Effect of Grape Seeds Extract in the Modulation of Matrix Metalloproteinase-9 Activity and Oxidative Stress Induced By Doxorubicin in Mice

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Abstract: The therapeutic value of doxorubicin as antitumor agent is limited by its cardiotoxicity. Matrix metalloproteinases activation is an early event in doxorubicin–induced cardiotoxicity. Because Matrix metalloproteinases are up-regulated by increased formation of reactive oxygen species, the present study was designed to tested whether the grape seeds extract could attenuate the increases in matrix metalloproteinase-9 activity and prevent the doxorubicin –induced cardiotoxicity in mice. Mice were dosed with a single injection of doxorubicin (20 mg/kg b.wt , i.p) with or without pretreatment of grape seeds extract. The protective role of grape seeds extract against doxorubicin induced-cardiac damage was evaluated on the aspects of the release of cardiac enzymes into serum, the formation of malondialdehyde, the activation of matrix metalloproteinase-9 and the histopathological changes in heart tissues. The results showed that doxorubicin led to increase in serum metalloproteinase-9 activity, heart injury as shown by increased serum creatine kinase, lactate dehydrogenase, alanin aminotranseferase and aspartat aminotransferase. Oxidative stress was also increased in cardiac tissue as shown by increased malondialdehyde and decrease of antioxidants (superoxide dismutase, catalase and reduced glutathione). This damage was accompanied with histopathological changes in the heart tissue. Pretreatment with GS extract (100mg/kg b.wt daily for 12 days) effectively hindered the adverse effect of doxorubicin and protect against cardiac damage via suppression of oxidative stress.
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Keywords: matrix metalloproteinase-9, reduced glutathione, lipid peroxidation, cardiotoxicity.

Abbreviations: Dox, doxorubicin; ROS, reactive oxygen species; MMPs, matrix metalloproteinases; MMP-9, matrix metalloproteinase-9; TIMPs tissue inhibitors of metalloproteinase; GS, grape seed; LDH, lactate dehydrogenase; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH , reduced glutathione.

1. Introduction

The anthracycline antibiotic doxorubicin [Adriamycin®, (Dox)] is one of the most effective chemotherapeutic agents against a wide variety of cancers, but its clinical use is limited by development of cardiotoxicity, which may ultimately lead to severe and irreversible cardiomyopathy[1]. The cause of Dox cardiotoxicity is multifactorial, even though most Dox- induced cardiac effects can be attributed to the formation of reactive oxygen species (ROS), which ultimately results in myocyte apoptosis (or programmed cell death)[2]. Several studies suggest that ROS play a major role in the activation of matrix metalloproteinases (MMPs). These are an endogenous family of zinc-dependent enzymes that have an important role in vascular dysfunction and tissue remodeling in many cardiovascular conditions as a result of increased oxidative stress[3,4]. MMPs are regulated at multiple levels including transcription, secretion, and activation of inactive zymogens while their activity is under the strict control of specific tissue inhibitors of metalloproteinase (TIMPs)[5]. Dox was found to be induced matrix metalloproteinase-9 (MMP-9) expression and activation in the heart [6]. A great deal of effort has been made to identify which agents might mitigate the cardiotoxic effects of Dox. Because of free radical plays important role in Dox - induce cardiotoxicity, it is logical to consider antioxidants as primary potential therapeutic agent to prevent such toxic effect. In fact, several compounds with antioxidant properties (carvedilol, sesame oil and statin) have been investigated with some degree of success[7-9]. In spite of up regulation of MMPs was reported in animal models of Dox -induced cardiotoxicity[6,10,11], few studies examined whether antioxidants could prevent the increases in MMP-9 level associated with Dox-induced cardiotoxicity[12].

Grape seed extract (GS extract) is a natural extract from the seed of grape [13]. It is a rich source of one of the most beneficial groups of plant flavonoids and pro-anthocyanidins oligomers[14]. It improves hepatic ischemia-reperfusion injury and reduces the size of the infarct in cardiac ischemia in

the rat[15]. GS extract involves in ameliorating the oxidative stress *in vitro* and *in vivo*[16]. The aim of the present study was to investigate the possible potential role of GS extract in the modulation of the increased activity of MMP-9 and oxidative stress induced by Dox in mouse model.

2. Material and Methods

Chemicals

Doxorubicin (Adriablastina) was purchased in a vial contains 10 mg powder from Pharmacia & Upjohn Co. S.P.A, Milan, Italy. GS extract (proanthocyanidins 95%) was obtained from Arab Company for Pharmaceuticals and Medicinal Plants (MEPACO-Egypt), which is patented as Gervital (patent ARE 312034-vrs1). All other chemicals and solvents that required for the biochemical assays were of highest purity and analytical grade and purchased from Sigma–Aldrich Chemic (Deisenhofen, Germany). Reagent Kits for MMP-9 was purchased from Quantikine, RnD Systems Co. (UK). Commercially available reagent kits for assays aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and creatine kinase (CK) were obtained from Stanbio Laboratory (USA). Reagent Kits for determination of malondialdehyde (MDA), reduced glutathione (GSH)), catalase (CAT) and superoxide dismutase (SOD) were purchased from Biodiagnostics (Egypt).

Animals and experimental design:

Forty eight male adult Swiss albino mice weighing (20-25g) were obtained from the Animal House in National Research Centre, Egypt. Animals were housed in plastic cages at an environmentally controlled room (constant temperature 25-27°C, with 12h light / dark cycle) for one week prior to starting the experiments and they were fed on standard feed, water *ad libitum*. All animals received humane care in compliance with the international guiding principles for animal research. The experimental procedure used in this study met the guidelines of the ethics committee of the National Research Centre. The animals were fasted for 16-18 hrs before sacrificing. Doxorubicin was reconstituted in 5ml of dist. water and injected intraperitoneally to animals at a single dose of 20 mg/ kg body weight which was evaluated to cause cardiotoxicity[8,9]. GS extract was dissolved in de-ionized water (5mg/ml) and administered to animals by oral gavages at a dose of 100 mg / kg b.w. The dose of GS extract used was selected on the basis of the previous studies [17].

The animals were randomly assigned into four groups containing twelve mice each one.

Control group: Mice were received 0.5 ml vehicle (distilled water) through oral intubation for 12 days,

GS extract group: Mice were received 0.5 ml (2.5 mg) of GS extract through oral intubation for 12 consecutive days.

Dox group: Mice were received vehicle for 7 days and afterwards treated with a single i.p injection of Dox, then vehicle treatment continued for 5 days.

(GS extract+Dox) group: The mice were administered with 0.5ml (2.5 mg) GS extract orally for 7 days prior to i.p injection of Dox followed by administration 0.5 ml (2.5 mg) of GS extract for other 5 days.

At the end of the experiment, the mice were sacrificed under anesthesia. Blood was collected and serum was separated by centrifugation at 3000 rpm. The serum was used for the determination of AST and ALT activities by colorimetric method[18]. LDH [19] and CK [20] activities were estimated by kinetic procedures. MMP-9 activity was assayed by Immunoassay method[5]. The hearts were dissected out and portions of them were preserved in 10% formalin (pH7.2) and subjected to histopathological examination[21].The remaining parts of the hearts were immediately washed in ice cold physiological saline and homogenized in 100 mM tris- HCl buffer (pH 7.4) to render 10% homogenate. Aliquots of homogenate were used for MDA [22], GSH [23], SOD [24] and CAT [25] estimation.

Statistical Analysis

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by student's "t" test. All analyses were performed using Statistical Package for the Social Sciences software (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were regarded as significant.

3. Results

Serum markers of heart damage: There was a significant ($P<0.001$) increase in the activities of LDH, CK and AST as well as a significant ($P<0.01$) increase for ALT activity in the Dox group as compared with control group. The administration of GS extract along with Dox significantly attenuated elevation of these enzymes (Table 1). There was insignificant difference in the activities of above enzymes between control and GS extract group.

Serum MMP-9 activity: As shown in Table 1, Dox injection produced a significant increase in the serum activity of MMP-9 (3.6000±0.8075 ng/ml; $P<0.001$) as compared with control group (0.8383±0.1585 ng/ml). This effect was significantly ($P<0.001$) modulated by the administration of GS

extract which deceased the activity of MMP-9 (0.7750 ± 0.2034 ng/ml) as compared with Dox group. Treatment with GS extract alone has no effect on MMP-9 activity.

Table (1): Effect of administration of Dox alone and along with GS extract on serum markers of cardiotoxicity and MMP-9 activity

Group	LDH U/L	CK U/L	AST U/ml	ALT U/ml	MMP-9 ng/ml
Control group	155.85 ± 35.55	112.71± 15.08	28.83 ±6.59	27.67 ±7.17	0.8383±0.1585
GS extract group	175.56 ± 16.66	119.16 ± 16.77	32.75 ±8.48	31.33 ±5.32	0.7367±0.1946
Dox group	538.09± 140.54[ac]	409.65± 124.76[ac]	90.67±15.36[ac]	43.83 ±9.84[ab]	3.6000±0.8075[ac]
(GS extract+Dox) group	222.29± 41.77[c]	172.56 ± 38.44[b]	44.50 ±8.78[c]	37.67 ±7.39	0.7750 ± 0.2034[ac]

Values are expressed as mean ± SD (n=12). P values: a < 0.05, b< 0.01, c < 0.001 vs Dox and aa <0.05, ab < 0.01, ac < 0.001 vs control (one way ANOVA).

Heart oxidative stress and antioxidant activity:

The concentrations of MDA (a marker of lipid peroxidation) & GSH (a non-enzymatic antioxidant) and the activities of SOD & CAT (enzymatic antioxidants) were shown in Table 2. GS extract administration alone did not alter the previous measurements. There was a significant (P<0.001) increase of MDA concentration (139.57±18.85 nmol/g) in the Dox-treated group as compared with control (73.67±9.22 nmol/g). Treatment with GS extract along with Dox significantly (P<0.001) reduced the MDA level (83.51±11.29 nmol/g) as compared to Dox group. The activity of SOD which was observed to be lower in the Dox group (354.24±106.14 U/g tissue ; P<0.05) as compared with control (615.71± 281.04 U/g) , was also attenuated by GS extract (636.99±136.85 U/g; P<0.001). Administration of Dox also produced a significant decrease in the activity of CAT (0.52±0.14 U/g; P<0.01) as compared to that of control group (1.98 ± 0.73 U/g). Treatment with GS extract significantly attenuated the CAT activity (0.99 ± 0.38; P<0.01) as compared to Dox group. The GSH level showed marked reduction in the Dox group (5.29±1.31 mg/g; P<0.001) as compared to control group (10.64±1.54 mg/g). Similarly, treatment with GS extract reversed this effect (9.06±0.87 mg/g; P< 0.001).

Table (2): Effect of administration of Dox alone and along with GS extract on biomarkers of oxidative stress

Group	MAD nmol/g tissue	SOD U/g tissue	CAT U/g tissue	GSH mg/g tissue
Control group	73.67 ±9.22	615.71± 281.04	1.98 ±0.73	10.64 ±1.54
GS extract group	72.23 ±7.23	726.23 ± 134.62	1.67 ±0.90	10.76 ±1.33
DOX group	139.57 ±18.85[ac]	354.24 ± 106.14[aa]	0.52 ±0.14[ab]	5.29 ±1.31[ac]
(GS extract + DOX) group	83.51±11.29[c]	636.99 ± 136.85[c]	0.99 ±0.38[b]	9.06 ±0.87[c]

Values are expressed as mean ± SD (n=12). P values: a < 0.05, b< 0.01, c < 0.001 vs Dox and aa <0.05, ab < 0.01, ac < 0.001 vs control (one way ANOVA).

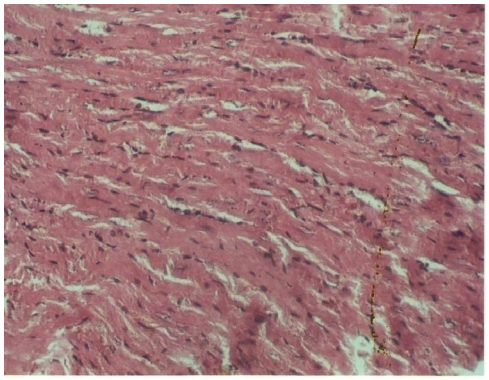

Fig (1): The cardiac muscle of control mouse revealing longitudinally sectioned cardiac muscle branching. The centrally located nucleus is surrounded by clear area. (H&E, X20).

Histopathological effects of Dox:

Fig.1 shows the light micrograph of cardiac muscle of control mice revealing longitudinally sectioned cardiac muscle branching. The centrally located nucleus is surrounded by clear area. Light micrograph of heart of mice treated with GS extract alone showed normal structure (Fig.2). Degenerations of the myofibrils, vacuolated cytoplasm , separation and focal hemorrhage in-between cardiac muscle fibers were clearly seen in the Dox treated group (Fig.3).Animals treated with GS extract along with Dox showed better-preserved appearance of cardiac muscle fibers with slight pyknosis of nucleus of cardiac muscle fibers(Fig.4).

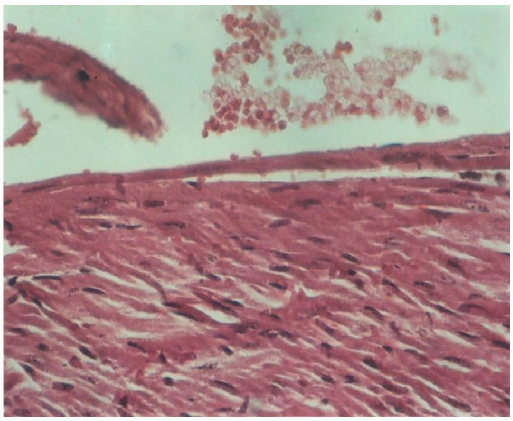

Fig (2): The cardiac muscle of a mouse after treatment with GS extract alone revealing normal structure (H&E, X40).

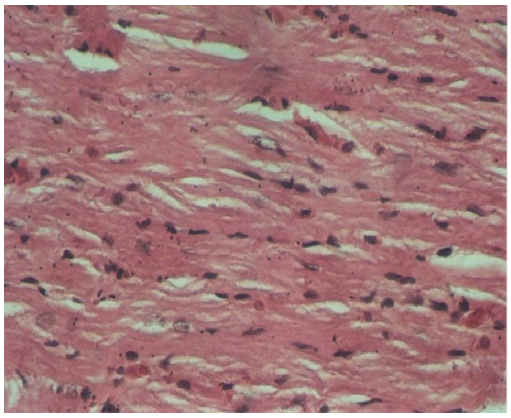

Fig (3): The cardiac muscle of a mouse after treatment with Dox showing degenerations of the myofibrils, vacuolated cytoplasm, separation and focal hemorrhage in-between cardiac muscle fibers (H&E, X40)

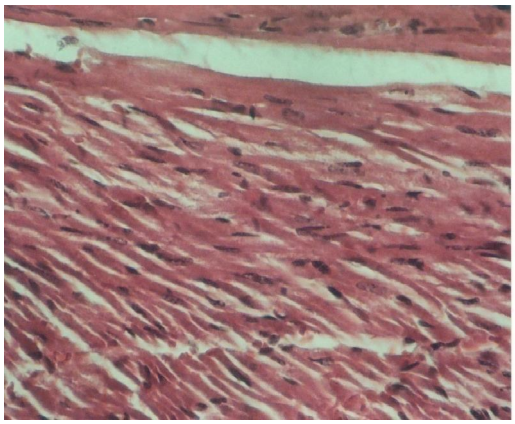

Fig (4): The cardiac muscle of a mouse after treatment with GS extract along with Dox revealing better-preserved appearance of cardiac muscle fibers with slight pyknosis of nucleus of cardiac muscle fibers((H&E, X40).

4. Discussion

The clinical use of Dox is marred by its unwanted side effects that include cardiomyopathy and congestive heart failure[1]. Many studies indicated that oxidative stress from Dox disposition is significantly correlated with the Dox -induced cardiotoxicity[8,9,26].

The present study demonstrates that GS extract administered along with Dox is an effective scavenger of toxic free radicals and inhibitor of lipid peroxidation. Oxidative stress is increased after Dox treatment due to overproduction of ROS and decreased efficiency of endogenous antioxidant defenses in heart, liver, kidney and brain [27]. In this study, intraperitoneal administration of Dox at a single dose of (20 mg/kg b.w.) induced cardiovascular changes by increase in free radical production as indicated by significant increase in LDH, CK, AST, ALT and MDA. These results are consistent with earlier studies [26,28]. In our study, the increase in serum CK and LDH might indicate the leakage of these enzymes through the membranes. The activities of serum CK and LDH have been widely used as parameters for the diagnosis of cardiac dysfunction and the increase of CK level in serum and in myocytes culture media as a result of possible cell damage is occurring in concert with the decrease of CK activity in the cardiac tissue [29].

The present study also confirmed that treatment with Dox significantly increased the MDA level (a marker of lipid peroxidation and an indicator of oxidative injury) and decreased the SOD and CAT activities and GSH level in cardiac tissues. These results are in accordance with the findings from other animal models treated with Dox[26,28]. The observed decrease in GSH level in our study group treated with Dox could be possibly due to its conversion to oxidized glutathione or due to decreased synthesis under oxidative stress. Comporti[30] reported that , the depletion of GSH resulted in enhanced lipid peroxidation, and excessive lipid peroxidation caused increased GSH consumption.

In the present work, the observed depletion in GSH levels and decreased activities of SOD and CAT can be correlated with significant increment in cardiac lipid peroxidation in Dox-treated mice and indicate higher susceptibility of myocardium to oxidative damage. SOD protects cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which gets further metabolized by CAT to molecular oxygen and water [31]. Lipid peroxidation which has been linked with altered membrane structure and inactivation of SOD and CAT resulting in accumulation of superoxide anion, which further damages the myocardium. Therefore, according to the present data, it seems that increased

CK and LDH in serum paralleled the inhibition of SOD and CAT activities and deceased GSH level and production of MDA in heart of Dox treated mice. Thus, the present findings might confirm that the heart damage resulting in leakage and increase in CK and LDH levels in serum as a consequence of cellular oxidative injury induced by Dox mediated ROS production.

The present study demonstrated that the levels of AST, ALT, CK and LDH were greatly attenuated in serum of mice receiving GS extract for twelve days during Dox treatment. This is accompanied by a marked protection against lipid peroxidation as well as amelioration of the inhibition of SOD and CAT activities and GSH level in heart. Furthermore, light microscopy of all the mice tissue sections treated with GS extract showed a well-preserved normal morphology of cardiac muscle. Such amelioration effect might be due to that the grape seeds are rich sources of monomeric phenolic compounds such as catechin, epicatechin, dimeric, trimeric and tetrameric proanthocynidins[32] which confers on them an antioxidant property and their protection against cardiac cell apoptosis via the induction of endogenous antioxidant enzymes which may be an important mechanism underlying the protective effects of GS extract observed with various forms of cardiovascular disorders[33]. Moreover, GS extract inhibits enzyme systems that are responsible for the production of free radicals [34].

The increase in serum MMP-9 activity in mice treated with Dox in this work are in accordance with the findings of Bai *et al*.,[6] and Li *et al*.,[12] who reported that MMPs activation is an early event in Dox-induced cardiotoxicity . Spallarossa *et al*.,[10] showed that low doses of Dox, which had previously been found to induce apoptotic, and not necrotic cell death in cardiomyocytes, also induce early transcription and activation of MMP-9 in these cells without changing the transcript levels of their inhibitors, TIMP-1 and TIMP-2. However ,when MMPs activity is increased with insufficient quenching activity by TIMPs, progressive disruption of the collagen network occurs, thus leading to progressive dilation and remodeling of the left ventricle[35]. Interestingly, the present study, revealed that GS extract reduced the activity of serum MMP-9 that induced by Dox treatment, thus offering a mechanistic insight into protective effect associated with antioxidant therapy against the alterations induced by Dox.

Conclusion

Our study suggests that GS extract produce cardioprotective effects against Dox- induced cardiotoxicity, which may be through re-modulation of MMP-9 and reducing oxidative stress, but further studies are needed to evaluate the possible effect of GS extract on TIMPs to confirm weather GS extract is possible as an early inhibitory therapy before myocardial injury induced by Dox treatment.

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