Doxorubicin-induced cardiotoxicity in mice; protection by silymarin

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Abstract

Background: despite its vast utility in clinical oncology, the use of doxorubicin is limited by a potentially fatal cardiomyopathy and congestive heart failure. Free radical formation and antioxidants depletion are mechanisms proposed for this cardiomyopathy. The aim of this study is to compare the potential antioxidative protective effect of silymarin on doxorubicin-induced cardiotoxicity in experimental mice.

Materials and methods: four groups (ten animals in each group) of experimental mice were used as follows: Group 1, mice received only saline (intraperitoneally) and served as a negative control group; Group 2, mice received doxorubicin (intraperitoneally, 5 mg/kg body weight) in three equal injections over a period of two weeks for a cumulative dose of 15 mg/kg body weight; Group 3, mice orally administrated silymarin (200 mg/day/kg body weight) respectively, through an intragastric feeding tube over a period of three weeks; Group 4, mice treated orally with silymarin plus intraperitoneally doxorubicin administration with the same protocol of groups 3 and 4. Serum lactate dehydrogenase (LDH), creatine phosphokinase (CPK), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), malondialdehyde (MDA), total nitric oxide (NO), cardiac reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were measured in all tested groups.

Results: doxorubicin elevated the activities of LDH, CPK, AST, ALT, MDA and NO in the cardiac tissue. Cardiac antioxidant enzymes activities SOD and CAT also increased while GPx activity was decreased. Pre-co-treatment with silymarin prevented the changes induced by doxorubicin administration. These findings demonstrate the cardio-protective effect of silymarin on cardiac antioxidant status during doxorubicin induced cardiac damage in mice.

Conclusion: silymarin could be recommended for further investigation as potentially new indication for clinical application.

Keywords: doxorubicin, cardiotoxicity, silymarin, antioxidant enzymes, oxidative stress.

Introduction

Doxorubicin (DOX), also called adriamycin is a potent antibiotic, widely used for the treatment of different solid and hematopoietic cancers. However, in addition to its anti-tumoricidal activity, it promotes several well-known side effects that include chronic and irreversible cardiotoxicity **(Asmis *et al.*, 2005; Patil *et al.*, 2008)**. DOX-induced cardiotoxicity had been explained by many mechanisms, including the affinity of DOX to lipids, calcium alterations and membrane depolarization, disorder of membranes, free radical production, injury due to its metabolite (doxorubicinol), and disturbances in iron metabolism **(Cummings *et al.*, 1991; Forrest *et al.*, 2000; Kwok and Richardson, 2002).** Free radical generation is potentially involved in the cytotoxicity of DOX, both in terms of antitumor effects and cardiotoxicity **(Gewirtz, 1999).** DOX can generate reactive oxygen species (ROS) either by forming semiquinone radical which participates in the inactivation of mitochondrial enzymes or by redox cycling with non-mitochondrial flavoenzymes such as NADPH-cytochrome P450 reductase, NADH-Cytochrome b reductase and nitric oxide synthases, and generates superoxide radicals **(Brunmark and Cadenas, 1989; Garner *et al.*, 1999; Muraoka and Miura, 2003**). The oxy-radicals cause damage to mitochondrial and other cytoplasmic organelle membrane structures through peroxidation of phospholipids, proteins and nucleotides **(Muraoka and Miura, 2003).**

On trying to prevent or attenuate the side effects of doxorubicin administration, several strategies have been followed as dosage optimization, synthesis and use of analogues or combined therapy like antioxidants. the combination of the drug delivery together with an antioxidant in order to reduce the toxic effects of doxorubicin by decreasing the oxidative stress without interference with its antitumor properties (**Singal *et al.*, 2000)**. Biological compounds with antioxidant properties may contribute to

the protection of cells and tissues against deleterious effects of ROS and other free radicals induced by ADR **(Deepa and Varalakshmi, 2003**). Flavonoids are naturally occurring substances in plants that possess various pharmacological actions and therapeutic applications which could be attributed due to their phenolic structures **(Toklu *et al.*, 2007)**.

Natural antioxidant (silymarin) is obtained from seeds of *Silybum marianum* (Family: *Composite*), have been used for centuries to treat liver, spleen and gallbladder disorders **(Rainone, 2005).** It is widely used in as an antioxidant flavonoid complex of: silibinin (its main, active component), isosilibinin, silydianin and silychristin **(Crocenzi and Roma, 2006)**, it possesses a powerful free radical scavenging properties **(de Groot and Raven, 1998; Kren and Walterova, 2005**).

The aims of this study were to evaluate the antioxidant potentiality of silymarin against acute cardiac toxicity induced in male albino mice by low-dose of DOX. Biochemical (myocardial marker enzymes, cardiac antioxidant enzymes, cardiac lipid peroxides and cardiac nitric oxide in mice) parameters will be assayed to evaluate the protective effect of silymarin.

Material and methods

Chemicals

Adricin® (doxorubicin hydrochloride) vials were purchased from EIMC united pharmaceuticals, Egypt.

Legalon® (Silymarin 140 mg) capsules were purchased from MADAUS Co., Germany.

Animals

Adult male albino mice, weighing about 22-25g, were purchased from Theodor Bilharz Research Institute, Ministry of Scientific Research and maintained at the animal house of Zoology Department-Faculty of Science (Damiette)-Mansoura University. The mice were housed at 23 ± 2°C and in daily dark/light cycle. They were maintained under standard condition and fed standard chow and water *ad libitum*.

Experimental design

Mice were divided into four groups of six animals in each group as follows: **Group 1**, Control; **Group 2**, Doxorubicin administered; **Group 3**, silymarin administered; **Group 4**, silymarin treated plus doxorubicin administered. Drug administration was as follows: silymarin were given orally (200 mg/kg body weight) through an intragastric feeding tube over a period of three weeks, one week prior to the doxorubicin administration and two weeks along with doxorubicin administered. Doxorubicin was given intraperitoneally (5 mg/kg bw) in three equal injections over a period of two weeks (4 days intervals) for a cumulative dose of 15 mg/kg body weight **(Siveski-Iliskovic *et al.*, 1994, 1995)**. Mice were sacrificed after 4 days of the last DOX injection. Blood samples were collected in clean, dry centrifuge tubes without anticoagulant and allowed to precipitate at room temperature for 30 minutes. Sera were then obtained by centrifugation for 10 minutes at 4000 rpm. These samples were kept preserved at -20°C until assayed.

Hearts were quickly excised, removed, washed in normal saline solution to remove excess blood and 30 mg of heart tissue was weighed and washed with normal saline, then it was homogenized in ice-cold a phosphate (0.05M - KCl 1.15% buffer, pH 7.40) **(Homsy et al. , 1995)** for 30 seconds twice, then the homogenate was diluted to yield a 5% (w/v) heart homogenate, after complete homogenization the homogenate was centrifuged at 13.000 r.p.m for 35 minutes at 4°C in a cooling centrifuge , the supernatant was then removed and stored on ice for immediate assay.

Biochemical parameters

LDH activity was assayed according to **(Weishaar *et al.*, 1975)**, CPK activity was assayed according to **(Horder *et al.*, 1989)**, ASAT and ALAT activities were assayed according to **(Reitman and Frankel, 1957)** using Diamond diagnostics kit method (Diamond diagnostics company, Egypt).

GPx activity was determined according to **(Paglia and Valentine, 1967)**, SOD activity was assayed by the method of **(Nishikimi *et al.*, 1972)**, CAT activity was assayed by according to **(Aebi, 1984 and Fossati *et al.*, 1980)**, MDA level was evaluated by using the method of **(Satoh, 1978)**, NO level was assayed according to the method of **(Montgomery and Dymock, 1961)**.

Statistical analysis of the data

Comparisons among different groups were performed by one way analysis of variance (ANOVA). It is a parametric statistical analysis that compares between-and within-groups variance to measure differences between two or more groups. All the grouped data were statistically evaluated with **SPSS**

software (version 17.0). P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as Mean ± S.D. for six animals in each group.

Results

Cardiac marker enzymes

Data represented in **Table (1)** shows the activities of marker enzymes such as LDH, CPK, AST, and ALT in the serum of control and experimental groups of mice. Marked significant elevations ($p < 0.05$) in the activities of these enzymes were observed in doxorubicin intoxicated mice when compared to the control group. Activities of these enzymes in serum significantly ($p < 0.05$) restored to near normal levels in mice pre-co-treated with silymarin.

Cardiac antioxidant enzymes

Data represented in **Table (2)** shows the activities of antioxidant enzymes SOD, GPx, and CAT, in the heart homogenate of control and experimental groups of mice treated with silymarin. Marked significant increase ($p < 0.05$) in the activities of antioxidant enzymes SOD and CAT along with non significant decrease in GPx were observed in doxorubicin intoxicated mice when compared to the control group. Pre-co-treatment with silymarin significantly prevented ($p < 0.05$) these alterations when compared to doxorubicin intoxicated mice; CAT was an exception, showed non-significant decrease in silymarin + DOX group in comparison with DOX intoxicated mice. Mice administered with silymarin extract alone did not show any changes when compared to the control mice.

Cardiac lipid peroxides (LPO; MDA)

Data represented in **Table (3)** shows the
level of lipid peroxides (LPO) in the heart homogenate of control and experimental groups of mice. Marked maximum induction of LPO was noticed in doxorubicin intoxicated mice when compared to control mice. The altered metabolic changes were significantly ($p < 0.05$) restored to near normal levels in the mice treated with silymarin consider as Pre-co-treatment. Mice administered with silymarin alone did not show any changes when compared to group 1, control mice.

Cardiac nitric oxide (NO)

Data represented in **Table (3)** shows the level of nitric oxide (NO) in the heart homogenate of control and experimental groups of mice. Marked maximum induction of NO was noticed in doxorubicin intoxicated mice when compared to control mice. The altered metabolic changes insignificantly ($p < 0.05$) restored to near normal levels in the mice treated with silymarin consider as Pre-co-treatment. Mice administered with silymarin alone did not show any changes when compared to control mice.

Discussion

Biological compounds with antioxidant properties may contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species (ROS) and other free radicals induced by DOX **(Prahalathan *et al.*, 2005).** Many antioxidants have been assayed with very different results. These includes vitamins as vitamin E **(Wahab *et al.*, 2000)**, vitamin C **(Kurbacher *et al.*, 1996)**, metal ion chelators like transferrins, low molecular-mass agents as bilirubin, sex hormones, melatonin, flavonoids, antioxidant components of virgin olive oil, and selenium…, etc. **(Quiles *et al.*, 2002).**

Herbal antioxidants are important for man, because of their high pharmacological potency. A great interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activity of polyphenolic compounds **(Diplock *et al.*, 1998)**. Due to their radical-scavenging and iron-chelating properties; flavonoids, they can be considered as potential protectors against chronic cardiotoxicity caused by doxorubicin **(Quiles *et al.*, 2002)**. So, the main objective of the present study was carried out to investigate the cardioprotective effect of natural antioxidant silymarin on cardiotoxicity induced by doxorubicin in male albino mice.

In relation to DOX-induced cardiotoxicity, increased activity of serum LDH, CPK, ASAT and ALAT is a well-known diagnostic marker of myocardial function and cardiotoxicity induced by doxorubicin was also manifested by increasing of these enzymes as well. The rapid cell swelling of sub-sarcolemmal bulbs and injured myocardium could facilitate the loss of intracellular enzymes. This could be the possible reason for the increased serum LDH and CPK activities in ADR administered mice. Also, ALAT and ASAT elevation suggests that doxorubicin may induce generalized toxicity in mice **(Monnet and Orton, 1999)**. In the present study, marked elevation in the activities of these enzymes in the serum of doxorubicin-intoxicated mice was observed. Pre-co-treatment with silymarin resulted in significant ($p < 0.05$) reduction in the levels of these enzymes towards near normal as compared with cardiomyopathy-induced mice.

The primary cause of DOX-induced cardiotoxicity proved by most experimental studies have been pointed to oxidative stress which is believed to be secondary to the generation of oxygen-derived free radicals; the protection of cell death by administration of antioxidants support this hypothesis **(L'Ecuyer *et al.*, 2004; Doroshow *et al.*, 1980).**

Yin *et al.* (1998) demonstrated that DOX increased the levels of mRNAs for Cu, Zn-SOD, catalase and GPx. However, only catalase activity was increased. In the present study, the decreased activity of GPx was observed in DOX intoxicated mice, which may be due to exhaustion in combating the oxidative stress **(Elberry *et al.*, 2010)**. Pre-co-treatment with silymarin shows prevention ($p < 0.05$) against GPx activity alteration in when compared with cardiomyopathy-induced mice.

Adachi *et al.* (1983) have reported the activities of SOD and CAT in the heart of mice were increased significantly by the i.p. administration of 15 mg/kg of DOX which agreed with our results, the elevation of SOD activity may be one of the mechanisms adopted by DOX treated mice to overcome oxidative stress exerted by the cardiac tissue under DOX treatment **(Reddy *et al.*, 2007)**, While the elevation of CAT activity could be due to enhanced free radical generation especially H_2O_2. This is in agreement with findings of the present study where doxorubicin intoxicated mice showed increased activities of SOD and CAT indicating the attempt to detoxify the oxygen free radicals induced by doxorubicin

The mice pre-co-treated with silymarin showed significantly ($p < 0.05$) normalization in SOD activity which suggest that the extract may have ability to prevent the deleterious effects induced by free radicals. On the other hand, pre-co-treatment with silymarin could not prevent ($p > 0.05$) the alteration of CAT activity when compared with cardiomyopathy-induced mice.

DOX can form a semiquinone free radical by a one-electron reduction that yields superoxide radicals ($O^{2\bullet-}$) through redox cycling of this semiquinone. Also, DOX can produce free radicals by a non-enzymatic mechanism that involves reactions with iron **(Halliwell and Gutteridge, 2007)**, which can in turn lead to the induction of lipid peroxidation. Increased levels of oxygen species due to doxorubicin have been detected by an increase in tissue MDA formation, which is a breakdown product of lipid peroxidation **(Minotti, 1990)**. Significant elevation in the level of LPO after doxorubicin administration was observed in the present study. The mice administered silymarin orally plus doxorubicin showed a significant ($p < 0.05$) decrease lipid peroxidation status when compared with doxorubicin intoxicated mice. This could be due to lipid peroxidative activity that help protecting the myocardium from lipid peroxidation and decrease the production of oxygen species and reduce concomitant tissue damage especially myocardium tissue.

Myocardial mitochondrion is a pivotal source of superoxide generation after DOX exposure and peroxynitrite ($ONOO^-$) through diffusion-limited reaction of iNOS-derived NO and superoxide is a major trigger/mediator of DOX-induced apoptotic cell death, which is a key component of DOX-induced cardiotoxicity **(Mukhopadhyay *et al.*, 2009)**. In agreement with this hypothesis, evidence is available demonstrating a significant contribution of increased RNS/ROS production and protein nitration in the progression of cardiovascular disease **(Turko and Murad, 2002)**. Significant elevation in the level of NO after doxorubicin administration was observed in the present study; these results were in agreement with **Guerra *et al.* (2005) and Reddy *et al.* (2007)**. The mice administered silymarin orally plus doxorubicin showed a significant decrease in NO status when compared to doxorubicin intoxicated mice.

The results of this work demonstrate that using of chemotherapeutic drugs such as anthracycline antibiotic doxorubicin (doxorubicin) in the treatment of a variety of human cancers leads to significant oxidative and nitrosative damage and a compromised antioxidant status as shown by increasing in LPO, NO, along with alterations in the activity of the key antioxidant enzymes like CAT, SOD and GPx . Also, it has affected the activity of myocardial enzyme markers such as cardiac enzymes (LDH, CPK and GOT) and liver enzyme (GPT) by increasing their levels in the serum. Oral administration of silymarin exerts a significant protective role against the oxidative stress in mice heart following the toxicity caused by doxorubicin.

Conclusion

In conclusion, the data of the present study show that silymarin may be a particularly useful agent as it could enhance myocardial antioxidants when compared to silymarin as a standard commercially available antioxidant, it significantly prevent the heart from doxorubicin induced oxidative stress,

inhibition of lipid peroxidation, all of which result in the recuperation of the biological parameters and the integrity of the tissue especially heart tissue. Therefore, it could offer a useful support to the therapy by acting as a cardioprotective agent and thus prevents the extent of cardiac damage during treatment of cancer.**Tables**

Table (1): The mean serum activities of lactate dehydrogenase (LDH), Creatine phosphokinase (CPK) in Units/L, Glutamate oxaloacetate transaminase GOT (ASAT) and Glutamic – Pyruvic Transaminase GPT (ALAT) in Units/ml of the four different groups, each group of 10 mice.

Parameter	**Group**
LDH (U/L)	1416.60 ± 154.90
CPK (U/L)	340.60 ± 21.38
ALAT(U/ml)	25.034 ± 3.65
ASAT (U/ml)	61.637 ± 3.50
Group 2: injected with Doxorubicin.	
	2282.00 ± 127.96[a*]
	1087.80±193.07 [a*]
	52.255 ± 3.16 [a*]
	103.09 ± 13.96 [a*]
Group 3: treated with silymarin.	
	1177.60 ± 147.95[a*]
	340.40 ± 22.34
	15.94 ± 1.92[a*]
	59.42 ± 4.15
Group 4: DOX injected mice and treated with silymarin.	
	1574.20 ± 200.52[b*]
	607.20 ± 33.84[b*]
	37.626 ± 1.89 [b***]
	93.85 ± 4.24[b*]

[*] Significant ($p<0.05$), where: [a] significance vs. control group; [b] significance vs. DOX group.
All data are expressed as mean ± S.D.

Table (2): the mean activities of cardiac catalase (CAT) and Glutathione peroxidase (GPx) in homogenate of the four different groups, each group of 10 mice.

Group

Parameter

	CAT (U/mg protein)	GPx (mU/mg protein)	SOD (U/mg protein)
Group 1(Control): treated with saline.	0.119 ± .025	9.81 ± 1.96	70.32 ± 12.12
Group 2: injected with Doxorubicin.	0.1867 ± 0.01 [a*]	7.95 ± 1.91	99.97 ± 5.65 [a*]
Group 3: treated with silymarin.	0.131 ± 0.019[b*]	22.228 ± 1.66	75.48± 5.16[b*]
Group 4: DOX injected mice and treated with silymarin.	0.1846 ± .062	13.10 ± 2.0 [b*]	90.34 ± 1.63[b*]

[*] Significant ($p<0.05$), where: [a] significance vs. control group; [b] significance vs. DOX group.
All data are expressed as mean ± S.D.

Table (3): The mean levels of Malondialdehyde in (µmol / mg protein) and Nitric oxide in (µmol / mg protein) in the heart homogenate of the four different groups, each group of 10 mice.

Group

Parameter

	MDA (µmol/mg protein)

NO (µmol/mg protein)

Group 1(Control): treated with saline.

0.896 ± 0.117
6.258 ± 1.842

Group 2: injected with Doxorubicin.

1.22 ± 0.099 [a***]
30.462 ± 13.17 [a***]

Group 3: treated with silymarin.

0.687 ± 0.016[a***]
6.23±2.5

Group 4: DOX injected mice and treated with silymarin.

0.835 ± 0.099[b***]
11.12±4.927[b***]

[*] Significant ($p<0.05$), [**] very significant ($p<0.01$) and [***] extremely significant ($p<0.001$).
Where: [a] significance vs. control group; [b] significance vs. DOX group.

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مية القلب المستحدثة من عقار الدوكسوروبيسين في الفئران :الوقاية باستخدام السيلامارين

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الملخص

يعتبر عقار الأدرياميسين)دوكسوروبيسين(، مضاد حيوى من عائلة الأنثراسيكلين، علاج طبى كيميائى ذو فاعلية كبيرة جداً فى معالجة العديد من الأورام السرطانية التى تصيب الإنسان، ولكن نظراً للآثار الجانبية التي يسببها والتي من أهمها السمية للحادة للقلب ، فإن استخدامه أصبح بشكل محدود ومقنن .تعد الشقوق الحرة وزيادة معدل الأكسدة، ونقص مضادات أكسدة القلب، من أهم الفروض التى تلعب دوراً هاماً والمسؤوله عن حدوث قصور فى وظائف القلب وموت القلب الإحتقانى كنتيجة لاستعمال عقار الأدرياميسين فى العلاج بشكل متكرر.

لهذا تم تصميم هذه الدراسة بهدف الكشف عن دوربعض مضادات الأكسدة الطبيعية مثل مستخلص السيلامارين (silymarin) من نبات الخرفيش (*Silybum marinum*) وإنزيمات مضادات (LDH, CPK, GOT, GPT) على إنزيمات خاصة بالقلب ونسبة أكسيد النيتريك فى القلب (LPO; MDA)، ومستوى الأكسدة (SOD, GPX, CAT)، الأكسدة الخاصة بالقلب (NO).

وقد تم حقن فئران بالغة ذكرية من نوع البينو بعقار الأدرياميسين بتركيز 5مجم/ كجم من وزن الجسم (والذى أعطى عن طريق الحقن فى تجويف البطن ثلاث مرات لمدة إسبوعين .وقد أوضحت نتائج البحث إرتفاع معدل الضرر الناتج من الأكسدة والتأثير الضار على مضادات الأكسدة ولوحظ ذلك من إرتفاع مستوى تأكسد الدهون (LPO; MDA)، وإرتفاع مستوى أكسيد النيتريك(NO)، ونقص في انزيم (SOD, CAT) وتغيرات في نشاط إنزيمات مضادات الأكسدة كزيادة (GPx).

وأيضاً زيادة فى مستويات إنزيمات القلب أوالكبد (LDH, CPK, GOT, GPT) إعتمدت فى مصل الدم وذلك فى المجموعة التى الفئران فيها على عقار الأدرياميسين .ومن خلال الدراسة وجد أيضاً ان إعطاء السيلامارين مع عقار الأدرياميسين، أظهر تأثيرً وقائياً ضد التأثير الضار لعقار الأدرياميسين حيث أعاد التغيرات التى ظهرت فى النتائج البيوكيميائية لمجموعة الأدرياميسين الى القيمة الطبيعية للمجموعة الضابطة (Control).

بناءاً على ذلك توصى الدراسة بإستخدام السيلامارين كعامل وقائى ضد الإصابة بتسمم القلب الإحتقانى الناتج من إستخدام عقار الأدرياميسين لما لة من تأثير إيجابى على المستوى الوظيفى البيوكميائى للقلب ووقايتة من الأثار الضارة لعقار الأدرياميسين والذى يستخدم بصورة كبيرة فى علاج كثير من الأورام السرطانية التى تصيب الإنسان.