

Cardioprotective Effect of Clarithromycin on Doxorubicin-Induced Cardiac Toxicity in Rats

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ABSTRACT

Doxorubicin is an anthracycline group antibiotic and has long been used as an antineoplastic agent. The most important side effect that limits the usage of doxorubicin is cardiotoxicity, which is observed at cumulative doses. We investigated the protective effect of clarithromycin that is known to have antioxidant and anti-inflammatory effects against Doxorubicin related cardiotoxicity. The aim of our study was to evaluate the effects of clarithromycin in the antioxidant enzyme status and myocardium of doxorubicin-treated rats. A total of 28 adult, male Wistar rats (200-250 g) were divided into 4 groups. Group 1 was the control group, rats in group 2 were administered doxorubicin, rats in group 3 were administered clarithromycin, and rats in group 4 were administered Doxorubicin + Clarithromycin. Following the scarification of all rats, antioxidant (glutathione) and oxidant (malondialdehyde) levels were measured in the cardiac tissue. Additionally, the cardiac muscles were evaluated histopathologically via hematoxylin eosin staining. The antioxidant amount (glutathione) was significantly higher in the treatment group compared to the doxorubicin group ($p= 0.025$), whereas the amount of oxidant (malondialdehyde) was significantly lower ($p= 0.022$). The histopathological examination revealed significant cardiotoxicity in the Doxorubicin group and significant reduction in the cardiotoxicity in the Doxorubicin + Clarithromycin group. The results obtained in this study provide evidence for the usefulness of the clarithromycin as a cardioprotective agent.

Keywords: Doxorubicin, Cardiotoxicity, Clarithromycin, Rat

ÖZET

Ratlarda Oluşturulan Doksorubisin'e Bağlı Kardiyotoksistede Klaritromisin'in Koruyucu Etkisi

Doksorubisin antrasiklin grubu antibiyotiktir ve uzun süredir antineoplastik ajan olarak kullanılmaktadır. Doksorubisin kullanımını kısıtlayan en önemli yan etkisi kümülatif dozlarda gözlenen kardiyotoksistedir. Antioksidan ve antiinflamatuvar etkileri bilinen klaritromisin'in, doksorubisin ilişkili kardiyotoksisteye karşı koruyucu etkisini araştırdık. Çalışmamızın amacı doksorubisin ile tedavi edilen ratlarda klaritromisin'in myokarda ve antioksidan enzimler üzerine etkilerini değerlendirmektir. Çalışmaya alınan toplam 28 yetişkin erkek Wistar ratı (200-250 g) 4 gruba ayrıldı. Grup 1 kontrol grubuydu, grup 2' de ki ratlara doksorubisin, grup 3' de ki ratlara klaritromisin ve grup 4' e doksorubisin+klaritromisin (tedavi grubu) uygulandı. Skarifikasyonu takiben tüm ratların kalp dokusunda antioksidan (glutatyon) ve oksidan (malodialdehid) düzeyleri ölçüldü. Buna ek olarak myokard dokusu hematoksilen-eozin ile boyanarak histopatolojik olarak değerlendirildi. Antioksidan (glutatyon) miktarı, tedavi grubunda doksorubisin grubuyla karşılaştırıldığında önemli derecede yüksekti ($p= 0.025$), oysa oksidan (malodialdehid) miktarı önemli derecede düşük saptandı ($p= 0.022$). Histopatolojik değerlendirmede doksorubisin grubunda ciddi kardiyotoksisteye saptanırken doksorubisin + klaritromisin uygulanan grupta kardiyotoksisteye de ciddi azalma belirlendi. Bu çalışmada elde edilen sonuç, klaritromisin'in doksorubisine bağlı kardiyotoksistenin engellenmesinde kullanılabilecek kardiyoproduktif etkiye sahip bir ajan olduğudur.

Anahtar Kelimeler: Doksorubisin, Kardiyotoksisteye, Klaritromisin, Rat

INTRODUCTION

Doxorubicin (DXR) is an anthracycline group antibiotic that is widely used in the treatment of haematological and solid tumours in human since late 1960s. The most feared and usage limiting side effect of DXR is cardiotoxicity.¹⁻³ This effect is observed in the cumulative doses. The time of cardiotoxicity onset may depend on the patients. The prognosis of DXR dependent cardiomyopathy is worse than that of ischemic cardiomyopathy. Currently, there is no treatment option other than heart transplantation in the terminal stage treatment of DXR related chronic heart failure.²⁻⁵ DXR-induced cardiotoxicity is mediated by the formation of free radicals that oxidatively damage many cellular components.^{1,2,5} DXR is enzymatically reduced to its semiquinone radical.⁶ This DXR semiquinone radical directly transfers its electron to molecular oxygen, generating a superoxide radical and hydrogen peroxide.⁷ As reactive oxygen species (ROS) play important roles in DXR-induced toxicity, many compounds with antioxidant properties have been examined as a potential therapeutic and/or protective agent. Among these compounds were p-coumaric acid⁸, garlic acid¹, erdosteine⁵, carvedilol², and lycopene.³ Many experimental studies have been conducted in order to prevent the cardiotoxic effect of DXR.⁹⁻¹¹ The agents used in these studies were usually antioxidants that were ceased at the animal study level, and did not take place in the clinical practice. Clarithromycin (CLT), which was used in our study, is U.S. Food and Drug Administration (a-FDA) approved drug with clinical usage and known effects and side-effects. Clarithromycin is a macrolide group antibiotic with a cardioprotective effect

and previously determined anti-inflammatory and antioxidant effects.¹²⁻¹⁵ Clarithromycin suppresses various transcription factors such as activator protein 1 and nuclear factor kappa B. Its cardioprotective, anti-inflammatory and antioxidant effects were mediated by the inhibition of proinflammatory cytokine production and matrix metalloproteinase (MMP) activity via suppressing these transcription factors. Based on this effect of clarithromycin, we investigated its mechanism of action in preventing the cardiotoxic effect of DXR.¹⁴

MATERIALS AND METHOD

Animals

Twenty-eight healthy male Wistar albino rats, weigh-

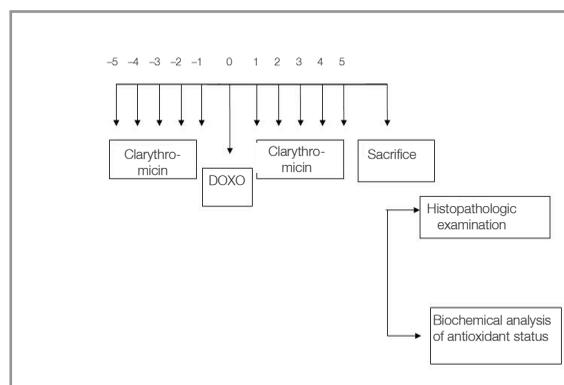


Figure 1. Experimental design of treatment group

ing 200-250 g and averaging 16 weeks old were utilized in this study. The animals were purchased from Pamukkale University Animal Care and Research Unit. Rats were fed on a standard rat chow and tap water ad libitum. In the windowless animal quarter automatic temperature (20-22°C) and lighting controls were used (12 hour light/12 h dark cycle). Humidity ranged from 50 to 55%. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the Institutional Animal Ethical Committee of Pamukkale University, Denizli, Turkey.

Experimental Design: The rats were randomly allotted to one of four experimental groups: control, DOX-treated, only clarithromycin and DOX-treated with clarithromycin; each group contained seven animals (Figure 1)

Experimental Procedures and Sample Collection: Rats were randomly divided into 4 groups; each group included 7 rats. The rats in group 1 were administered a single dose of 5 ml/kg distilled water intraperitoneally for 10 days (control group). The rats in group 2 were administered a single dose of 5 ml/kg distilled water for the first 5 days and a single dose of doxorubicine (Adriblastina 50 mg/mL/Deva/Turkey) 15 mg/kg in the 6th day intraperitoneally. Then 5 ml/kg distilled water were given for another 5 days (placebo group). The rats in-group 3 were administered a single dose of clarithromycin (diluted with distilled water) (Klacid, 50 mg/mL, Abbott Laboratories, Istanbul, Turkey) 40 mg/kg intraperitoneally for 10 days [clarithromycin group]. The rats in group 4 were

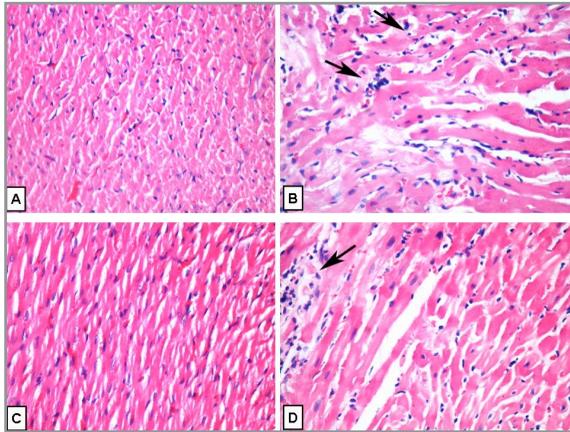


Figure 2. Photomicrograph of the heart tissues of control (a) showing the normal myofibrillar structure with striations and branched appearance. Heart tissues of DXR-treated rats (b) showing focal necrosis of muscle fibers with eosinophilia in the cytoplasm. Heart tissues of DXR-treated rats also show deformations of muscle fibers with focal hemorrhage and inflammatory cell infiltrations (arrows). Rats treated with CLT+DXR (d), exhibiting relatively normal myocardial cells with vascular dilatation and moderate degeneration of some fibril cells. (hematoxylin and eosin, x200.). The heart tissues of clathromycin group (c) showing the normal myofibrillar structure with striations and branched appearance.

administered clarithromycin 40 mg/kg diluted with distilled water intraperitoneally for the first 5 days. On the 6th day, a single dose of doxorubicin 15 mg/kg diluted with distilled water was administered intraperitoneally. Following the administration of doxorubicin, clarithromycin was administered intraperitoneally for an additional 5 days (treatment group). At the end of treatments and after the last clarithromycin and distilled water administration, blood and heart tissues were collected from all groups and stored at -20°C for further processing (Figure 1)

Histopathologic Examination: Heart tissues were harvested from the sacrificed animals, fixed in 10% neutral formalin and embedded in paraffin blocks. Sections of 5mm were obtained, deparaffinized and stained with hematoxylin and eosin. The heart tissue was examined, evaluated and photographed in random order under blind conditions with standard light microscopy (Nikon Optiphot 2, Tokyo, Japan). The severity of changes was quantitated as none (-) to severe (+++) based on the degree of cytoplasmic vacuolization, myocardial disorganization and myofibrillar loss. The scoring system was as follows: (-) no damage, (+) mild, (++) moderate, and (+++) severe damage.

Analysis of Malondialdehyde (MDA) Levels: The levels of 2-thiobarbituric acid-reactive substances (TBARS) were determined following the procedure reported by Ohkawa et al. After thawing, each sample was weighed and homogenized 10 times (w/v) using a 0.15 N potassium chloride solution (Sigma-Aldrich); 0.4 ml of homogenate was mixed with 1.5 ml thiobarbituric acid (0.8%), 1.5 ml acetic acid (pH: 3.5, 20%) and 0.2 ml sodium dodecyl sulfate (8.1%) (Sigma-Aldrich). After mixing, all samples and standards were heated at 100°C for 1 h. Absorbance was recorded at 532 nm and compared with that of the TBARS standards.¹⁶

Quantification of Reduced Glutathione Levels: Reduced glutathione (GSH) was estimated using a modification of the procedure described by Moron et al. as follows: After homogenization of tissue samples with 0.15 N potassium chloride, 0.5 ml homogenate was mixed with 3 ml of deproteinization solution (sodium chloride, metaphosphoric acid, EDTA, and distilled water) and 1.5 ml potassium chloride solution (SigmaAldrich). Each sample was centrifuged at $1000 \times g$ for 5 min, and 0.5 ml of the supernatant was added to 2 ml of Na_2HPO_4 and 0.5 ml of Ellman's reactive (DTNB; dithiodinitrobenzoic acid, sodium citrate, distilled water) (Sigma-Aldrich Corp). The absorbance of supernatants was recorded at 412 nm and compared with GSH standards.¹⁷

Statistical Analysis

All statistical analyses were carried out using SPSS statistical software (SPSS PASW statistics 18.0). All data are presented in mean (\pm) standard deviations (SD). Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test. These differences were considered significant when probability was less than 0.05.

RESULTS

Histopathological effects on the cardiac tissue

No pathological change was observed in the histopathological examination of the control or clarithromycin group, whereas significant cytoplasmic vacuolization, myofibrillar disorganization and myofibrillar loss were detected in the DXR group. This negative effects was detected to be significantly decreased in the DXR + clarithromycin group (Table 1) (Figure 2).

Table 1. Effect of clarithromycin (clt) on morphological changes as assessed by histopathological examination of heart in the doxorubicin (DXR)-treated rats

GROUPS	Inflammation	Myocardial disorganization	Interstitial fibrosis
Control	(-)	(-)	(-)
DXR	(+) to (++)	(++) to (+++)	(+) to (++)
CLT	(-)	(-)	(-)
DXR+CLT	(-) to (+)	(-) to (+)	(-) to (+)

(-): none; (+): mild; (++): moderate; (+++): severe

Effects on the MDA and Glutathione levels in the cardiac muscle

The MDA level [oxidant system] within the cardiac tissue in DXR group was significantly higher than that of the control ($p= 0.002$) and clarithromycin groups ($p= 0.008$), whereas it was not higher in the treatment group than that of the control group ($p= 0.886$) (Table 2). The MDA level was significantly lower in the clarithromycin group than the control group ($p= 0.014$). These results indicate once more that clarithromycin has a reductive effect on the oxidant system (Table 2) (Figure 3).

The glutathione [GSH] level within the cardiac tissue in the DXR group was significantly lower than that of the control ($p:0,028$) and clarithromycin groups ($p= 0.022$). However, no significant reduction was observed in the DXR + clarithromycin group compared to the control group with regard to the GSH level ($p= 0.520$). The GSH level in the clarithromycin group was significantly higher compared to the control group ($p= 0.022$). These results indicate the antioxidant effects of clarithromycin (Table 2) (Figure 4).

The antioxidant amount (glutathione) was significantly higher in the treatment group compared to the doxorubicin group ($p= 0.025$), whereas the amount of oxidant (malondialdehyde) was significantly lower ($p= 0.022$) (Table 2).

DISCUSSION

In our study, the efficiency of clarithromycin was investigated in the prevention of cardiotoxic effects of DXR. There are many studies that demonstrate the cardiotoxic effects of DXR mediated by oxidative stress.¹⁸⁻²¹

Oxidative stress is an important biological process that is believed to cause severe damage to cell membranes.²² Oxidative stress has also been implicated as the major contributing factor to the DXR-induced deformation of heart tissues.^{2,5,23} In the present work, DXR-induced oxidative stress is confirmed by the elevation of oxidized lipids (MDA). Two different ways of free radical formation by DXR have been described. The first way implicates the formation of a semiquinone free radical by the action of several NADPH-dependent reductases that produce a one-electron reduction of the DXR to the corresponding DOX semiquinone. In the presence of oxygen, redox cycling of DXR-derived quinone-semiquinone yields superoxide radicals. In the second way, DXR free radicals come from a non-enzymatic mechanism that involves reactions with iron. DXR not only increases free radical production in the tissue, but also decreases its ability to detoxify reactive oxygen species.²⁴

DXR reduced the TAC content, Glutathione and the

Table 2. Comparison levels of MDA and GSH in study groups

	Control	Doxorubicine	Clarithromycin	Doxorubicine-Clarithromycin
Malondialdehyde	18.07±1.56 ^a	21.00±3.48 ^{ab}	14.09±3.14	17.36±2.30 ^b
Glutathione	12.25±3.00 ^c	10.10±2.36 ^{cd}	12.75±1.93	13.50±3.26 ^d

^a: ($p= 0.022$) comparison between doxo and doxo- clarithromycin groups (MDA)

^b: ($p= 0.025$) comparison between doxo and doxo- clarithromycin groups (GSH)

^c: ($p= 0.002$) comparison between control and doxo groups (MDA)

^d: ($p= 0.028$) comparison between control and doxo groups (GSH)

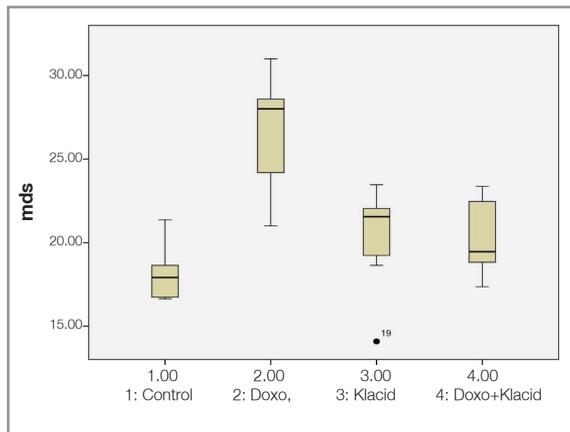


Figure 3. Comparison with MDA levels in groups.

antioxidant activity of SOD enzyme in the heart. Free radicals have been shown to exhaust the antioxidant defence system and hence elevate the oxidation process of both lipids and proteins in heart tissues of DXR-treated rats.^{2,5,23} In accordance with the previous studies, the glutathione level was found to be reduced in the DXR treatment group of our study as well, due to the increased functioning of the antioxidant system in order to compensate the oxidative stress and related increase in the consumption of glutathione.²³

DXR has been reported to cause cardiomyopathy in various animal species [3,4]. Similarly to many other studies, the DXR-induced myocardial damage reported here was associated with clear myocardial histopathological lesions (myocyte necrosis, degeneration, and inflammatory cell infiltrations).^{3,4,5,25} Similar to these studies, significant cytoplasmic vacuolization, myofibrillar disorganization and myofibrillar losses were observed in the histopathological examination of the cardiac tissues in the DXR administered rats compared to the control group, in our study.

There are many other studies demonstrating the antioxidant and anti-inflammatory effects of clarithromycin.¹²⁻¹⁵ Recent studies show that this macrolide antibiotic has anti-inflammatory activity, which likely depends on its ability to prevent the production of proinflammatory mediators and cytokines, and suggest that this agent can exert therapeutic effects independent of its antibacterial activity.¹²⁻¹⁵ The MDA level was significantly decreased and GSH level was significantly increased in the clarithromycin group compared to the control group in our study. The MDA level was significantly decreased and the GSH level was significantly increased in the treatment group compared to the DXR group. These results are evi-

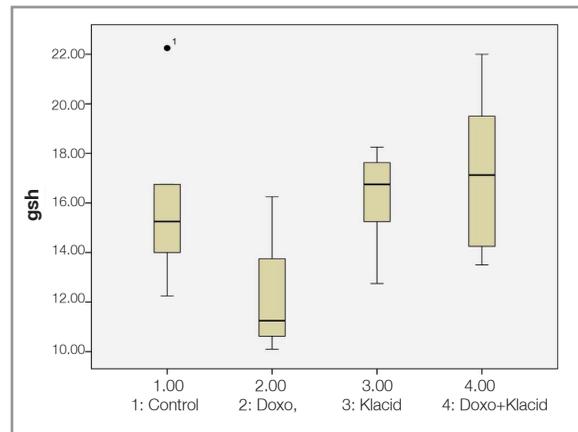


Figure 4. Comparison with GSH levels in groups.

dences of the antioxidant effects of clarithromycin.

The cardiotoxic effect in the treatment group was shown significantly reduced at a histopathological level compared to the DXR group. Based on these results, the cardioprotective effect of clarithromycin against DXR was evidently demonstrated at a histopathological level.

Cardiotoxic effect of Clarithromycin is rarely seen as ventricular arrhythmia and torsades de pointes in patients with long QT syndrome, but it does not have toxic effects on cardiac functions. In 2010, Guo D et al. published a review of 48 patients reported in literature. Eighteen of those 48 patients were reported as clinical study and 30 of them reported as case report and it was reported that all of those patients has had long QTc syndrome as adverse effect. In this review, it was reported that advanced age, high dosage, fast application of drug and concomitant cardiac pathology increase cardiac toxicity. Lack of our study is, we could not take ECG records of rats. However to prevent the doxorubicin toxicity clarithromycin usage with ECG recordings before and after drug admission could be more appropriate.²⁶

Many herbal agents that are known to be antioxidants have been used in previous studies in order to reduce the cardiotoxic effects of doxorubicin, and positive results were obtained.²⁷ However, none of these agents were introduced in the medical usage, and therefore, the positive and negative effects on human are not clearly understood. Thus, these agents could only reach the animal study level.^{3,5,28} Clarithromycin on the other hand, is an antibiotic with known antioxidant and anti-inflammatory effects for a long time. Therefore the effects and side effects of the drug is clearly known. We believe that clarithromycin

is a more proper agent compared to the agents used in other studies due to this effect. As a conclusion, clarithromycin is a directly effective drug in the prevention of DXR related cardiotoxicity.

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