal physiological conditions, the tissue concentration of free radicals is limited due to the existence of a delicate balance between the generation of activated oxygen species and the antioxidant defense system [44].

However, if this balance is disturbed either through an enhanced production of free radicals or via a reduction in the endogenous antioxidant defense system or both. Thus, changes in myocardial antioxidant status and oxidative stress may have profound effects on cardiac structure and function [45]. In the present study a significant increase in inflammatory cytokine (TNF-α, IL-1β) level ($P<0.05$) was found in the cardiotoxic rats as compared with sham group. The increase in TNF-α levels is due to ROS generation, which occurs upstream of NF-κB activation and acts as an intracellular signal by changing the redox status of the cell.

The resulting translocation of NF-κB into the nucleus is proposed to be proinflammatory, either directly or indirectly leading to a significant increase in TNF-α production as well as inducing the inflammatory response [46]. Matook et al. 2012 [47], their results revealed that chronic DOX administration induced an elevation in serum levels of the inflammatory mediator, TNF-α. The results in the present study are in agreement with that reported by

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Mukhergee et al. 2003 [48], their results showed that marked TNF-α expression in the subendocardial region, myocardial cells and intramyocardial vessel wall of DOX -treated rat.

IL-1β is an initiator cytokine that plays a central role in the regulation of immune and inflammatory responses [49]. Sauter et al. 2011 [19] results support the idea that proinflammatory responses to anthracycline chemotherapeutic agents are mediated, at least in part, by promoting the processing and release of IL-1β, and that some of the adverse inflammatory consequences that complicate chemotherapy with anthracyclines may be reduced by suppressing the anthracycline-mediated release of IL-1β. Zhu et al. 2010 [50] showed that serum levels of IL-1β were increased in doxorubicin-treated mice relative to their untreated counterparts.

In the present study a significant increase in anti- inflammatory cytokine (IL-10) level ($P<0.05$) was found in the cardiotoxic rats as compared with sham group. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses [19]. That the net effect of the inflammatory response is determined by a delicate balance between pro- and anti-inflammatory cytokines [51].

In the present study a significant increase in inflammatory marker(hs-CRP) level ($P< 0.05$) wasfound in the cardiotoxic rats as compared with sham group. As a global inflammatory marker and a critical component of the immune system, high-sensitivity C-reactive protein (hsCRP) is widely used to assess patient health [24]. Lipshultz et al. 2005 [52] showed that the survivors of childhood acute lymphoblastic leukemia (ALL) had significantly elevated levels of hsCRP when compared to levels in sibling controls. In this experiment, MDA levels were significantly increase ($P<0.05$) in the cardiotoxic rats as compared with sham group. El-Sayed et al. 2011[39] showed that cardiotoxicity induced by doxorubicin was further confirmed by significant increase in each of serum and cardiac malondialdehyde (MDA) level. In the present study, a significant increase in plasma level of cTn-I ($P<0.05$) was found in the doxorubicin treated rats as compared with sham group. Lobna et al. 2000 [53] observed that serial monitoring of serum cTnI could be of value as an early sensitive detector for anthracycline induced acute myocyte injury.

Further, increased serum cTn-I level can be detected in the early stages of anthracycline therapy and it is associated with diastolic dysfunction of the left ventricle [54]. Therefore, serum cTn-I level could be a useful measure for early detection of anthracycline-induced cardiotoxicity. Teng et al.2010 [55] showed significant decrease in LVEF with the administration of doxorubicin. The results in the present study are in agreement with Chen et al.2013 [56] who observed that the values of cardiac output significantly reduced in the DOX-treated rats as compared with the control rats. Chan et al. 2011 [57] confirmed that the acute- and chronic doxorubicin animal models resulted in significant left ventricular functional impairment as indicated by significantly decreased cardiac output, their results are in accordance with our study.

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In our study, a significant loss in body weights and there was an increase in heart/body weight ratio in DOX treated rats (cardiotoxic group) compared with sham group. Rašković et al. 2011 [58] found greatest decrease in body weight in experimental group treated with doxorubicin (Dox), their results are in agreement with our study. Matook et al.2012 [59] demonstrated that DOX treated group showed significant increase in the heart weight and the heart weight ratio when compared to control group.

In the present study, no structural changes demonstrated microscopically in the heart of doxorubicin intoxicated rats as compared with sham group. Tan J 2005 [60] (CAPE) directly blocked calcium-induced cytochrome c release from mitochondria so the levels of inflammatory proteins IL-1beta and TNF-alpha expressed in the area at risk were significantly reduced with (CAPE) treatment. These data demonstrate that (CAPE) has potent cardioprotective effects against I/R injury, which are mediated, at least in part, by the inhibition of inflammatory and cell death responses. Motawi et al. 2010 [61] demonstrated that significant reduction of the elevated levels of serum tumor necrosis factor-alpha (TNF-α) was achieved after (CAPE) pretreatment of lipopolysaccharides induced cardiac stress rats.

The present study shows that the effect of caffeic acid (CA)administration with DOX caused significant lowering ($P<0.05$) in serum MDA level. Kumaran and Prince 2010 [62] revealed that caffeic acid ameliorates cardiac damage in isoproterenol-induced myocardial infarction by maintaining lipid peroxide metabolism due to its free radical scavenging and antioxidant effects. A diet containing caffeic acid may be beneficial to myocardial infarction. Oktar et al.2010 [63] stated that lipid peroxidation is an important index of oxidant injury in isoproterenol- induced necrotic damage of the heart CAPE treatment significantly decreased the MDA levels by preventing formation of lipid peroxides from fatty acids. Fadillioglu et al. 2004 [64] showed that the administration of Dox alone resulted in higher lipid peroxidation, and concluded that CAPE pretreatment significantly attenuated DOX-induced cardiac injury.

Author Contributions

ZA, NA wrote the paper. BM, MY, NH, provided substantial revision. All authors reviewed and approved the final version of the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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