Hesperidin Alleviates Doxorubicin-Induced Cardiotoxicity in Rats

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ABSTRACT

Background: Doxorubicin (DOX) is a potent chemotherapeutic agent used in the treatment of several tumors but its cardiac toxicity prevents its use at a maximum dose, representing an important problem. Increased reactive oxygen species (ROS) and imbalance in nitric oxide (NO) production have been implicated in the cardiotoxicity of doxorubicin. Hesperidin is a citrus bioflavonoid that possesses a potent antioxidant and NO modulating activities.

Objectives: Therefore, the aim of this study was to investigate the possible protective role of hesperidin against doxorubicin-induced cardiac toxicity.

Methods: Four groups of animals were used in this study. First group served as a control and injected with the vehicle. Second group was given 200 mg/kg of hesperidin orally for seven consecutive days. The third group was injected with a single dose (20 mg/kg) of doxorubicin intraperitoneally and was sacrificed after 48 h. The fourth group was treated with hesperidin for seven days but on day five, 1-hour after hesperidin treatment, rats were injected with the single dose of doxorubicin. On day seven, the rats were sacrificed by decapitation. Blood was collected and processed for determination of serum lactate dehydrogenase (LDH), creatine kinase (CK) and NO. The hearts were removed and processed for both histopathological examination and determination of oxidative stress parameters like reduced glutathione (GSH), lipid peroxide (TBARS) levels and superoxide dismutase (SOD) activity.

Results: Our results showed that doxorubicin produced severe cardiotoxicity as indicated from increase in serum LDH, CK activities and NO level. Histopathological examination of DOX-treated rats revealed degenerative changes in heart tissues. The significant decrease in GSH levels, SOD activity and increase in TBARS levels, indicated that DOX-induced cardiotoxicity was mediated through ROS generation. On the other hand, pretreatment of rats with hesperidin protected cardiac tissues against the cardiotoxic effects of doxorubicin as evidenced from amelioration of histopathological changes and normalization of cardiac biochemical parameters.

Conclusion: Hesperidin may have a protective effect against DOX-induced cardiotoxicity.

Key Words: Doxorubicin – Hesperidin – Cardiotoxicity – Nitric oxide – Reactive oxygen species.

INTRODUCTION

Doxorubicin (DOX) is a commonly employed anti-neoplastic agent for treatment of solid and hematologic tumors [1]. Doxorubicin-induced cardiomyopathy may result in progressive heart failure after anti-neoplastic therapy, thus limiting the application of this potent chemotherapeutic agent [2]. In a study with 399 patients, the incidence of congestive heart failure from patients who had received doxorubicin was more than 18% [3]. The molecular basis of doxorubicin-induced cardiotoxicity is attributed to several different molecular events such as generation of reactive oxygen species (ROS) and degradation of doxorubicin to its toxic metabolite doxorubicinol [4,5]. The vulnerability of the heart to ROS is further intensified by doxorubicin inhibition of ROS neutralizing enzymes [6]. Moreover, it was reported that nitric oxide (NO) is a free radical that has been implicated in the etiology of doxorubicin-induced cardiotoxicity [7,8]. Therefore, the use of one compound with a potent antioxidant activity and has the ability to modulate nitric
oxide production may be of value in the protection against doxorubicin-induced cardiotoxicity.

Hesperidin (5,7,3’-trihydroxy-4’-methoxyflavanone7-rhamnoglucoside) [9] is a flavanone glycoside abundantly found in sweet orange and lemon and is an inexpensive by-product of citrus cultivation [10]. It has several biological and pharmacological properties. Various preliminary studies have revealed that hesperidin reduces cholesterol [11] and blood pressure [12] in rats. Large doses of hesperidin decrease bone density loss in mice [13]. It also showed protective effects against sepsis in animals [14]. Hesperidin has anti-inflammatory effects [15]. Hesperidin is also a sedative, possibly acting through opioid or adenosine receptors [16]. A number of researchers have examined and proved the antioxidant activity and radical scavenging properties of hesperidin using a variety of assay systems [17,18]. Another mechanism whereby the citrus flavonoid, hesperidin may influence proliferation and suppress cell apoptosis is through its ability to reduce NO overproduction as a result of its inhibitory effects on the expression and activity of inducible nitric oxide synthase (iNOS) [19].

Accordingly, the aim of this study was to investigate the cardioprotective effects of hesperidin against doxorubicin-induced cardiomyopathy through evaluation of serum cardiac enzymatic parameters such as lactate dehydrogenase (LDH) and creatine kinase (CK) activities as well as serum nitrite/nitrate level (as an indicator of NO production). In addition, oxidative stress biomarkers such as glutathione (GSH), thiobarbituric acid reactive substance (TBARS) level, superoxide dismutase (SOD) activity and histopathological changes were evaluated in cardiac tissues of rats treated with doxorubicin alone or pretreated with hesperidin before doxorubicin.

**MATERIAL AND METHODS**

**Animals:**

Thirty-two adult male Wistar albino rats weighing 180-200 g were selected for this study. The animals were obtained from the Animal House, Faculty of Medicine, Assiut University (Assiut, Egypt), they were fed standard diet and water ad-libitum. During the study, rats were maintained at 12 h light/dark cycle.

**Drugs and chemicals:**

Doxorubicin hydrochloride (adriblastina) was obtained from Pharmacia Italia (Milan, Italy). Hesperidin, vanadium trichloride, thiobarbituric acid, N-1-(napthyl) ethylenediamine dihydrochloride (Griess reagent), Ellman’s reagent (DTNB) (5,5’-dithio-bis-(2-nitrobenzoic acid) and pyrogallol were supplied from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest quality and analytical grade.

**Experimental protocol:**

The animals were divided into 4 groups each of 8 rats: Group 1: Rats in this group (control) were given distilled water orally by intragastric gavage. Group 2: Rats in this group were orally treated with 200 mg/kg of hesperidin for seven consecutive days [20]. Hesperidin was suspended in distilled water for oral administration. Group 3: Rats in this group were treated intraperitoneally with a single dose (20 mg/kg) of doxorubicin and were sacrificed after 48h [21]. This dose of doxorubicin was reported to be cardiotoxic [21]. Group 4: Rats in this group were received hesperidin daily for seven days and on day five, 1-hour after hesperidin treatment; rats were injected intraperitoneally with a single dose (20 mg/kg) of doxorubicin. On day seven, the rats were sacrificed by decapitation; blood samples were collected into tubes and allowed to clot at room temperature. Thereafter, serum was separated by centrifugation at 4000 rpm for 15 min at 4°C for determination of serum lactate dehydrogenase, creatine kinase activities and total nitrite/nitrate level. Hearts were removed, cleaned and washed in ice-cold physiological saline then fixed in 10% buffered formalin solution in the room temperature for histopathological evaluation. Other cardiac tissue samples were homogenized (10% w/v) in 0.15 M KCl at 4°C then centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was used for determination of oxidative stress biomarkers.

**Biochemical analysis:**

**Determination of Serum Lactate Dehydrogenase (LDH) activity:**

Lactate dehydrogenase activity was estimated in serum by commercially available LDH
kit (Linear Chemicals, S.L., Spain) according to the method of Whitaker [22]. Using this method, lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH) at pH 7.5. The reaction is monitored kinetically at 340 nm using an UV-visible spectrophotometer (Shimadzu, Japan) by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD$^+$ which is proportional to the activity of LDH present in the sample.

**Determination of Serum Creatine Kinase (CK) activity:**

Creatine kinase activity was estimated in serum by commercially available CK assay kit (BioAssay Systems, USA) adopting the method of Bishop et al. [23]. This assay is based on the conversion of creatine phosphate and ADP by creatine kinase to creatine and ATP. The ATP and glucose are then converted to ADP and glucose-6-phosphate by hexokinase (HK). Glucose-6-phosphate dehydrogenase (G6PD) then oxidizes glucose-6-phosphate and reduces the nicotinamide adenine dinucleotide (NAD) to NADH. The rate of NADH formation, measured at 340 nm, is therefore, directly proportional to creatine kinase activity.

**Determination of Serum total Nitrite/Nitrate Contents:**

Nitric oxide production was determined in serum by measuring its stable metabolites nitrite (NO$_2^-$) and nitrate (NO$_3^-$) according to the method of Miranda et al. [24] and modified by Motawi et al. [25]. The total concentration of oxidative end-products can be determined by reduction of nitrate to nitrite using vanadium trichloride. Then diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines to form chromophore which can be measured colorimetrically at 540 nm. In detail, 0.5 ml of serum was treated with 50µl 30% ZnSO$_4$ for protein precipitation. Then precipitated protein was removed by centrifugation at 3000 rpm for 15 min. One hundred µl of the resulting supernatant was diluted to 300µl with water and treated with 300µl vanadium trichloride (0.8 g % in 1 M HCl), followed by rapid addition of 150µl sulfanilamide (2% in 5% HCl) followed by 150µl N-1-(naphthyl) ethylenediamine dihydrochloride (0.1%). The mixtures were then incubated at 37°C for 30 min then cooled. The absorbance of the formed pink chromophore was measured at 540 nm.

**Estimation of oxidative stress biomarkers in cardiac tissues:**

Glutathione (GSH) content of cardiac tissues homogenate was determined using Ellman’s reagent according to the method described by Ellman, [26]. Lipid peroxide levels were measured by colorimetric determination of thiobarbituric acid reactive substance (TBARS). It is based on the reaction of one molecule of malondialdehyde with 2 molecules of thiobarbituric acid at low pH (2-3) according to the method of Mihara and Uchiyama, [27]. The enzymatic activity of superoxide dismutase (SOD) was determined by mixing equal volumes of 10% tissue homogenate and Tris–EDTA (100 mM Tris and 0.2 mM EDTA) buffer, pH 7.6, followed by centrifugation at 6000 rpm at 4°C for 15 min. The resultant supernatant is the cytosolic fraction used for the determination of protein content and superoxide dismutase activity according to the method of Marklund, [28] by computing the difference between auto-oxidation of pyrogallol alone and in presence of superoxide dismutase enzyme.

**Histopathological evaluation:**

The heart of each animal was dissected out then fixed in 10% formalin for 24 h and processed for histopathological examination. Four micrometer-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination using conventional protocol [29]. A minimum of 8 fields for each heart section were examined and assigned for severity of changes by an observer blinded to the treatments of the animals.

**Statistical analysis:**

Data analysis was achieved using the GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Results were expressed as means and standard deviation (SD). All groups (n=8/group) passed Kolmogorov-Smirnov normality test using Prism program. Statistical significant difference was determined by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. A
probability value of $p<0.05$ was considered to indicate statistical significance.

**RESULTS**

Effects of hesperidin on doxorubicin-induced alterations in serum lactate dehydrogenase (LDH), creatine kinase (CK) activities and serum nitrite/nitrate concentrations in rats.

Doxorubicin produced severe cardiotoxicity as indicated from the elevation in serum cardiac enzymes biomarkers. Serum lactate dehydrogenase activity (LDH, U/L) was higher in DOX treated rats ($617\pm24.12$) compared to control ($251.3\pm23.36$) ($p=0.0001$) (Fig. 1:A). Similarly, after 48 h of DOX injection, serum creatine kinase (CK, U/L) activity was more pronounced in DOX group ($448.8\pm23.47$) than in untreated control one ($201.3\pm34.81$) (Fig. 1:B). In addition, DOX-treated group had a higher serum nitric oxide expressed as nitrite/nitrate ($60\pm10.4$) ($\mu$M) compared to that of control group ($30\pm5.2$) ($\mu$M) ($p=0.0006$) (Fig. 1:C). On the other hand, the measured serum activities of cardiac enzymes (LDH and CK) were found to be lower in hesperidin pre-administered groups ($302.5\pm24.3$ for LDH and $257.5\pm24.1$ for CK) compared to DOX-treated groups (Figs. 1:A, B). Similarly, rats pretreated with hesperidin before DOX had a lower level of serum nitric oxide ($40\pm9.8$) compared to that of DOX-treated rats ($p=0.002$) (Fig. 1:C). Administration of hesperidin alone had no effect on serum LDH, CK activities and NO levels ($p=0.084$, 0.072 and 0.09 respectively) (Figs. 1:A-C).

**Effects of hesperidin on doxorubicin-induced alterations in oxidative stress biomarkers (glutathione, thiobarbituric acid reactive substance, superoxide dismutase) in cardiac tissues:**

In this study, doxorubicin treated group had a lower ($p=0.0001$) GSH levels ($1.4\pm0.42\mu$mol/g tissue) in cardiac tissues compared to that of control group ($2.4\pm0.39\mu$mol/g tissue). However, administration of hesperidin to rats before DOX maintained the GSH content at a higher level compared to that of DOX-treated rats ($p=0.003$). The DOX-induced decline in GSH content was inhibited and the level of GSH was increased by 64% to $2.5\pm0.312$ in hesperidin pretreated group (Fig. 2:A). Free oxygen radicals can induce lipid peroxidation in cells; the lipid...
peroxide expressed as thiobarbituric acid reactive substance (TBARS) is formed during oxidative degeneration and accepted as an indicator of lipid peroxidation. The level of lipid peroxide, TBARS, was found to be higher in DOX group (6 ± 0.65 µmol/g tissue) compared to control group (3.2 ± 0.42 µmol/g tissue) \( p = 0.0005 \). Hesperidin prevented the DOX-induced elevation in TBARS levels and decreased its elevated levels by 40% to 3.6 ± 0.45 µmol/g tissue (Fig. 2:B). Superoxide dismutase activity (U/mg protein) was found to be lower in cardiac tissues of DOX-treated rats (8 ± 1.97) compared to control rats (16 ± 3.96). However, hesperidin augmented the superoxide dismutase activity and kept it higher (increased by 75% to 14 ± 3.22) in hesperidin pre-administered group compared to DOX-treated group \( p = 0.04 \) (Fig. 2:C). Administration of hesperidin alone did not show any significant effects on glutathione, thiobarbituric acid reactive substance contents or superoxide dismutase activity \( p = 0.187, 0.09 \) & \( 0.087 \) respectively (Fig. 2:A-C).

**Histopathological analysis:**

Sections from control and hesperidin-treated groups showed normal histological structure of the myocardial muscle cells in the myocardial bundles (Fig. 3:A,B) respectively. Doxorubicin produced severe toxicity in the cardiac tissues as seen from the sections of doxorubicin-treated rats. The pericardium of doxorubicin-treated rats showed edema with inflammatory cells infiltration associated with hyalinization in the underlying myocardium (Fig. 3:C). Moreover, severe congestion was observed also in the pericardial blood vessels of the edematous pericardium with focal hemorrhage in the myocardium (Fig. 3:D). Hesperidin pretreatment reversed most of the histopathological alterations induced by doxorubicin as revealed from sections of hesperidin-doxorubicin treated rats. Photomicrographs from hesperidin-doxorubicin group revealed normal myocardial structure with absence of inflammatory cells infiltration and the hyalinization in the underlying myocardium (Fig. 3:E). Hesperidin also alleviated the edema and congestion in the blood vessels that were considered to be severe in the myocardium tissues of doxorubicin group (Fig. 3:E). In addition to its protective effects, hesperidin alone was found to be safe and did not induce any histopathological changes in the cardiac tissues (Fig. 3:B).
DISCUSSION

Doxorubicin is an anthracycline, which is widely used for the treatment of various cancers. The clinical use of doxorubicin is limited by acute and chronic cardiotoxicity, which often leads to progressive heart failure with impaired contractility, arrhythmias, or sudden death [30]. Retrospective analysis of three clinical trials with 630 breast carcinoma and small-cell lung carcinoma patients indicated that at a cumulative dose of 550 mg/m², an estimated 26% of patients experienced DOX-related congestive heart failure [31]. The mechanisms of cardiac toxicity are not fully understood and are thought to include heightened oxidative stress status leading to apoptosis of endothelial cells and cardiomyocytes [32].
In agreement with previous studies and in line with the findings derived from human studies, doxorubicin in our model led to severe cardiomyopathy as indicated from the increase in serum activities of cardiac enzymes such as lactate dehydrogenase and creatine kinase [33, 34]. These enzymes are present in sufficiently high content in myocardial tissue so that the death of a relatively small amount of tissue results in a substantial increase in measured enzyme activity in serum. Recent studies suggest that mitochondria are the target organelle of doxorubicin-induced free radical toxicity in myocytes [35,36]. An important factor, which can mediate the damaging action of DOX in myocardial tissues, especially in mitochondria, is high affinity binding of DOX to cardiolipin, an anionic phospholipid in the inner mitochondrial membrane [36] leading to dissociation of cardiolipin-associated peripheral proteins from the inner mitochondrial membrane, like cytochrome c and mitochondrial creatine kinase resulting in initiation of programmed cell death [37].

Nitric oxide, an unstable free radical produced by the action of the enzyme NO synthase on L-arginine, is a mediator of multiple physiological functions, and may also mediate local inflammation and tissue destruction [38]. Several evidences suggest that NO is a well-established participant in the regulation of vascular tone and may play important roles in cardiac function and disease. While basal production of NO via constitutive nitric oxide synthase (cNOS) modulates cardiomyocytes contractility and blood flow distribution, [39] high levels of NO production via inducible nitric oxide synthase (iNOS) are associated with dilated cardiomyopathy and congestive heart failure [40]. Doxorubicin is able to evoke synthesis of NO and reactive oxygen species such as superoxide radical [41]. In agreement with the pervious finding, an elevation in serum nitric oxide (expressed as nitrite/nitrate) was observed in our study after doxorubicin injection.

There are several hypotheses to explain the mechanisms of doxorubicin-induced cardiotoxicity, oxidative damage to membrane lipid and other cellular components is believed to be a major factor [32]. Different mechanisms of free radical formation by DOX have been postulated. One of these mechanisms implicates the formation of a semiquinone that yields superoxide radicals in the presence of oxygen [42]. Another possible pathway is that DOX free radicals are produced by a non-enzymatic mechanism that involves reaction with iron. Iron-DOX complex can reduce oxygen to superoxide anion and hydrogen peroxide [43]. Hydrogen peroxide subsequently leads to the formation of hydroxyl radicals, which is considered to be most damaging, greatly enhances lipid peroxidation [43]. In our study, increased lipid peroxidation and depletion of tissue non-protein sulphhydryl compounds as well as reduction in superoxide dismutase activity in response to DOX administration all together support an oxidative mechanism of DOX-toxicity. Our finding was consistent with that of Su et al. [44] who reported that doxorubicin administration to rats significantly increased lipid peroxide expressed as TBARS, and decreased both glutathione peroxidase and superoxide dismutase activities in cardiac tissues. Furthermore, in this study, the biochemical alterations induced by doxorubicin in rats were further confirmed by histopathological studies of heart tissues of doxorubicin treated rats which showed edema with inflammatory cells infiltration associated with hyalinization in the underlying myocardium. In addition, severe congestion with focal hemorrhage was seen in the myocardium of doxorubicin-treated group.

As a powerful antioxidant, hesperidin has demonstrated remarkable protection against carcinogens and acts as a scavenger that searches for and engulfs the free radicals that cause the cell damage produced by unstable oxygen molecules [45]. Hesperidin is known to be deglycosylated in the gut to its aglycone hesperetin by intestinal microflora [46]. Hesperitin also is a potent antioxidant and free radicals scavenger [47]. In our study, the prior administration of hesperidin before DOX to rats resulted in significant reduction in the elevated activities of serum cardiac enzymes like lactate dehydrogenase and creatine kinase indicating that hesperidin has a true cardioprotective effect. These results could be in accord with several other researches, which reported that, compounds with antioxidant properties like gamma-glutamylesteine ethyl ester and resveratrol could ameliorate DOX-induced cardiotoxicity [48-49]. Sakata et al. [19] have reported that hesperidin has the ability to reduce the elevated
nitric oxide level in mouse microphage cell line, an effect that could be attributed to inhibition of expression of inducible nitric oxide synthase (iNOS). In this study, the elevated nitric oxide level which is harmful to cardiomyocytes was inhibited by hesperidin.

In addition to improvement of serum cardiac enzymes like LDH and CK, hesperidin also ameliorated the altered oxidative stress biomarkers. Hesperidin markedly increased the reduced glutathione (GSH) levels and augmented the superoxide dismutase (SOD) activity in heart tissues that was attenuated by doxorubicin treatment. On the other hand, hesperidin decreased the elevated lipid peroxide (TBARS) levels in doxorubicin-treated rats suggesting that the cardioprotective effect of hesperidin at least in part due to its antioxidant and free radicals scavenging activity. Our results are matched with that of Tirkey et al. [20] who reported that hesperidin improves the oxidative stress markers (GSH, TBARS, SOD) in carbon tetrachloride treated rats. The amelioration in the biochemical enzymatic cardiac and oxidative stress markers in this study was accompanied by an alleviation of histopathological changes (like absence of inflammatory cells infiltration and mild congestion of blood vessels) induced by doxorubicin in rats.

In conclusion, the present study revealed that the toxicity of doxorubicin on rat hearts is mediated through oxidative stress mechanisms and pretreatment with hesperidin reversed most of these negative effects induced by DOX as evidenced biochemically and histologically.

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