Increased cardiac endothelin-1 and nitric oxide in adriamycin-induced acute cardiotoxicity: Protective effect of Ginkgo biloba extract

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Cardiotoxicity and congestive heart failure are the major factors that limit the use of anti-neoplastic drug adriamycin (ADR). There is increasing experimental evidence that endothelin-1 (ET-1) and nitric oxide (NO) are vasoactive mediators that regulate cardiac performance. The present study was undertaken to investigate the role of ET-1 and NO in ADR-induced acute cardiotoxicity and to evaluate the protective effect of Ginkgo biloba extract (EGb761) in rats. A single dose of ADR (20 mg/kg i.p.) caused a significant increase in the cardiac enzyme activities of aspartate transaminases (AST), lactate dehydrogenase (LDH) and creatine phosphokinase isoenzyme (CK-MB) in the serum of animals. This was accompanied by significant increase in cardiac malondialdehyde (MDA), total antioxidant capacity (TAC), tumor necrosis factor-alpha (TNF-α), ET-1 and nitrite/nitrate (NOx) levels. On the other hand, reduced glutathione (GSH) was significantly depressed. Histopathological examination of heart tissues showed hyalinization of the myocardium, with interstitial edema and inflammatory exudates. Pre-treatment of the animals with EGb761 (100 mg/kg, orally) 10 days before and 5 days after ADR treatment reversed the cardiac enzyme levels to normal value, decreased cardiac MDA, TAC, TNF-α, ET-1 and NOx, increased GSH and reversed the histopathological damage induced by ADR. In conclusion, the cardioprotective effects of EGb761 on markers of ADR-induced acute cardiotoxicity appeared to have been mediated by the regulation of inflammatory and vasoactive mediators, as well as the inhibition of membrane lipid peroxidation. Thus, EGb761 may find use as promising adjuvant therapy to ameliorate cardiotoxicity of ADR.

Keywords: Adriamycin, Endothelin-1, Ginkgo biloba extract, Cardiotoxicity, Nitric oxide, Tumor necrosis factor-α.

Adriamycin (ADR) is an anthracycline antibiotic that has been used for a long time in therapy of an array of human malignancies, either alone or combined with other cytotoxic agents. The clinical usefulness of ADR, however, has been hampered by its detrimental cardiotoxicity. Several mechanisms have been postulated to account for the effects of ADR, both in terms of anticancer potential and cardiotoxicity. These proposed mechanisms include: inhibition of the DNA synthesis, formation of free radicals and superoxides (O₂⁻) which can damage cells by lipid peroxidation, modification of mitochondrial membrane function, interaction with intracellular calcium homeostasis and the involvement of the C-13 hydroxy anthracycline metabolites, the primary circulating metabolite of ADR, which is considerably more potent than the parent compound as myocardial depressant and as inhibitor of ATPase of sarcoplasmic reticulum, mitochondria, and sarcolemma. Other studies have shown the implication of nitric oxide (NO) and endothelin-1 (ET-1) in ADR-induced toxicity in cardiac tissues.

NO is a volatile diatomic free radical that plays physiological roles in normal as well as tumor tissues. It has been reported to contribute to ADR’s antitumor effect and NO donors could increase the effectiveness of anti-neoplastic agents and inhibit the development of drug resistance. Furthermore, both increased oxidative stress and dysregulation of NO have been implicated in the cardiotoxicity of ADR. On the other hand, ET-1 is a 21-amino acid vasoconstrictive peptide produced by many types of cells, including vascular endothelial and smooth muscle cells, as well as cardiomyocytes. ET-1 is the main representative of the endothelin family and is known to be a multi-functional peptide. In the heart, ET-1 contributes to myocardial contractility,
chronotropy, arrhythmogenesis and cardiac remodeling after congestive heart failure. Circulating ET-1 and big ET-1 levels are increased in heart failure and are prognostic indicators of survival in patients with heart failure. Moreover, it is shown that cardiac-specific over-expression of ET-1 is combined with inflammatory characteristics, such as increased tumor necrosis factor-α (TNF-α) content and interstitial inflammatory infiltrates, leading to cardiotoxicity.

Standardized *Ginkgo biloba* extract (EGb761) derived from leaves and nuts of *Ginkgo* tree is a valuable therapeutic drug for a variety of disorders. A number of studies have provided evidence for a role of EGb761 in the regulation of production of NO and ET-1. Since the ET-1 and NO appear to be more important in ADR-induced cardiotoxicity, we postulate that EGb761 can exert a protective effect on cardiac injury induced by ADR through the regulation of production of NO and ET-1. Thus, the present study has been undertaken to verify the above hypothesis and to elucidate EGb761 protective mechanisms related to cardiotoxicity induced by ADR. To achieve this, some biochemical anomalies that have been proposed to reflect the mechanism of development of ADR-induced cardiotoxicity have been investigated in rats. These include lipid peroxidation, measured as malondialdehyde (MDA) and the antioxidant system of the cell represented by evaluating reduced glutathione (GSH) and total antioxidant capacity (TAC). Increased NO synthesis is measured by determining its main metabolites content nitrite and nitrate. In addition, the cardiac content of inflammatory mediators TNF-α and ET-1 has been assessed. The activities of serum aspartate transaminases (AST), lactate dehydrogenase (LDH) and creatine phosphokinase isoenzyme (CK-MB) as markers of myocardial damage have also been measured.

**Materials and Methods**

**Chemicals**

Adriamycin supplied as ampoules (Adriablastina) was purchased from Pharmacia (Milan, Italy). EGb761 commercially known as Tanakan® was purchased from Amriya for Pharmaceutical industries (Alexandria, Egypt). The extract consisted of two groups of major substances — flavonoids compounds and terpenoids (24% heterosides Ginkgo and 6% Ginkgolides-bilobalide). Reduced GSH, N-1-(naphthyl)-ethylene diamine, 5,5-dithiobis-2-nitrobenzoic acid, 2-thiobarbituric acid and vanadium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the finest analytical grade.

**Animals**

Male Wistar albino rats (200 – 250 g) were obtained from the animal house of Faculty of Medicine, Cairo University, Cairo, Egypt. They were housed in the animal facility of Faculty of Pharmacy, Cairo University, Cairo, Egypt. Rats were kept under standard conditions of 25 ± 2°C and 12-h light/dark cycle each day. They were fed standard chow diet and provided with water ad libitum along the period of the study. The study was approved by the Ethical Committee for Animal Experimentation at the Faculty of Pharmacy, Cairo University.

**Experimental design**

Animals were randomly divided into normal control (NC) group (n = 8) receiving appropriate volume of saline i.p. and ADR-induced toxicity group (n = 16) receiving a single dose of ADR (20 mg/kg i.p.) dissolved in saline. The ADR-treated animals were then divided into two groups (n = 8, each), one kept without further treatment and termed ADR-group and the other received EGb761 (100 mg/kg, orally) administered 10 days before and 5 days after ADR injection. This group was termed ADR + EGb761 group. Five days following the ADR injection, animals were sacrificed, blood was collected and serum was separated for the estimation of AST, LDH and CK-MB activities.

For determination of biochemical parameters in cardiac tissue, hearts were quickly excised, washed with cold saline, blotted dry with filter paper and weighed. Portions of the hearts were taken for histopathology and the remaining were homogenized for 1 min in ice-cold deionized water using an ice-cold Teflon homogenizer (Potter Elvehjem type). Homogenization was carried out as 20% w/v. An aliquot of each homogenate was mixed with an ice-cold medium appropriate to the parameter being measured.

**Determination of cardiac enzymes in serum**

The level of serum AST was determined using commercially available kits (Quimica Clinica Aplicada, Spain), while LDH and CK-MB were
determined using Stanbio kits (San Antonio, TX, USA). Briefly, the AST was determined spectrophotometrically at 505 nm in presence of α-ketoglutarate, aspartate, 2,4-dinitrophenylhydrazine and NaOH. LDH activity was measured by monitoring the rate of increase in absorbance at 340 nm for 3 min as a result of NAD reduction into NADH. The CK-MB assay employed a coupled enzyme assay, which ultimately monitors the formation of NADPH spectrophotometrically at 340 nm.

**Determination of cardiac biochemical parameters**

**GSH**
A portion of homogenate was mixed with an ice-cold 7.5% sulfosalicylic acid (1:1), centrifuged at 600 × g at 4°C for 10 min using a Dupont Sorvall Combiplus ultracentrifuge. The GSH content was measured according to Beutler et al. based on the reaction of GSH with 5,5-dithiobis-2-nitrobenzoic acid, forming a product that has a maximal absorbance at 412 nm. The results were expressed as mg/g tissue.

**MDA**
Another portion of homogenate was mixed with an ice-cold 2.3% KCl (1:1), centrifuged at 600 × g at 4°C for 15 min and the MDA level was measured as described previously. Briefly, MDA reacts with thiobarbituric acid in acid medium giving a pink-colored complex that can be measured spectrophotometrically at 520 and 535 nm using 1,1,3,3-tetramethoxypropane as standard. MDA level was expressed as nmol/mg protein.

**TAC**
Another portion of homogenate was mixed with an ice-cold phosphate buffer centrifuged at 800 × g at 4°C for 15 min and the level of TAC was measured according to Koracevic et al. This method depends upon the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H$_2$O$_2$) causing its decomposition. The residual H$_2$O$_2$ is determined by an enzymatic reaction which involves the conversion of 3,5-dichloro-2-hydroxybenzensulphonate to a colored product measured colorimetrically at 505 nm. TAC level was expressed as µmol/mg protein.

**NOx (NO$_3^-$/NO$_2^-$)**
They were determined by the method of Braman and Hendrix in the supernatant, resulting from centrifugation of homogenate at 21,000 × g at 4°C for 10 min. The assay is based on the reduction of NO$_3^-$ into NO$_2^-$ using vanadium chloride then diazotization of sulfanilic acid with the total NO$_2^-$ in the medium with subsequent coupling with N-(1-naphthyl)-ethylenediamine. The azo dye produced was measured colorimetrically at 540 nm. NOx level was expressed as nmol/mg protein.

**TNF-α and ET-1**
A portion of the heart was weighed (~50 mg) and homogenized in 0.8 ml lysis buffer (25 mM HEPES, 5 mM MgCl$_2$, 1 mM EGTA, 0.5% Triton X100, 5 mM DTT, 1 mM pefablock), pH 7.4. The lysate was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was taken for estimation of TNF-α and ET-1 levels. TNF-α and ET-1 were measured by solid phase sandwich ELISA using two kinds of highly specific antibodies. Tetramethyl benzidine was used as chromogen. The strength of color measured at 450 nm was proportional to the quantities of rat TNF-α and ET-1 that were expressed as pg/mg protein.

**Protein**
The protein content of different fractions resulting from ultracentrifugation of heart homogenate was determined by the method of Lowry et al. using bovine serum albumin as standard.

**Histopathological examination**
Heart specimens were fixed in 10% formaldehyde. They were subsequently embedded in paraffin and sliced into slices of 4 µm thickness, followed by staining with hematoxylin and eosin (H & E) and examination under light microscope.

**Statistical analysis**
Statistical analysis was performed by GraphPad InStat software. Means and standard error of means were calculated and statistical significance was tested by one-way ANOVA. The strength of association between pairs of variables was assessed by Pearson correlation coefficient. The level of significance was set at P<0.05.

**Results**
**Toxicological effects of ADR in normal rats**
ADR cardiotoxicity was established by a significant increase in serum AST, LDH and CK-MB by 30, 91 and 106% respectively, as compared to the
normal group (Fig. 1). Increased oxidative stress was evident by the significant increase in cardiac MDA, reaching about 1.6-fold the basal level accompanied by a marked elevation in TAC levels (23%) and a reduction in GSH (35%) as compared to normal group (Fig. 2). These alterations were associated with an elevation in the levels of NOx, TNF-α and ET-1 that reached 114, 318 and 108%, respectively, as compared with the basal levels (Fig. 3). These results were confirmed by histological examination, showing focal hyalinization in the myocardium in heart of ADR-treated rats (Fig. 4B), focal inflammatory cells in the pericardium, as well as edema in both pericardium and underlying myocardium (Fig. 4C) as compared to the control group (Fig. 4A).

Modulatory effects of EGb761 in ADR-treated rats

EGb761 treatment caused significant lowering in the elevated serum cardiac enzymes to reach the normal level (Fig. 1). Significant reduction in cardiac MDA was demonstrated by EGb761 treatment, accompanied with apparent reduction in TAC content by 12% of the corresponding untreated group. Moreover, EGb761 also increased the depressed GSH content (Fig. 2). EGb761 treatment also resulted in significant reduction in the levels of NOx, TNF-α and ET-1 by 37, 65 and 35% respectively, as compared to ADR group (Fig. 3). The protective effect of EGb761 on ADR-induced cardiac tissue damage was confirmed by the histological examination (Fig. 4D), as EGb761 ameliorated all the pathological changes caused by ADR.
The linear regression analysis levels showed a significant positive correlation between cardiac TNF-α and serum cardiac enzyme AST, LDH and CK-MB ($R^2 = 0.420, 0.285, 0.256$, respectively), as well as between cardiac TNF-α and cardiac MDA ($R^2 = 0.731$). A significant negative correlation between cardiac TNF-α and cardiac GSH was observed ($R^2 = -0.339$) (Fig. 5A). Moreover, significant positive correlations were observed between ET-1 and NO, MDA, as well as TNF-α levels ($R^2 = 0.340, 0.345, 0.363$, respectively) and significant negative correlation was observed between ET-1 and GSH levels in cardiac tissue ($R^2 = -0.460$) (Fig. 5B).

**Discussion**

In the present study, 20 mg/kg of ADR induced acute cardiotoxicity, manifested biochemically by a significant increase in serum AST, LDH and CK-MB 5 days later. These results were consistent with previous studies supporting ADR-induced cardiotoxicity in normal rats. Increased cardiac enzymes could be due to an increase in their release, following ADR-induced damage of cardiac membranes. ADR administration resulted in a disturbance of the oxidative state of cardiac tissue, as revealed by sharp increase in MDA content and TAC capacity along with a marked depression of GSH. The cytotoxic activity of ADR was partly related to its quinone structure. The drug is converted to a
semiquinone free radical by NADPH-cytochrome P-450 that subsequently interacts with molecular oxygen and initiates a cascade of reaction, producing reactive oxygen species (ROS). These in turn might have stimulated the activity of antioxidant enzymes, as shown in increase of TAC in cardiac tissue in our results. Additionally, ROS can react with polyunsaturated phospholipids that ultimately lead to the formation of MDA. The overproduction of free radicals can be detoxified by the endogenous antioxidants causing their cellular stores to be depleted. This was in agreement with the present data showing decreased GSH levels. It could be further explained via their scavenging action towards the elevated level of NOx.

The data presented here revealed marked increase in cardiac NOx level upon ADR administration. The observed elevation in cardiac NOx was in line with earlier reports and might support the important contribution of NOx in the cytotoxic action of ADR. NOx may influence several aspects of tumor biology, including modulation of cell growth, apoptosis, differentiation, metastatic capability and tumor-induced immunosuppression. These actions of NOx are attributed to its high reactivity with other radicals to form cytotoxic agents. Although the source of NOx remains undefined, however, the three isoforms of nitric oxide synthetase (NOS) have been identified in cardiac myocytes. These NOS isoforms are able to undertake ADR redox cycling to produce O2·− and other damaging free radical species that are thought to play a pivotal role in ADR-induced cardiotoxicity. On the other hand, it is possible that ADR directly or indirectly stimulates NOx production. Directly, ADR might stimulate NOx production as a consequence of increased free radical generation or indirectly through the production of cytokines.

The balance between ET and NOx is important for maintaining adequate myocardial blood flow. Therefore, any increase of these inflammatory mediators after ADR either in combination or alone may exert detrimental effects and would be associated with cardiac myocyte damage. Increased ET-1 levels in heart have been previously demonstrated in ADR-induced cardiomyopathy. In the present study, cardiac ET-1 level was increased more than twice by ADR treatment. ET-1 is synthesized in several steps from a precursor preproendothelin. ET-1 expression in endothelial and vascular smooth muscle cultured cells can be induced by several substances, including ROS. Hydrogen peroxide (H2O2) increases the synthesis of ET-1 by induction of endothelin-converting enzymes (ECE), which catalyze the conversion of the inactive precursor “big endothelin” to the biologically active peptide ET-1. However, O2·− inhibits ECE, reducing ET-1 synthesis. These results show that the role of ROS in the control of ET-1 synthesis is quite complex. The local balance between O2·−, NO and H2O2 would determine the ET-1 levels. In smooth muscle cells and blood vessels, ET-1 activates NADP(H) oxidase, suggesting a possible activation in the heart as well. The NADP(H) oxidase could be a possible source of the increased ROS, leading to lipid peroxidation. This finding is supported by the discovered polymorphisms of NADP(H) oxidase, which are associated with anthracyclin-induced cardiotoxicity. These data suggest that the increased ET-1 release after ADR may play a role in cardiac cellular injury. Indeed, the present study showed a significant correlation between the elevated cardiac ET-1 and NOx level (Fig. 5 B).

 Accumulating evidences indicate that ET-1 may play a role in the regulation of TNF-α expression and that both are involved in ADR-induced cardiotoxicity. In this study, myocardial TNF-α and ET-1 levels were positively correlated (Fig. 5B) and increased significantly after ADR administration. TNF-α has been detected in several human cardiac-related conditions, including congestive heart failure and septic cardiomyopathy and its overexpression has been shown to induce cardiomyopathy in transgenic mice. A close relationship has also been reported between TNF-α levels and heart or liver dysfunction. In conformity, in the present study, the elevated TNF-α levels were significantly correlated with the increased plasma levels of cardiac biochemical markers AST, LDH and CK-MB. This suggested that the increased TNF-α release after ADR might also play a role in cardiac cellular injury (Fig. 5A). TNF-α itself is cardiotoxic and induces the depression of cardiac function, as it possesses a depressant effect on myocardial contractility and may also cause auto-destructive inflammation. TNF-α is also able to induce programmed cell death (apoptosis) of cardiomyocytes in experiments such as ischemic conditions of heart. Cardiovascular abnormalities have been reported in canines receiving different intravenous doses of human recombinant TNF-α with
the death rate showing increase with increasing dose of TNF-α.

The EGb761 dose regimen used in this study could protect heart against ADR-induced cardiac injury as manifested by decreased release of serum enzymes AST, LDH and CK-MB. A primary consideration in the assessment of the efficacy of a potential therapeutic agent for cardiac injury was its effect on heart histology. EGb761 group had showed a remarkable improvement in pathological damage compared to ADR group. These results suggested that EGb761 effectively ameliorated the ADR-induced cardiac injury and were in agreement with earlier studies.

The standardized EGb761 has two main components — 6% terpenoids and 24% flavonoids. Terpenoids can improve microcirculation, inhibit platelet activation factor, lower blood triglycerides and prevent vascular sclerosis. The flavonoids have the effects of modulation, free radicals removal and antioxidation. For the effectiveness of EGb761 on improving cardiac microcirculation and alleviating cardiotoxicity, the following mechanisms might be involved. Firstly, EGb761 scavenges free radicals and ROS, inhibits lipid peroxidation and lowers MDA levels. Panda and Naik have shown that EGb761 treatment significantly decreases MDA levels by preventing formation of lipid peroxides and significantly elevates the levels of depressed GSH in heart of isoproterenol-induced myocardial necrosis in rats. They suggested that EGb761 may either enhance the GSH synthesis or to improve glutathione reductase activity. Secondly, EGb761 prevents the pathological elevation of NOx. It is reported that EGb761 directly acts as a NO scavenger and concomitantly inhibits the expression of iNOS mRNA in myocardial tissues, thus improving the recovery of post-ischemic cardiac function after myocardial ischemia/reperfusion. In addition, EGb761 inhibits NO production in lipopolysaccharide/gamma interferon (LPS/IFN-γ)-activated macrophages via the same postulated mechanism. Although the experimental models employed in previous studies are different from the present study, these findings could, at least in part, support our current conclusion that the protective effect of EGb761 on cardiac injury might be attributed to its suppression on the iNOS/NO-dependent pathway. Importantly, kaempferol, quercetin and isorhamnetin are the main flavonoid of EGb761. Quercetin reduces NOx generation in LPS-induced shock in rat brain. Similarly, pre-treatment with kaempferol and apigenin dose-dependently inhibit nitric oxide synthase in murine macrophage cells. Thirdly, EGb761 prevents the pathological elevation of ET-1. Previous studies have shown that EGb761 can significantly decrease ET-1 level in chronic liver injury induced by CCl₄. EGb761 can also alleviate cardiomyopathy by acting as ET-1 antagonist in patients with chronic hepatitis B and antagonize the overproduction of ET-1 both in plasma and in brain tissue. Fourthly, EGb761 can lower cardiac TNF-α. It is also shown that EGb761 depresses elevated serum TNF-α level in mercury-induced cardiovascular oxidative damage in rats. It is also reported that EGb761 can trap adriamycyl radical in an in vitro model.

In conclusion, EGb761 is capable of alleviating ADR-induced cardiotoxicity by removing oxygen free radicals and NOx, preventing the pathological ET-1 and TNF-α elevation and protecting cardiac endothelial cells. Thus, EGb761 may be used as adjuvant drug to reduce the serious and potentially fatal complications of ADR. However, this work warrants further investigation with an adequate clinical trial to test the interaction of EGb761 and ADR or whether EGb761 may be interfering in the anti-neoplastic properties of ADR.

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References