EFFECT OF TAXIFOLIN ON DOXORUBICIN-INDUCED OXIDATIVE CARDIAC DAMAGE IN RATS: A BIOCHEMICAL AND HISTOPATHOLOGICAL EVALUATION

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ABSTRACT

Doxorubicin is a widely used anthracycline-derived broad-spectrum antitumoral antibiotic drug. However, cardiotoxicity due to doxorubicin treatment has warranted dose reduction or complete discontinuation in certain cases. The role of oxidative stress in the pathogenesis of doxorubicin-induced cardiotoxicity has been previously demonstrated. Against this background, this study aimed to investigate the protective effect of the potent antioxidant flavone taxifolin against possible oxidative heart damage biochemically and histopathologically induced by doxorubicin. Albino Wistar male rats were divided into three groups: healthy controls (HG), a group given doxorubicin alone (DG), and a group given taxifolin + doxorubicin (TDG). Taxifolin was administered orally at a dose of 50 mg/kg via gavage. Doxorubicin was injected intraperitoneally at a dose of 5 mg/kg. This procedure was repeated for 7 days. The results of the biochemical experiment showed that taxifolin significantly inhibited doxorubicin-induced malondialdehyde increases and glutathione decreases in heart tissues. In addition, taxifolin significantly suppressed the increases in cardiac damage markers, such as serum troponin I, creatine kinase, and creatine kinase-MB, induced by doxorubicin. Taxifolin treatment has also been histopathologically shown to alleviate doxorubicin-induced heart tissue damage. Accordingly, the results of the present study suggest that taxifolin may be useful in the treatment of doxorubicin-induced oxidative heart damage.

Keywords: Antioxidant, cardiotoxicity, doxorubicin, flavonoid, taxifolin.

INTRODUCTION

Doxorubicin (DXR) is a broad-spectrum antineoplastic agent belonging to the anthracycline group of antibiotics. Anthracyclines were first produced in 1963 from the culture of Streptomyces peucetius variete caesiu, a fungus species (Singal et al. 2000; Khan et al. 2022). DXR is the most widely used member of this group and was introduced into chemotherapy in 1967–1969 (Chabner et al. 1975). DXR is frequently used in the treatment of tumors, such as leukemia, lymphoma, soft tissue and bone sarcomas, Wilms tumor, neuroblastoma, and hepatoblastoma (Muggia et al. 1991). DXR has clinical side effects, such as alopecia, bone marrow suppression, mucositis, extravasation injury, radiosensitization, gastrointestinal toxicity, nephrotoxicity, and cardiotoxicity (Chabner et al. 1975; Bryant et al. 2007). DXR-induced cardiotoxicity is the most challenging side effect that has led to dose reductions (Singal et al. 2000). The mechanism of cardiotoxicity formation has not yet been fully elucidated. However, reactive oxygen species (ROS) formation, a decrease in the myocardial antioxidant enzyme level, lipid peroxidation, and programmed cell death (apoptosis) are considered to be responsible for the condition (Lipshultz et al. 2006; Takemura et al. 2007). The lack of antioxidant enzyme content in the heart tissue renders it incapable of scavenging against ROS insults. As a result, heart tissue may become vulnerable to damage after DXR treatment (Basser et al. 1993). In the existing literature, a variety of parameters, such as malondialdehyde (MDA), plasma cardiac troponin I (cTnI), and creatine kinase isoenzyme MB (CK-MB), have been used to evaluate cardiotoxicity based on oxidative stress (Polat et al. 2015).

Taxifolin (3,3',4',5,7-pentahydroxyflavanone) is a flavanone found in abundance in onion, milk thistle, French maritime pine bark, and Douglas fir bark (Wang et al. 2017). In addition to its proven antioxidant activity (Topal et al. 2016), taxifolin has also been reported to suppress various types of cancer (Chen et al. 2018; Haque et al. 2018).
Information from past studies indicates that taxifolin can reduce cardiac toxicity without suppressing the anticancer effect of DXR and may even potentiate its anticancer activity. However, we could not find any studies in the existing literature showing that taxifolin reduces DXR-induced cardiotoxicity. Accordingly, the present study aimed to biochemically and histopathologically investigate the effect of taxifolin on DXR-induced oxidative cardiac damage in rats.

MATERIALS AND METHODS

Experimental Animals: Male albino Wistar rats (N=30) weighing between 258–270 g were procured from Atatürk University Medical Experimental Application and Research Center. The rats were kept at normal room temperature (22°C) in an appropriate laboratory environment and were fed ad libitum. The protocols and procedures were approved by the local Atatürk University Animal Experimentation Ethics Committee (Meeting date: 29.08.2018; Decision no. 2018/9).

Chemicals: Thiopental sodium (IE Ulagay, Turkey), doxorubicin hydrochloride (Kocak Farma, Turkey), and taxifolin (Evalar, Russia) were used as chemical agents in the experiment.

Experimental groups: The animals were divided into three groups, each of which included 10 rats: a healthy control group (HG), a group receiving doxorubicin (DG), and a group receiving taxifolin + doxorubicin (TDG).

Experimental procedure: Taxifolin at a dose of 50 mg/kg was administered to the TDG (n = 10) via oral gavage. The same volume of distilled water was orally administered to the HG (n = 10) and DG (n = 10) as a solvent with a gavage. The rats in the TDG and DG groups were injected with 5 mg/kg DXR intraperitoneally 1 h after the administration of taxifolin and distilled water. This procedure was repeated once daily for seven days. At the end of this period, all the rats were sacrificed with high-dose thiopental sodium (50 mg/kg thiopental), and the hearts were removed. In the next step, cTnI, creatine kinase (CK), and creatine kinase-MB (CK-MB) levels were measured in the blood samples taken before the animals were sacrificed. Malondialdehyde (MDA) and total glutathione (tGSH) levels were measured in the removed cardiac tissues. In addition, the cardiac tissues were analyzed histopathologically. Biochemical and histopathological findings obtained from the HG, DG, and TDG were evaluated through comparisons.

Biochemical analysis

MDA analysis: MDA measurements were based on the method used by Ohkawa et al. (Ohkawa et al. 1979), which included spectrophotometrically measuring the absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA. The serum/tissue-homogenate sample (0.1 mL) was added to a solution containing 0.2 ml of 80 g/L sodium dodecyl sulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate, and 0.3 mL of distilled water. The mixture was incubated at 95°C for 1 h upon cooling, and 5 mL of n-butanol: pyridine (15: 1) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained using 1,1,3,3-tetramethoxy propane. The MDA concentration in the cardiac tissue was calculated as nmol/g protein.

tGSH analysis: According to the method defined by Sedlak and Lindsay (Sedlak et al. 1968), 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) disulfide was chromogenic in the medium, and DTNB was reduced easily by sulfhydryl groups. The yellow color that appeared during the reduction was measured with spectrophotometry at 412 nm. To complete this measurement, a cocktail solution consisting of 5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB, 3.75 mL 1 mM nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and 80 µL 625 U/L glutathione reductase was prepared. Before performing the measurement, 0.1 mL meta-phosphoric acid was added to 0.1 mL serum/tissue-homogenate and centrifuged for 2 min at 2000 rpm for deproteinization. The 0.15 mL of cocktail solution was added to 50 µL of supernatant. The standard curve was obtained using glutathione disulfide (GSSG). Cardiac tissue tGSH concentration was calculated as µmol/g protein.

cTnI analyses: cTnI levels in the blood samples from the rats were measured with a VIDAS troponin I ultra assay kit using the enzyme-linked fluorescent assay (ELFA) technique. All steps of the test were performed automatically on the VIDAS device using the test reagents available in the kit. The sample was transferred to a well that included anti-cardiac cTnI antibodies labeled with alkaline phosphatase (conjugate). The sample-conjugate mixture was transferred to the solid-phase receptacle and left there, which facilitated binding to the cTnI and conjugate, which were themselves bound to the inner wall of the antigen solid-phase binder. The unbound content was removed by washing. The conjugate enzyme catalyzed the hydrolysis of 4-methyl umbelliferyl phosphate, the substrate, to 4-methyl umbelliferon, a product with a fluorescence of 450 nm. The intensity of the fluorescence was in proportion to the antigen concentration in the sample. The blood serum cTnI concentration was calculated in terms of µg/L.
**CK analyses:** CK levels in the blood samples of the animals were measured photometrically with the Roche/Hitachi Cobas c 701 system. All steps of the test were carried out in accordance with the procedure and by using the test reagents available. A UV- test was performed according to the following reactions. Equal molar amounts of NADPH and ATP occurred at the same rate, and the rate of NADPH formation measured photometrically at 340 nm was directly proportional to CK activity. Blood serum CK concentration was calculated as U/L.

**CK-MB analyses:** CK-MB levels in the animal blood samples were measured using the Roche/Hitachi Cobas c 701 system. All steps of the measurement process were carried out using an immunological UV test, in accordance with the procedure used for the ready-made test reagents. The CK-MB isoenzyme included two subunits of CK-M and CK-B, both of which had active locations. The catalytic activity of CK-M subunits in the sample was inhibited by up to 99.6%, without affecting the CK-B subunits with the help of specific antibodies. The remainder of the CK-B activity corresponding to half of the CK-MB activity was determined using the total CK method. The blood serum CK-MB concentration was calculated in terms of U/L.

**Histopathological examination:** Tissue samples were fixed with 10% formaldehyde for 72 h and washed with tap water for 24 h following the fixation process. The tissue samples were then washed under tap water in cassettes for 24 h. The samples were then treated with a conventional grade of alcohol (70%, 80%, 90%, and 100%) to remove the water within the tissues. The tissues were then passed through xylol and embedded in paraffin. Four to five micron-thick sections were cut from the paraffin blocks, and hematoxylin–eosin (H&E) staining was administered. Sections were photographed and assessed using the DP2-SAL firmware program and a light microscope (Olympus Inc., Tokyo, Japan). From the serial sections taken, one central area and five peripheral areas were selected for semi-quantitative scoring, and the degeneration criteria were scored in the selected areas for each subject. Cardiac muscle tissue damage was defined as the presence of general tissue degeneration, myofiber atrophy, and dilatation/congestion. Each sample was scored in terms of each criterion, with 0 indicating no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage. Histopathological assessments and scoring were performed by a pathologist who was blinded to the experimental groups.

**Statistical analysis:** The results obtained from the experiments were expressed as mean values ± standard deviations (mean ± SD). The statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., released in 2013, Armonk, NY, USA). The normality of the distribution for the continuous variables in the biochemical test results was checked by the Shapiro–Wilk test. The significance of the differences between the groups was determined using a one-way ANOVA. Levene’s test was performed to determine whether a homogeneity of variances was achieved. Afterwards, Tukey’s honest significant differences (HSD) test was performed because the homogeneity of the variances was assumed. The probability value of p < 0.05 was indicative of statistical significance. For the histopathological data, the difference between the groups was determined by the Kruskal–Wallis test, a nonparametric test, and the group that generated the difference was determined by the Mann–Whitney U test (p < 0.05).

**RESULTS**

**Biochemical findings:** A comparison of the groups according to the biochemical results is presented in Figure 1A–E and Table 1. In the DG, MDA, cTnI, CK, and CK-MB levels were significantly higher (p < 0.001), and tGSH levels were significantly lower (p < 0.001) compared to the HG. When the DG and TDG were compared, the MDA, cTnI, CK, and CK-MB levels of the TDG were significantly lower (p < 0.001), and the tGSH levels were significantly higher than those of the DG (p < 0.001). We found no statistically significant difference between TDG and HG in terms of MDA, cTnI, CK, and CK-MB levels (p > 0.05). However, tGSH levels were significantly lower in the TDG (p = 0.010).
Figure 1. The levels of cardiac tissue (A) MDA, (B) tGSH, and blood serum (C) cTnI, (D) CK, (E) CK-MB in experimental groups

Footnotes: Bars are mean±SD. a means p<0.001 according to the HG group, b means p<0.001 according to the DG group, and c means p<0.05 according to the HG group.

Abbreviations: HG: healthy control group; DG: doxorubicin group; TDG: taxifolin+doxorubicin group; MDA: malondialdehyde; tGSH: total glutathione; cTnI: cardiac troponin I; CK: creatinine kinase; CK-MB: creatinine kinase-MB.

Table 1. Mean and standard deviation values of the groups in terms of biochemical test results (p-values of post-hoc comparisons between experimental groups are given for variables).

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>HG</th>
<th>DG</th>
<th>TDG</th>
<th>HG vs. DG</th>
<th>HG vs. TDG</th>
<th>DG vs. TDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>1.74±0.41</td>
<td>5.59±0.38</td>
<td>2.11±0.49</td>
<td>&lt;0.001</td>
<td>0.149</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>tGSH</td>
<td>4.62±0.27</td>
<td>1.41±0.32</td>
<td>4.18±0.34</td>
<td>&lt;0.001</td>
<td>0.010</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cTnI</td>
<td>0.01±0.00</td>
<td>0.26±0.04</td>
<td>0.03±0.01</td>
<td>&lt;0.001</td>
<td>0.300</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CK</td>
<td>98.50±2.26</td>
<td>182.67±6.80</td>
<td>104.50±5.47</td>
<td>&lt;0.001</td>
<td>0.147</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CK-MB</td>
<td>269.67±17.4</td>
<td>583.00±53.6</td>
<td>287.33±27.0</td>
<td>&lt;0.001</td>
<td>0.681</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: HG: healthy control group; DG: doxorubicin group; TDG: taxifolin+doxorubicin group; MDA: malondialdehyde; tGSH: total glutathione; cTnI: cardiac troponin I; CK: creatinine kinase; CK-MB: creatinine kinase-MB.

Footnotes: Tukey's honest significant difference (HSD) post-hoc test was used for pairwise comparisons. Results are presented as mean±standard deviation.
Histopathological findings: The structure and morphology of the cardiac muscle fibers and blood vessels were normal in the HG (Figures 2A and 2B). A microscopic examination of the DG showed obvious cardiac muscle tissue damage. Dilated myofibers, marked fiber size variability, and locally atrophic myofibers were prominent across the tissue. The cardiac muscle myofibers contained several atrophic fibers and mostly congested blood vessels (Figure 2C). In the TDG, prior treatment of the rats with taxifolin demonstrated marked improvement throughout the heart tissue and mostly normal blood vessels (Figure 2D). The histopathological evaluation results can be seen in Table 2.

Table 2. Histopathological evaluation results.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Degeneration</th>
<th>Myofiber atrophy</th>
<th>Congestion / Dilatation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>0.00±0.00a</td>
<td>0.00±0.00a</td>
<td>0.00±0.00a</td>
</tr>
<tr>
<td>DG</td>
<td>2.69±0.25b</td>
<td>2.34±0.26b</td>
<td>2.78±0.13b</td>
</tr>
<tr>
<td>TDG</td>
<td>1.64±0.12ab</td>
<td>0.89±0.40ab</td>
<td>0.98±0.48ab</td>
</tr>
</tbody>
</table>

Abbreviations: HG: healthy control group; DG: doxorubicin group; TDG: taxifolin+doxorubicin group.

Footnotes: Groups marked with the same letter in the same column are statistically similar (p>0.05), while there is a statistical difference in groups with different letters (p<0.05). Kruskal Wallis test was used for statistical analysis and Mann Whitney U test was used as post hoc. Results are presented as mean±standard deviation.
DISCUSSION

The present study biochemically and histopathologically investigated the effect of taxifolin on DXR-induced cardiotoxicity in rats. The biochemical experimental results showed that DXR increased MDA, cTnI, CK, and CK-MB levels and decreased tGSH levels. Previous studies have demonstrated that DXR disrupts the antioxidant balance by inducing ROS overproduction, which results in oxidative damage in heart tissue (Alam et al. 2018; Zhao et al. 2018). ROS has been known to accelerate lipid peroxidation (LPO) in cell membranes (Su et al. 2019). MDA is the toxic end-product of LPO that causes cross-binding to membrane components, which ultimately exacerbates oxidative cell damage (Ayala et al. 2014). Many studies have reported that DXR increases MDA levels in cardiac tissue by accelerating LPO (Polat et al. 2015; Alam et al. 2018). In the present study, the high MDA tissue levels in the DG suggest that our experimental results are in line with the findings in the existing literature.

GSH is a tripeptide consisting of a combination of glutamate, cysteine, and glycine and is one of the most important antioxidants in living tissues (Lushchak 2012). GSH protects cells from ROS damage by detoxifying organic peroxides, hydroxyl radicals, and hydrogen peroxide (Lushchak 2012; Kurutas 2016). Previous studies have associated DXR-induced cardiac damage with a reduction in GSH (Polat et al. 2015; Alam et al. 2018). The present study revealed that tGSH levels were decreased in the DG. Specifically, high MDA levels and low tGSH levels in the DG indicated that the oxidant/antioxidant balance in the heart tissue changed in favor of oxidants.

An increase in the serum levels of cTnI, CK, and CK-MB is considered to be an indicator of cardiac myocardial damage (Polat et al. 2015; Abdelatty et al. 2021; Basit et al. 2022). Disruption of cell membrane integrity leads to elevated cTnI, CK, and CK-MB enzyme levels in systemic circulation (Ahmed et al. 2021; Elblehi et al. 2021). Furthermore, DXR-induced ROS causes the degeneration of cell membrane phospholipids (Ahmed et al. 2021). After DXR administration, the cTnI, CK, and CK-MB levels in rat serum samples increased proportionally to the increase in oxidant parameters (Polat et al. 2015). In the present study, the increase in cTnI, CK, and CK-MB levels in rat serum samples after DXR administration suggests compatibility with the findings reported in the existing literature.

Another key finding is that taxifolin, a powerful antioxidant, significantly suppressed the increase in MDA and significantly prevented the decrease in the tGSH level induced by DXR. Tang et al. demonstrated taxifolin’s cardioprotective effect by showing that it significantly suppressed the increased MDA levels in cardiac ischemia-reperfusion in rats (Tang et al. 2019). Furthermore, Obeidat et al. reported that taxifolin protects heart tissue against oxidative damage by antagonizing the increased MDA and decreased GSH levels in rat heart tissue in cases of isoproterenol-induced cardiac damage (Obeidat et al. 2022). Our study’s results and the information obtained from the literature show that taxifolin protects cardiac tissue against oxidative damage, possibly by strengthening the cellular antioxidant defense and preventing LPO. Moreover, it was found that the cTnI, CK, and CK-MB levels approached normal values as a result of taxifolin administration. Consistent with previous studies, taxifolin administration exhibited a preventive effect on heart damage by bringing cTnI, CK, and CK-MB levels closer to those of the HG (Tang et al. 2019; Obeidat et al. 2022). Overall, these results indicate that taxifolin protects cardiac tissue from DXR-induced oxidative damage by stabilizing the cell membrane.

Our histopathological findings were consistent with the biochemical test results. DXR treatment caused dilatation of myofibrils, variation in fiber size, and locally atrophic myofibrils. Cardiac muscle myofibers contained several atrophic fibers and mostly congested blood vessels. In line with the present study, Polat et al. reported histopathological findings, such as vessel congestion, cytoplasmic vacuolization, myofibrillar loss, necrotic areas, and pycnotic nuclei, in DXR-administered rat heart tissue (Polat et al. 2015; Zhao et al. 2018). Our findings are also consistent with the study by Obeidat et al., who reported that taxifolin prevents isoproterenol-induced oxidative heart damage (Obeidat et al. 2022). Likewise, in this study, treatment with taxifolin prevented DXR-induced damage and restored cardiac tissue integrity.

Conclusion: The results of the present study biochemically and histopathologically showed that DXR-induced oxidative heart damage. To our knowledge, this study is the first to demonstrate that taxifolin treatment is able to reverse increased oxidative stress and provide protection against cardiac damage in a DXR-induced cardiotoxicity model in rats. Overall, our experimental results suggest that taxifolin may be useful in the treatment of DXR-associated cardiac damage. To further elucidate this mechanism, studies on the molecular foundations behind this process are needed. Specifically, total oxidant, total antioxidant, and pro-inflammatory cytokine levels must be measured to clarify the action mechanism of taxifolin against DXR-induced cardiac damage.

Conflict of interest: The authors declare that there is no conflict of interest.

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Author’s Contribution: MNA and HS conceived and designed the study. RM, BC, and BY conducted the experiments. GNY and MG analyzed the serum and tissue samples. MNA, AS, and RC analyzed data. MNA, AVK, AS, and HS wrote
the manuscript. All authors read the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

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