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NADPH OXIDASE INHIBITION PROTECTS AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY AND INFLAMMATION IN RATS

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Abstract: Cardiac injury is a major limitation generated by the oxidative stress induced through clinical use of the anticancer doxorubicin. This study investigates whether the inhibition of NADPH oxidase by apocynin can protect against doxorubicin-induced cardiotoxicity in rats and compare it to the powerful antioxidant (-)-epigallocatechin-3-gallate (EGCG). Male Sprague Dawley rats were treated with apocynin (10 mg/kg/daily, i.p.) or EGCG (50 mg/kg/daily, i.p.) for 7 days. Doxorubicin (20 mg/kg, i.p.) single bolus was given on day 4. Blood samples and hearts were collected for measurement of serum lactate dehydrogenase (LDH), creatine kinase-MB isozyme (CK-MB), cardiac Troponin I (cTnI), C-reactive protein (CRP), cardiac malondialdehyde (MDA) and histopathological examination. Doxorubicin induced significant elevations in CK-MB, LDH, and cTnI by 290, 309 and 3750% respectively compared to control. Inflammation, indicated by CRP level, and lipid peroxidation, indicated by cardiac MDA level, were elevated by doxorubicin to 580 and 195% respectively. There were no significant changes in histopathological examination of the heart after doxorubicin administration. Apocynin significantly attenuated the doxorubicin-induced elevations in CK-MB, LDH, cTnI, CRP and MDA. EGCG had no significant effect on LDH, cTnI and CRP, but significantly attenuated the doxorubicin-induced elevation in CK-MB and MDA. These data indicates that inhibition of NADPH oxidase, as the major source of reactive oxygen species (ROS) in the cardiovascular system, by apocynin can protect against doxorubicin-induced cardiotoxicity in rats. Conversely, scavenging of doxorubicin-induced ROS by the antioxidant EGCG is less effective and cannot prevent doxorubicin-induced cardiotoxicity in rats.

Keywords: Doxorubicin, cardiotoxicity, apocynin, (-)-epigallocatechin-3-gallate (EGCG), oxidative stress, inflammation, rats.

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INTRODUCTION

Doxorubicin (Adriamycin) is one of anthracyclines that are highly effective antineoplastic agents used in treatment of solid and hematopoietic tumors. A main limitation of its clinical use is the irreversible cardiotoxicity [1]. Although the exact mechanism responsible for cardiotoxicity of doxorubicin is still unknown, accumulating evidence indicates that doxorubicin-induced cardiomyopathy is mainly caused by increased oxidative stress [2-4].

One of the proposals to ameliorate oxidative stress associated with doxorubicin is to reduce reactive oxygen species (ROS) production by inhibition of the NADPH oxidase. Cardiac apoptosis which is developed by doxorubicin has been attributed to excessive production of ROS by the mitochondrial NADPH oxidase [4]. A previous study has shown that the cardiotoxicity induced by doxorubicin in cardiomyocytes was inhibited by the NADPH oxidase inhibitor apocynin [2]**.** However, the effect of apocynin on doxorubicin-induced cardiotoxicity *in vivo* is still unclear.

Another proposal to ameliorate cardiotoxicity associated with doxorubicin is to scavenge ROS with antioxidants. Several antioxidants, such as alpha-tocopherol [5], flavonoids [3] and resveratrol [6] have been used to counteract doxorubicin-induced cardiotoxicity. However, there are only few investigations of the cardioprotective effects of the active components of green tea against doxorubicin-induced oxidative stress and cardiotoxicity in rats [7-10]. Most of these studies were performed on isolated cardiomyocytes but not *in vivo*. The beneficial effect of green tea is due to antioxidant properties of polyphenols particularly catechins among which (-) epigallocatechin gallate (EGCG) was the most effective. EGCG was demonstrated to increase antioxidant activity, hence the resistance to oxidative injury in cardiomyocytes increases [10;11].

The present study investigates two pathways for preventing doxorubicin-induced cardiotoxicity in rats. The first pathway is through preventing the production of ROS by using the NADPH oxidase inhibitor apocynin. The second pathway is through scavenging the preformed ROS by using the antioxidant EGCG.

MATERIALS AND METHODS

Materials

Apocynin was purchased from Sigma-Aldrich (MO, USA) and dissolved in ethanol then diluted with saline; doxorubicin from Pharmacia (Italy); EGCG from Zhejiang Yixin Pharmaceutical Co. (China) and suspended in 0.5% Carboxymethylcellulose (CMC); other chemicals from El-Nasr Chemical Co. (Abou-Zaabal, Cairo, Egypt).

Animals

Male Sprague-Dawley rats (150-200 g) were obtained from Mansoura University Animal House, Mansoura, Egypt. All animal care and experimental procedures were approved by the Animal Ethics Committee of Mansoura University, Mansoura, Egypt (Approval No. 2013-86) which is in accordance with the Principles of Laboratory Animals Care (NIH publication No. 85–23, revised 1985).

Experimental protocol

Animals were injected with apocynin (10 mg/kg/daily, i.p.) [12] or EGCG (50 mg/kg/daily, i.p.) [13] for 7 days. Doxorubicin was injected as a single i.p. bolus of 20 mg/kg [5] on the fourth day. Separate groups for control (vehicle only), EGCG or apocynin alone were used. Blood samples were collected at the beginning and the end of the experiment from retro-orbital sinus. Blood samples were allowed to clot for 90 min before centrifugation at 3000 g to obtain serum. All samples were analysed freshly in the same day.

Determination of serum CK-MB isozyme activity

Creatine kinases (CK) are dimeric molecules composed of M and B subunits and exist as the isoenzymees MM, MB, and BB [14]. The CK-MB isozyme is distributed primarly in the heart muscle and its elevation represents an indicator of myocardial dysfunction [15]. CK-MB activity was determined according to the method of Wurzburg *et al.* [16] using a commercial kit (Stanbio, USA). The method is based on measuring CK activity in the presence of an antibody to the CK-M monomer. The CK-MB activity was measured at 340 nm wavelength and expressed as IU/L.

Determination of serum LDH activity

LDH activity is an indicator for cytotoxicity. Elevations in serum LDH occur from myocardial infarction, liver disease, anemias, pulmonary emboli, malignancies, and muscular dystrophy. A combined analysis of LDH and CK-MB provides diagnosis of acute myocardial infarction [17]. LDH activity was assessed at 340 nm wavelength according to the method of Henry *et al.* [18] using a commercial kit (Stanbio, USA) and expressed as IU/L.

Measurement of serum cTnI

Troponin (Tn) is a component of thin filaments (along with actin and tropomyosin), and is the protein to which calcium binds to accomplish the regulation of calcium through muscles contraction and relaxation. Tn subunit I (cTnI) is specific marker for cardiac dysfunction [19]. cTnI concentration was determined according to the method of Larue *et al.* [20] using a solid-

phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 1000 TnI, Siemens Medical Solutions Diagnostics, California, USA). The solid phase is coated with monoclonal murine anti-TnI antibody. The liquid phase consists of alkaline phosphatase conjugated to polyclonal goat anti-TnI antibody (Immulite 1000 TnI, Siemens). cTnI concentrations were expressed as (µg/L).

Measurement of serum CRP

CRP is a recognized acute-phase protein and a very sensitive marker of systemic inflammation and tissue damage. CRP was measured using agglutination test kit (Omega diagnostics LTD, Scotland, UK) according to the method of Pepys *et al.* [21].The CRP reagent is a suspension of polystyrene latex particles, coated with goat IgG anti-CRP. When CRP is present in the serum, the presence of agglutination indicates a level of CRP equal to or greater than 6 mg/L.

Preparation of heart homogenate

Heart tissues were collected at the end of the experiment, and a 5% w/v tissue homogenate was made in ice-cold 0.9% NaCl solution using mini handheld homogenizer (Omni international, USA). Tissue homogenates were centrifuged (1000 g, 4 ºC, 10 min) and supernatants were collected to measure the lipid peroxidation.

Determination of Lipid peroxidation in heart homogenate

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant of heart homogenate. Briefly, 1.0 mL of 20% trichloroacetic acid and 1.0 mL of 1% TBARS reagent were added to 100 µL supernatant, then mixed and incubated at 100 ºC for 80 min. After cooling on ice, samples were centrifuged at 1000 g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using 1,1,3,3-tetramethoxypropan as a standard.

Histopathological examination:

At the end of the experiment, the hearts were rapidly dissected out and washed immediately with saline and fixed in 10% buffered formalin. The fixed tissues were embedded in paraffin and serial sections (5 µm thick) were cut. Each section was stained with hematoxylin and eosin (H&E). The analyses were performed microscopically (Leica Imaging Systems, Cambridge, UK).

Data analysis

Data are expressed as mean± standard error of mean (SEM), where (n) equals the number of rats. Statistical analysis and graphing were carried out using Graphpad Prism software

(Graphpad Software Inc., San Diego, USA) and significant differences between groups were determined with one-way ANOVA followed by Tukey-Kramer's multiple comparisons *post-hoc* test. The level of significance was set at ($p< 0.05$).

RESULTS

Characterization of the optimal doses of doxorubicin, apocynin and EGCG:

Initial experiments using 10, 15 and 20 mg/kg i.p. injection of doxorubicin showed that 20 mg/kg is the only dose that causes significant elevations in serum CK-MB, LDH and cTnI. The effect of EGCG (20, 50 and 100 mg/kg) and apocynin (1, 5 and 10 mg/kg) on normal and on doxorubicin-induced elevations of biochemical parameters were tested. We found that EGCG (50 mg/kg) and apocynin (10 mg/kg) were the optimal protective doses in our model without having any effect on normal biochemical parameters (data not shown).

The effect of apocynin and EGCG on serum CK-MB changes induced by doxorubicin:

Cardiac dysfunction induced by doxorubicin was assessed by measuring the increase in the levels of the cardiac enzyme CK-MB (an indicator of myocardial cell injury). Injection of doxorubicin significantly ($p<0.05$, $n=5$) increased the serum CK-MB by 290% (Figure 1A). Pretreatment with apocynin and EGCG significantly ($p<0.05$, $n=5$) decreased the doxorubicin-induced elevations of CK-MB (Figure 1A) where CK-MB level decreased to 174% and 219% respectively. However, these levels of CK-MB after treatment with apocynin and EGCG did not reach the control levels.

The effect of apocynin and EGCG on serum LDH changes induced by doxorubicin:

Cardiac dysfunction was additionally assessed by measuring the increase in the levels LDH (an indicator of cytotoxicity, and muscle dysfunction). Injection of doxorubicin significantly ($p<0.05$, $n=5$) increased the serum LDH by 309% (Figure 1B) due to cytotoxicity. Pretreatment with apocynin significantly ($p<0.05$, $n=5$) decreased this elevation in LDH (Figure 1B) where the doxorubicin-induced elevation of LDH level decreased to 174% compared to control. On the other hand EGCG did not significantly affect this increase in LDH level.

The effect of apocynin and EGCG on serum cTnI changes induced by doxorubicin:

The level of cTnI was measured as a one of the most important and sensitive key parameters indicating cardiotoxicity. Injection of doxorubicin significantly ($p<0.05$, $n=5$) increased the serum cTnI by 3750% (Figure 1C) indicating the sensitivity of cTnI as a marker of cardiac dysfunction. Pretreatment with apocynin significantly ($p<0.05$, $n=5$) inhibited the increase in cTnI (Figure 1C) where the cTnI level dropped to only 188% compared to control. There was no

significant difference in cTnI levels between control and doxorubicin+ apocynin groups. On the other hand EGCG did not significantly affect this doxorubicin-induced increase in cTnI level.

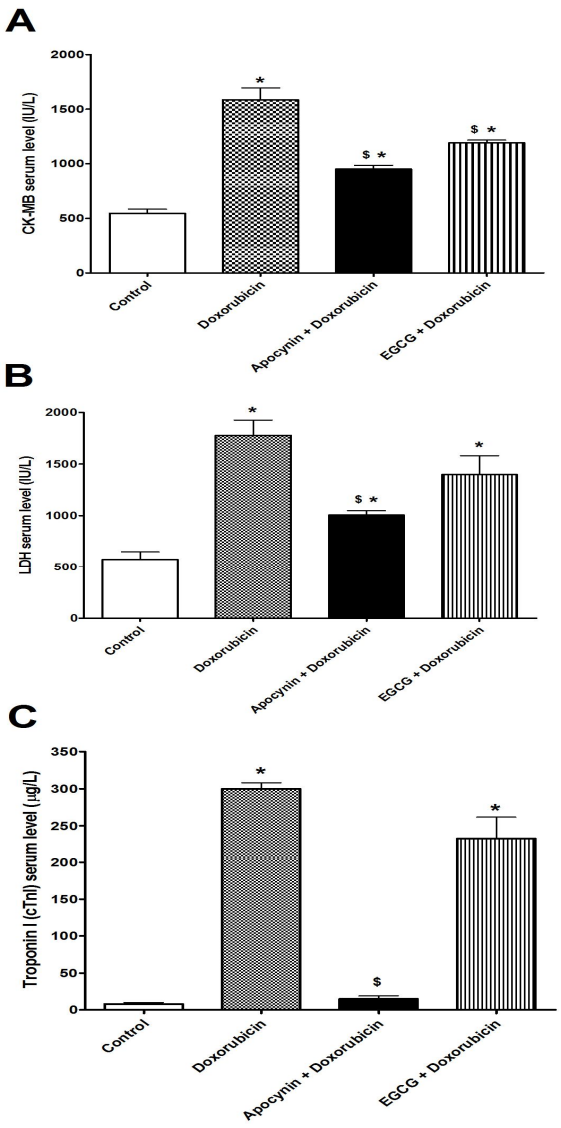

Figure 1: E*ffect of apocynin and EGCG on serum CK-MB, LDH and cTnI changes induced by doxorubicin*.

Serum CK-MB (A), LDH (B) and cTnI (C) were determined in rats treated with apocynin (10 mg/kg/daily, i.p.) or EGCG (50 mg/kg/daily, i.p.) for 7 days with a single bolus injection of doxorubicin (20 mg/kg, i.p.) given on the fourth day. Data are expressed as mean±S.E.M. (n=5).

* $p < 0.05$ compared to control group; $ $p<0.05$ compared to Doxorubicin group using one-way ANOVA with Tukey-Kramer's multiple comparisons *post-hoc* test.

The effect of apocynin and EGCG on serum CRP changes induced by doxorubicin:

The level of CRP was measured as a very sensitive marker of systemic inflammation and tissue damage. Injection of doxorubicin significantly ($p<0.05$, n=5) increased the serum CRP by 580% (Figure 2A) compared to control group. Pretreatment with apocynin significantly ($p<0.05$, n=5) decreased this elevation of CRP (Figure 2A) to 374% compared to control. EGCG pretreatment had no significant effect this doxorubicin-induced increase in CRP level.

The effect of apocynin and EGCG on cardiac MDA changes induced by doxorubicin:

The oxidative stress in heart homogenates was assessed by measuring lipid peroxidation indicated by the MDA level. Injection of doxorubicin significantly ($p<0.05$, n=5) increased the cardiac homogenate level of MDA by 195% (Figure 2B) compared to control group. Pretreatment with apocynin significantly ($p<0.05$, n=5) inhibited this increase in MDA level (Figure 2B) where the MDA level was 98% compared to control. EGCG showed a less effect than apocynin as EGCG significantly ($p<0.05$, n=5) decreased the elevations of MDA to 136% compared to control.

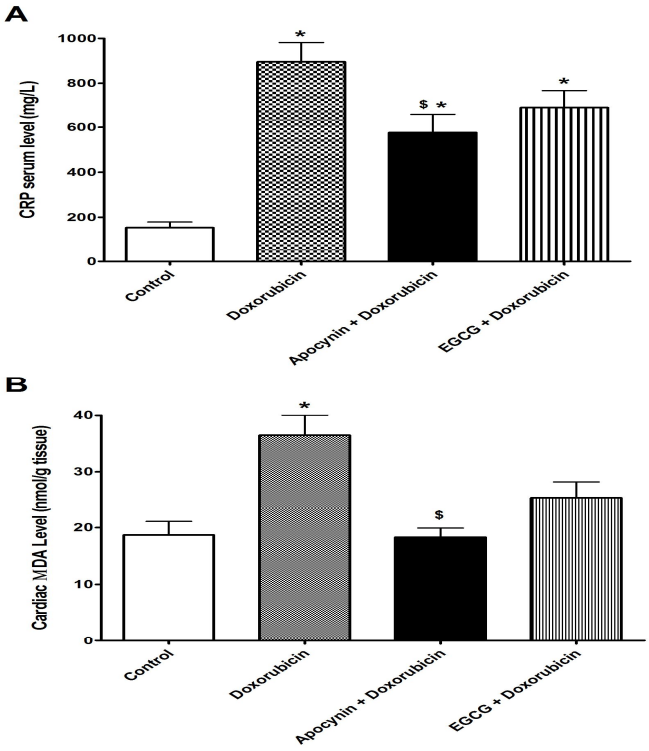

Figure 2: ***Effect of apocynin and EGCG on serum CRP and cardiac MDA changes induced by doxorubicin.***

Serum CRP (A) and cardiac MDA (B) were determined in rats treated with apocynin (10 mg/kg/daily, i.p.) or EGCG (50 mg/kg/daily, i.p.) for 7 days with a single bolus injection of doxorubicin (20 mg/kg, i.p.) given on the fourth day. Data are expressed as mean±S.E.M. (n=5).

* $p < 0.05$ compared to control group; $ $p<0.05$ compared to Doxorubicin group using one-way ANOVA with Tukey-Kramer's multiple comparisons *post-hoc* test.

***Histopathological changes*:**

As a further confirmation for any effect of doxorubicin on heart tissue, we evaluated histopathological changes induced by doxorubicin. Rats treated with doxorubicin (20 mg/kg, i.p), showed normal cardiac muscle without any pathological changes (Figure 3).

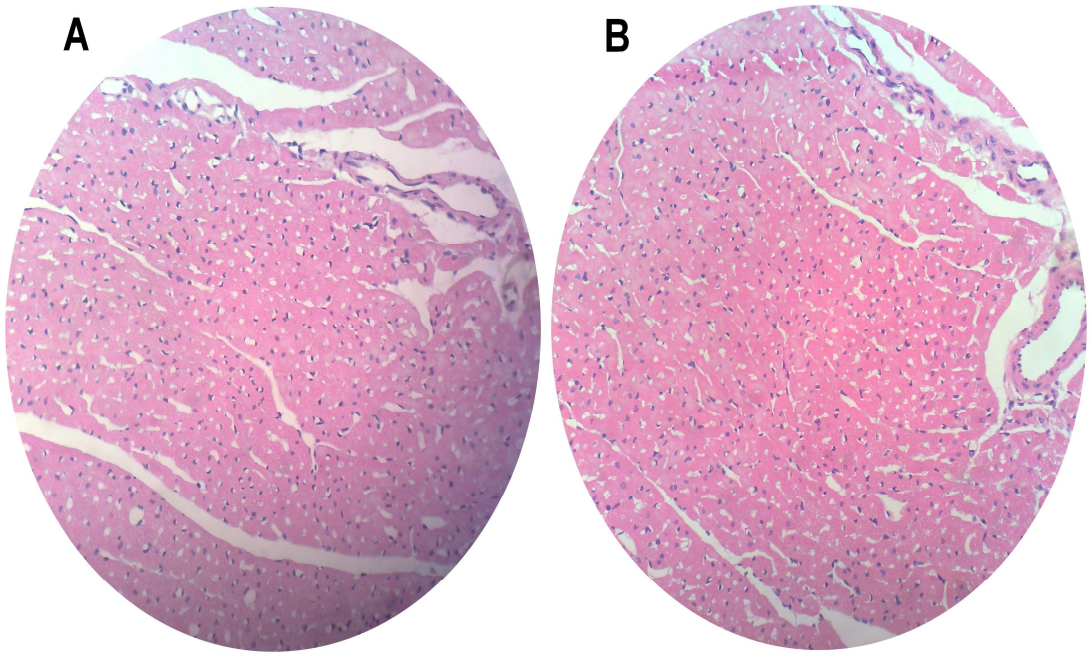

Figure 3: *Histopathological changes*

Rats treated with doxorubicin (20 mg/kg, i.p) showing normal cardiac muscle without any pathological changes after 3 days (H&E, 200X).

DISCUSSION

This study presents the cardioprotective effect of the NADPH oxidase inhibitor apocynin against doxorubicin-induced cardiotoxicity in rats and compares it to the powerful antioxidant EGCG.

Doxorubicin is one of the most important antitumor drugs for treating several types of solid tumor, leukemia and lynphomas. However, doxorubicin is a well-known cardiotoxic agent due to its ability to destruct myocardial cells [1], which is a main limitation to its clinical use. As a result of this myocardial cells destruction, cellular enzymes such as LDH, CK-MB and cTnI are released into blood stream and serve as the diagnostic markers of myocardial tissue damage. To confirm doxorubicin-induced cardiotoxicity, serum CK-MB, LDH and cTnI were assessed in our rat model. All of these cardiac markers were elevated by doxorubicin indicating cardiotoxicity. Additionally, doxorubicin induced inflammation as indicated by the elevated level of CRP.

Although these biochemical markers of cardiac dysfunction and inflammation were elevated, however, histopathological examination of rats treated with doxorubicin showed normal cardiac muscle without pathological changes. This discrepancy may be attributed to the short time of doxorubicin-induced changes in our model (3 days) which may be insufficient to induce remarkable cardiac tissue pathological changes.

The mechanism of cardiotoxicity induced by a doxorubicin may be attributed to increased oxidative stress, possibly through induction of NADPH oxidase, the major enzyme responsible for the formation of ROS in the cardiovascular system [2;4]. The oxidative stress induced by doxorubicin in heart homogenates was assessed by measuring lipid peroxidation. Cardiac MDA level was increased after doxorubicin injection, confirming doxorubicin-induced oxidative stress.

To prevent doxorubicin-induced cardiotoxicity and inflammation, we evaluated ROS inhibition pathway by using the NADPH oxidase inhibitor apocynin and compare it to the ROS scavenging pathway by the antioxidant EGCG. Pretreatment with apocynin significantly decreased the doxorubicin-induced elevations of CK-MB, LDH and cTnI. Moreover the inflammation induced by doxorubicin was also attenuated by apocynin which decreased CRP level. Additionally, apocynin efficiently decreased the doxorubicin-induced cardiac tissue lipid peroxidation by decreasing cardiac MDA level. A previous study has shown that doxorubicin-induced toxicity in cardiomyocytes was inhibited by apocynin [2]**.** Since doxorubicin is documented to induce NADPH oxidase [4], therefore our data indicates the usefulness of NADPH oxidase inhibitors, such as apocynin, as important pathway to prevent doxorubicin-induced cardiotoxicity.

We further evaluated the other pathway of preventing doxorubicin-induced cardiotoxicity by using the antioxidant EGCG. The most effective component in green tea is EGCG, which has a powerful antioxidant activity [10;11]. EGCG pretreatment in our model had no significant effect on doxorubicin-induced elevations of LDH, cTnI, and CRP, but significantly attenuated the

increase of CK-MB and MDA. These results indicate that EGCG is less effective in preventing doxorubicin-induced cardiotoxicity in rats compared to apocynin.

Since ROS are rapidly-acting and highly reactive, therefore inhibition of their production may be a better strategy than scavenging them after their induction. However, further studies are required to elucidate the molecular mechanism of doxorubicin-induced NADPH oxidase activation with subsequent ROS production that leads to cardiac dysfunction.

CONCLUSIONS

In conclusion, cardiac injury and inflammation induced by doxorubicin is dependent on oxidative stress that can be prevented more efficiently by the NADPH oxidase inhibitor apocynin but not with the ROS scavenger EGCG.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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