Potential effect of ferulic acid on NF-κB in a rat model of doxorubicin toxicity

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ABSTRACT

“Adricin” or Doxorubicin (Dox) is an anthracycline antibiotic agent used in the treatment of solid and haematopoietic tumors, but it has cardiac, renal and hepatic toxicities. Oxidative stress has an important role in the pathogenesis of Dox toxicity. This study investigated the role of Ferulic acid (FA), a natural antioxidant agent, in protection against Doxorubicin-induced toxicity. 40 albino rats were used, and divided into four groups, Group I (control group), Group II: Dox intraperitoneal for two weeks, Group III: Ginkgo biloba (GB) + Dox, Group IV: FA +Dox. Biochemical assays for serum creatinine, serum lactate dehydrogenase, creatine phosphokinase, AST and ALT were done. Immuno histochemistry examination of the kidneys, heart and liver and the levels of MDA, GSH, SOD and catalase were assessed. Results: Treatment with FA resulted in improvement of oxidative stress, and decrease in expression of NF-κB, that may be a promising natural adjuvant therapy, potentially ameliorating Dox toxicity in clinical practice. Conclusion: FA can be considered a good candidate for offering protection against the deleterious toxicity of Dox.

Keywords: Dox toxicity, Ferulic acid, Doxorubicin, Oxidative stress

1. INTRODUCTION

Doxorubicin is one of the most effective chemotherapeutic agents for the treatment of various types of cancer. A one of the serious adverse effect of doxorubicin is cardiomyopathy. Since cellular apoptosis are at least partially responsible for the pathogenesis of doxorubicin cardiac toxicity, different studies have been done involving anti-apoptotic remedies to manage this adverse effect
(Tsun-Jui et al., 2008). The exact causal mechanisms of Dox induced toxicity remain unclear and various mechanisms have been proposed to interpret the toxicity, (Oliveira et al., 2004; Kalender et al., 2005; Wang et al., 2009).

Ferulic acid is an organic acid. It is found in plant cell wall. It is found in plant seeds as rice, wheat, oats, as well as coffee, apple, artichoke, peanut, orange and pineapple and can be extracted from wheat and maize bran. Like many phenols, it is an antioxidant in vitro in the sense that it is reactive toward free radicals, quenching lipid peroxidative chains (Fereidoon & Marian, 2004; Yogeeta et al., 2006; Barone et al., 2009; Ashraf et al., 2010; Shen et al., 2011).

The aim of the study is to evaluate the efficacy of Ferulic acid and Ginkgo biloba in counteracting doxorubicin toxicity.

2. MATERIAL & METHODS

Drugs and Chemicals
Doxorubicin (Dox): it was purchased as Adricin vial [50mg/ml Doxorubicin hydrochloride, EMC, United Pharmaceuticals, Cairo, ARE (EUP)].
Ginkgo biloba (GB): was purchased as Ginkgolide powder (50 mg) [Fluka (St. Louis, USA)]. It was dissolved in distilled water and administered to the animals via gastric gavage.
Sodium Ferulate: It is Ferulic acid (Ferulate) salt and was purchased as a powder from Sigma–Aldrich Chemical Co. (St. Louis, USA). It was dissolved in distilled water and administered to the animals via gastric gavage.
Serum CK was determined using CK NAK liquid-UV kit, Human, Germany.
Serum LDH was determined using Human Diagnostics, Wiesbaden kit, Germany
Creatinine, MDA, GSH contents, SOD and CAT were determined using diagnostic kits purchased from Biodiagnostic Company (Cairo, Egypt).

Animals
Fourty albino rats (males) weighing between 150 - 250mg were used in this study. The rats were obtained from the animal unit of the Medical Experimental Research Centre of Faculty of Medicine, Mansoura University. They were kept under standard conditions of temperature (25 ± 5ºC) and with a 12 h light: 12 h dark schedule. Experiments were performed according to the local committee of Animal Care and Use Committee protocols, and guided by THE Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, no. 85-23, revised 1996).

Methods
Animals were equally divided into four groups, ten rats in each group.
Group I (control group): it was given 1ml/kg saline.
Group II: it was given 2.5 mg/kg Doxorubicin intraperitoneal three times weekly for two weeks (cumulative dose 15mg/kg) (Yalçın et al., 2010).
Group III: it was given Ginkgo biloba alone at an oral dose of 100mg/kg/day (Abd-Ellah & Mariee, 2007) one week before giving doxorubicin them with doxorubicin for two weeks, GB daily and intraperitoneal injection of 2.5mg/kg Doxorubicin three times weekly after it by 1 hour.
Group IV: it was given FA in a dose of 110 mg/kg/day (Zhao et al., 2004) orally for one week then with doxorubicin for two weeks, FA daily and intraperitoneal injection of 2.5mg/kg doxorubicin three times weekly after it by 1 hour.

Animals were sacrificed 48 hours following the last Doxorubicin dose by cervical dislocation. Intra-cardiac blood sample was taken at the time of scarification. Blood samples were centrifuged at 2000 rpm for 5 minutes at 4°C and the sera were frozen at -40°C until analyzed for levels of creatinine, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), ALT (alanine transaminase) and AST (aspartate transaminase). Heart, liver and Kidneys were removed immediately, washed in ice-cold physiological saline then each organ was sectioned, half of it was fixed in 10% formalin for immunohistochemistry examination and the remaining was homogenized separately in 10% phosphate-buffered saline at 4°C and the supernatants were collected for determination of the levels of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (Palanisamy et al., 2012).

Determination of myocardial MDA content: according to Draper & Hadley, (1990).
Determination of myocardial GSH content, according to Beutler et al., (1963)
Determination of myocardial SOD activity, according to Nishikimi et al., (1972)
Determination of catalase level, according to Aebi, (1984)
Determination of ALT (alanine transaminase) and AST (aspartate transaminase) according to (Retiman & Frankel, 1957).
Determination of Creatinine according to (Seeling & Wust, 1969)

**Tissue NF-KB immunohistochemical (IHC) analysis**
The tissues were fixed in 10% formalin for 24 h, processed routinely. Four micrometer-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination. A minimum of 8 fields for each organ section were examined and assigned for severity of changes by an observer blinded to the treatments of the animals. Two slides from each rat were examined by an observer blinded to the treatments. The changes of the kidney, heart and liver tissues on the light microscopy were graded as follows: normal (0); mild (1+); moderate (2+); severe (3+) (Cecen et al, 2011). Thus, a 1+ lesion represented an involvement of less than 25% of the tissues, 2+ lesion represented an involvement of 25% to 50% of the tissues while 3+ lesion indicated that more than 50% of the tissues are involved.

Deparaffinized sections were put with 0.3% hydrogen peroxide in methanol (30 minutes) and microwave heated (30 minutes) in EDTA buffer solution, pH 8.0. Slides were immunostained with antibodies against nuclear factor kappa-B using indirect immunoperoxidase technique. Primary antibody was left to react for 30 minutes at room temperature was performed. Immuno Pure Ultra-Sensitive ABC Peroxidase (catalog no. 32052; Thermo Scientific, UK), was used (Immuno peroxidase method), and diaminobenzidine as chromogen was used too. The stained sections were analysed using the following score: score 0, no nuclear or cytoplasmic staining; score 1, mild nuclear or cytoplasmic staining; score 2, moderate nuclear or cytoplasmic staining; score 3, strong nuclear or cytoplasmic staining (Jenkins et al., 2007).

**Statistical Analysis**
Means and standard deviations were calculated from the ten replicates per each group. One way ANOVA test with the Tukey posthoc test were performed. P is considered significant if <0.05.

**Image analysis**
For semi-quantitative assessment of nuclear factor kappa B (NF-κB), images were digitally imported to image analyzer software. All sections were randomly evaluated under blindfold manner. NF-κB was examined (× 100 magnification) in ten separate fields of its stained sections (at different time periods the experiment). The mean NF-κB percentage area per examined field was calculated as the average of the pooled readings from these fields in each specimen and represented in the figure below.

3. **RESULTS**

**Effects of Ginkgo biloba and Ferulic acid on serum ALT, AST, creatinine, CPK and LDH**
Compared to control group, serum ALT, AST, creatinine CPK and LDH were significantly higher in Doxorubicin group (p<0.05). The increment of these parameters was significantly attenuated in both GB-treated and FA treated group (p<0.05) (table 1). However, there were no statistical significant differences between Gb-treated and FA treated groups.

**Table 1** Effects of Ginkgo biloba and Ferulic acid treatments on ALT, AST, creatinine, LDH, CPK, MDA, GSH, SOD and catalase in serum and tissues

<table>
<thead>
<tr>
<th></th>
<th>Control mean ±SD</th>
<th>Dox mean ±SD</th>
<th>Dox+ GB mean ±SD</th>
<th>Dox + FA mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (alanine transaminase) (U/L)</td>
<td>24.3 ± 3.2</td>
<td>61.02 ± 11.9*</td>
<td>33.2 ± 9.9$</td>
<td>30.1 ± 7.5$</td>
</tr>
<tr>
<td>AST (aspartate transaminase (U/L)</td>
<td>66.0 ± 17.3</td>
<td>150.0 ± 33*</td>
<td>70.8 ± 9.5$</td>
<td>63.9 ± 16.1$</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.44 ± 0.11</td>
<td>3.9 ± 0.09$</td>
<td>0.50 ± 0.02$</td>
<td>0.7 ± 0.05$</td>
</tr>
<tr>
<td>Lactic dehydrogenase (LDH) (U/L)</td>
<td>145.0 ± 0.19</td>
<td>400.0 ± 96.2*</td>
<td>120.0 ± 23$</td>
<td>123.0 ± 20.8$</td>
</tr>
<tr>
<td>Creatine phosphokinase (CPK) (U/L)</td>
<td>86.0 ± 10.51</td>
<td>300.0 ± 33.1*</td>
<td>101.0 ± 21.9$</td>
<td>95.0 ± 30.2$</td>
</tr>
<tr>
<td>MDA nmol/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>2.44 ± 0.67</td>
<td>4.55 ± 0.79*</td>
<td>3.27 ± 1.18$</td>
<td>3.01 ± 0.45$</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.72 ± 0.52</td>
<td>3.00 ± 0.30*</td>
<td>2.26 ± 0.45$</td>
<td>1.91 ± 0.19$</td>
</tr>
</tbody>
</table>
Effects of Gingko biloba and Ferulic acid on markers of oxidative stress, Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase

Table 2 shows the results of oxidants and antioxidants in liver, kidney, heart and serum. Compared to control group, MDA was significantly higher in the Dox group in liver, kidney, heart, and serum (p<0.05). This increase in MDA was significantly attenuated in GB-treated group and FA-treated group (p< 0.05). Also, compared to control group, GSH was significantly decreased in serum and tissues in Dox group (p<0.05), and this decrease was significantly increased in ginkgo-treated and FA-treated group (p<0.05) except in liver of GB-treated group which was non-significant (P>0.05).

Table 2 Histopathological scoring of organs toxicity in different groups

Compared to the control group, SOD activity was significantly decreased in the Dox group in all tissues and serum except the heart which showed non-significant decrease (table 3). This decrease was significantly increased in FA-treated group in serum and liver only (p ≤ 0.05). Whereas, the change in SOD in GB-treated group was not significant in all tissues and serum compared to Dox.
group (P>0.05). Lastly, compared to control group, catalase enzyme was significantly decreased in Dox in tissues and serum (p ≤ 0.05). This decrease was significantly increased in both GB-treated group and FA-treated group compared to Dox group (p ≤ 0.05).

**Table 3** Effects of Ferulic acid treatments on NF-κB expression (measured by digital quantification of staining intensity of NF-κB)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dox</th>
<th>Dox + GB</th>
<th>Dox + FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.0±0.0</td>
<td>16.91±2.81*</td>
<td>7.74±1.15$</td>
<td>4.78±0.69$</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0±0.0</td>
<td>10.79±0.89*</td>
<td>6.07±1.04$</td>
<td>2.79±0.69$</td>
</tr>
<tr>
<td>Renal</td>
<td>0.0±0.0</td>
<td>13.55±2.34*</td>
<td>9.21±1.55$</td>
<td>5.29±1.81$</td>
</tr>
</tbody>
</table>

*= significant relative to control (p<0.05), $= significant relative to Dox (p<0.05).

One way ANOVA test with Tukey post hoc test

**Effects of ferulic acid on NK-κB expression in tested organs**

Dox treated showed significant increase in mean of NF-κB expression in kidney, liver and heart cells (staining fraction) as compared to non treated group (fig. 1, 2, 3). Treated groups caused significant decrease in mean NF-κB expression in the cells (staining fraction) compared to non treated group (fig. 1, 2, 3).

**Figure 1** IHC of NF-κB in different organs (kidney- heart -liver), the staining intensity of control rats is very weak, cytoplasmic with few nuclear staining.
Figure 2 Staining intensity is increased significantly in the diseased different organs (kidney - heart - liver).

Figure 3 Treated rats with ferulic acid showed significant reduction of staining intensity in the three organs respectively.
4. DISCUSSION

Adriamycin is an anticancer chemotherapeutic effective agent. However, different organ toxicity compromises the clinical effect of the drug. Therefore, the search for an effective and safe antidote of doxorubicin toxicity was done (Tsun-Jui et al., 2008; Ukwubule et al. 2019). Ferulic acid (FA) is a polyphenol substance in vegetables and maize bran. It has been used as a food additive to prevent lipid peroxidation (Sreenivasan et al., 2007). It induces up-regulation of cytoprotective enzymes (Barone et al., 2009). The aim of the present work, is exploring the possible counteracting mechanism of ferulic acid against doxorubicin induced nuclear factor kappa B (NF-κB).

Dox administration induced elevation of serum LDH and CK-MB which are important markers of cardiac injury. This is in agreement with that of Vijay et al., (2011), Ragavendran et al., (2012) and Saratchandran & Cherupally (2012) who demonstrated similar increase in cardiac enzyme activities. The increase in serum CK-MB and LDH following Dox is due to oxidative stress and lipid peroxidation in the heart (Zhang et al., 2006). Ferulic acid significantly attenuated the levels of serum ALT and AST that were elevated in DOX group. This protective effect might be due to stabilization of hepatocyte membranes with the consequent decrease in the leakage of liver enzymes.

Histopathological results revealed affection of kidneys, liver and heart in Dox group in different degrees. The organ damage resulting from Dox administration is due to ability of the drug to produce free radicals and reduce the antioxidant defense mechanism. Free radicals are known to damage several macromolecular and cellular components (Zhang et al., 2006; Lai et al., 2007). Mitochondrial apoptosis pathways are induced by the classic cytotoxic chemotherapeutic agents, including anthracyclines (Hossein et al., 2010). There was an improvement of Dox pathological effects in ginkgo biloba and ferulic acid group. This confirms the results of Saratchandran & Cherupally (2012) who deduced that FA has some protective effect on the kidneys of diabetic rats. Dietary FA was previously found to be protective against carbon tetrachloride-4-induced toxicity in rat kidney and this effect is associated with increase in glutathione peroxidase and superoxide dismutase levels (Srinivasan et al., 2005; Chan et al., 2007; Aluise et al., 2009; Yeh & Bickford 2009; Bradley et al., 2011; Tatliidede et al., 2009).

The present study showed that MDA level was increased in Dox group and this is supported by El-Shitany et al., (2008) and El-Sayed et al., (2011). On the other hand, GSH, SOD and catalase activity were decreased. Hossein et al., (2010) explained that Dox-induced oxidative stress in tissues by the alterations in antioxidant defense systems which are enzymatic and non-enzymatic. In their study, Dox reduced significantly the GSH content, besides the enzymatic activities of SOD and Glutathione-S-transeferase associated with marked increase in lipid peroxidation as manifested by increased malondialdehyde levels in rat. Regarding SOD, Dox caused a significant decrease in its activity in tissue and serum. Dox-induced free radical production accompanied by exhaustion of the antioxidant enzyme, SOD, which is responsible for scavenging the liberated, toxic free radicals. These results are proved with previous studies by Yoneko et al., (2007), Ayla et al., (2011) and Nahla (2012) who attributed these findings to tissue damage and cell membrane destruction by free radicals resulted from DOX administration.

The present results showed that there was an amelioration of biochemical oxidative stress markers induced by Doxorubicin in rats which well correlated with the alleviation in the histopathological changes, a significant decrease in MDA and the increase in GSH levels in FA group. However in another study of FA effects on age-related changes, found that dietary FA increased GSH in renal tissue of rats (Erdogan et al., 2009; Jung et al., 2009). FA significantly improved SOD and catalase activity. This agrees with Saratchandran and Cherupally (2012) who proved the efficacy of FA in the improvement of enzymatic and oxidative damage extent.

The NF-κB is best known for its immunoregulatory functions of genes involved in many biological processes such as inflammation, immune response, cell differentiation and growth, carcinogenesis and apoptosis. In particular, NF-κB plays a central role in regulating the production and activation of TNF-α, IL-1β, adhesion molecules, and other cytokines and chemokines. In non-activated cells, NF-κB is reserved in an inactive state in the cytoplasm by direct binding to NF-κB inhibitory protein (IκB). In response to inflammatory stimuli, including ROS and TNF-α, IκB proteins are phosphorylated and degraded unmasking the nuclear localization sequence of NF-κB subunits, allowing NF-κB to translocate to the nucleus and bind specific promoter elements to induce gene transcription. NF-κB, has been recognized to be fundamental in the cascade concerning with KC activation (Yoshidome et al., 1999, Tornatore et al., 2012). The major effect of TNF-α is to induce liver cell injury through neutrophil activation, ROS production, and mitochondrial toxicity (Tian et al., 2006). TNF-α also triggers expression of NF-κB which in turn upregulates the production of TNF-α in a positive feedback loop that can amplify the inflammatory response and extend the duration of inflammation (Lawrence, 2009). In this study, IHC staining of NF-κB was performed in formalin-fixed, paraffin-embedded tissue sections via utilizing antibodies directed against the p65 (F6) component of the NF-κB complex (Barone et al., 2009). The staining intensity of NF-κB was analyzed and quantified via digital analysis of the acquired images using specialized image analysis software. In our study, the staining of NF-κB in control rats was very scanty and was mainly cytoplasmic with few nuclear shadows. After Dox,
there was significant increase in staining intensity of NF-κB in non-treated rats which became mainly nuclear indicating translocation of NF-κB from the cytoplasm to the nucleus. Ferulic acid treated group showed significantly smaller staining fractions of NF-κB. Reports suggested that agents that ROS generation also decrease NF-κB activation (Bowie & O’Neill, 2000). These results were further confirmed by histopathologic examination of tissue sections being the “gold standard” assessment method of tissue injury. The overall improvement in histology is the result of combined inhibition of ROS production, inhibition of cytokine release and prevention of NF-κB expression under the effect of dox.

5. CONCLUSION
Treatment with FA resulted in improvement of oxidative stress, and decrease in expression of NF-κB, that may be a promising natural adjuvant therapy, potentially ameliorating Dox toxicity in clinical practice. Overall, FA can be considered a good candidate for offering protection against the deleterious toxicity of Dox.

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Author Contributions
Sameh Abdel Ghany, Sanad ELKholy, Hala Abdelmalek & Sahar EL Dakrory shared in creating the hypothesis, writing, doing the experimental design & work and the statistics were done by Sameh Abdel Ghany. Amira EL Hawary was responsible for the pathology and IHC.

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Conflict of Interest
The authors declare that there are no conflicts of interests.

Ethical approval
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

REFERENCES AND NOTES


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Data and materials Availability
All data associated with this study are present in the paper.

Peer-review
External peer-review was done through double-blind method.