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ORIGINAL ARTICLE

Protective effects of arbutin against doxorubicin-induced cardiac damage

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Abstract

Background Doxorubicin is an effective antineoplastic agent but has limited clinical application because of its cumulative toxicities, including cardiotoxicity. Cardiotoxicity causes lipid peroxidation, genetic impairment, oxidative stress, inhibition of autophagy, and disruption of calcium homeostasis. Doxorubicin-induced cardiotoxicity is frequently tried to be mitigated by phytochemicals, which are derived from plants and possess antioxidant, anti-inflammatory, and anti-apoptotic properties. Arbutin, a natural antioxidant found in the leaves of the bearberry plant, has numerous pharmacological benefits, including antioxidant, anti-bacterial, anti-hyperglycemic, anti-inflammatory, and anti-tumor activity.

Methods and results The study involved male Wistar rats divided into three groups: a control group, a group treated with doxorubicin (20 mg/kg) to induce cardiac toxicity, a group treated with arbutin (100 mg/kg) daily for two weeks before doxorubicin administration. After treatment, plasma and heart tissue samples were collected for analysis. The samples were evaluated for oxidative stress parameters, including superoxide dismutase, malondialdehyde, and catalase, as well as for cardiac biomarkers, including CK, CK-MB, and LDH. The heart tissues were also analyzed using molecular (TNF-α, IL-1β and Caspase 3), histopathological and immunohistochemical methods (8-OHDG, 4 Hydroxynonenal, and dityrosine). The results showed that arbutin treatment was protective against doxorubicin-induced oxidative damage by increasing SOD and CAT activity and decreasing MDA level. Arbutin treatment was similarly able to reverse the inflammatory response caused by doxorubicin by reducing TNF-α and IL-1β levels and also reverse the apoptosis by decreasing caspase-3 levels. It was able to prevent doxorubicin-induced cardiac damage by reducing cardiac biomarkers CK, CK-MB and LDH levels. In addition to all these results, histopathological analyzes also show that arbutin may be beneficial against the damage caused by doxorubicin on heart tissue.

Conclusion The study suggests that arbutin has the potential to be used to mitigate doxorubicin-induced cardiotoxicity in cancer patients.

Keywords Arbutin · Doxorubicin · Oxidative stress · Inflammation · Apoptosis · DNA/RNA damage

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Introduction

Doxorubicin (DX) is a potent antitumor agent that has been effectively used to treat a variety of cancers, such as breast, ovarian, lung, and uterine cancers, as well as soft-tissue sarcomas [1–3]. Unfortunately, its clinical use is restricted because of its cumulative toxicities, especially its cardiotoxicity, which has been extensively studied [4, 5]. The harmful effects of DX-induced cardiotoxicity comprise lipid peroxidation, oxidative stress, genetic impairment, inhibition of autophagy, apoptosis, and disruption of calcium homeostasis [6]. NADPH-cytochrome P-450 enzymes metabolize DX, producing superoxide anions and hydroxyl radicals that damage cellular membranes [7]. Additionally, excessive DX exposure has been linked to cardiac inflammation [8, 9].

DX is an effective chemotherapeutic drug, preventing and treating its associated cardiotoxicity remains a major research focus. Dexrazoxane, an efficacious agent utilized in combating the cardiotoxic effects induced by DX, stands as a cornerstone in oncological care [10]. However, its utilization comes with inherent limitations and drawbacks. Notably, its administration is tethered to specific patient demographics, such as pediatric and geriatric populations, and necessitates judicious dosing regimens. Moreover, while dexrazoxane mitigates cardiotoxicity, its employment may introduce rare adverse effects and pose risks of drug interactions [11]. The common side effects that culminate from dexrazoxane use include dose-limiting myelotoxicity (neutropenia, leukopenia, granulocytopenia, and thrombocytopenia). Thus, despite its therapeutic utility, the imperative for novel drug development persists. The quest for innovative pharmacotherapeutics arises from the ambition to devise agents that surpass the constraints of existing options, striving for enhanced efficacy, broader applicability across patient cohorts, and minimized adverse effects. In essence, while dexrazoxane remains a stalwart in cardioprotection, the exigency for pioneering pharmaceutical endeavors remains paramount in elevating the standards of oncologic care [10, 12].

One promising approach is the use of phytochemicals, which are small molecules derived from plants that possess antioxidant, anti-inflammatory, and anti-apoptotic properties [13]. Natural compounds have demonstrated their potential in the treatment of cardiovascular diseases. As a result, many phytoconstituents have been studied and have been shown to effectively mitigate DX-induced cardiotoxicity [14].

Arbutin (ARB) is a natural antioxidant that is primarily found in bearberry plant leaves [15]. It is well-known for its numerous pharmacological benefits, such as antioxidant, anti-bacterial, anti-hyperglycemic, anti-inflammatory, and anti-tumor activity [16, 17]. In addition to these benefits, ARB has also demonstrated its positive effects in treating isoproterenol-induced cardiac hypertrophy in animals [18]. In light of these findings, we conducted a study to investigate the potential beneficial impact of ARB in mitigating DX-induced cardiotoxicity in animals.

Materials and methods

Chemicals

We obtained DX (CAS No.: 25316-40-9) and ARB (CAS No.: 497-76-7) from Sigma-Aldrich, Germany.

Animals

In compliance with the "Guide for the Care and Use of Laboratory Animals" and the approval of the Ataturk University-Ethical Committee, all tests were conducted (2200370605). The study subjects consisted of male Wistar rats that were pathogen-free and were seven weeks old. The rats were housed at the Animal Experimental Center of Ataturk University and had access to food and water ad libitum. The facility was maintained at a temperature of 25 °C, and the rats were exposed to a 12-hour light and 12-hour dark cycle.

Methodology for conducting experiments

After being fed a specific diet for one week, the rats were separated randomly into three groups, each containing six rats. Group I: The control group was given sterile saline solution in the same amount as the experimental groups. Group II: In accordance with previous studies, rats in Group II (DX) were treated with a single intraperitoneal dose of 20 mg/kg DX that was dissolved in 0.9% normal saline to induce acute cardiac toxicity [19, 20]. In Group III (DX + ARB), the rats were given 100 mg/kg ARB [18, 21, 22] daily for two weeks and then administered a single dose of 20 mg/kg DX. Following two days of DX treatment, the rats were given sodium thiopental anesthesia, then euthanized and subjected to various experimental procedures.

Collection of plasma and heart tissue samples for analysis

Once the serum was centrifuged at 3000 rpm for 15 min, it was collected for the evaluation of Creatine Kinase (CK), Creatine Kinase-Myocardial Band (CK-MB), and Lactate Dehydrogenase (LDH) using ELISA kits based on the instructions provided by the manufacturer. Measurements of LDH, CK and CK-MB, were conducted using the standard protocol provided by the commercially available kits (Cat

No: E-EL-R2547, E-EL-R0274, and E-EL-R1327 respectively, Elabscience, USA) The heart tissue was immediately dissected and was removed and washed in cold saline. Next, the heart was carefully dissected into two sections. The one half of the heart was mixed and homogenized in PBS solution and then immediately stored at a temperature of − 20 °C for later analysis of oxidative stress parameters including superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT) (Cat No: E-EL-R1424, E-EL-0060, and E-BC- K031-M respectively, Elabscience, USA) and molecular analysis including TNF-α, IL-1β and caspase 3. All absorbance measurements were conducted at 450 nm with a spectrophotometer (BIOTEK Instruments, USA) [23, 24]. The other half of the heart tissue were placed in a solution of 10% formal saline for future investigation using histopathological and immunohistochemical techniques as previously described [24, 25].

Real-time quantitative PCR analysis

RT-PCR analysis was conducted following the methods outlined in previous literature [26]. The relative mRNA expressions of TNF-*α*, IL-1β, and caspase-3 mRNA in heart tissue were assessed by real time polymerase chain reaction (RT-PCR) system (QIAGEN-Rotor-Gene Q) (Hilden-Germany). Quadruplicate determinations were performed for each tissue sample, utilizing a 96-well optical plate. Each reaction included 2.5 µl of cDNA (100 ng), 1 µl of TaqMan gene expression assay, 10 µl of TaqMan PCR MasterMix (supplied by Applied Biosystems), and 6.5 µl of RNase-free water, totaling 20 µl per reaction. The plates were subjected to initial heating at 50 °C for 2 min followed by 10 min at 95 °C. Subsequently, 40 cycles were run, consisting of 15 s at 95 °C and 60 s at 60 °C for each cycle [27]. The obtained target gene expression levels were normalized to the housekeeping gene *β*-actin. The PCR primers utilized are listed in Table 1. The results were obtained with the $2^{-\Delta\Delta Ct}$ method.

Tissue analysis of heart using histopathological methods

Samples of heart tissues were taken from all rats in each group, and then preserved in a solution called neutral buffered formalin. They were then processed according to a standard protocol. Thin sections of the tissues, about 5 micrometers in thickness, were cut and stained with Hematoxylin and Eosin (H&E) dye. The stained sections were then examined under a light microscope. A pathologist who did not know which treatment the rats had received, looked at the samples to see if there were any abnormal changes in the tissues [23].

Immunohistochemical analysis

The heart sections were exposed to primary antibodies against 8-OHDG, 4 Hydroxynonenal (4-HNE), and dityrosine (DT), which were diluted 1:200. Diaminobenzidine tetrachloride (DAB) was used to visualize the immune reaction. The staining was graded as negative, weak, moderate, or strong depending on the intensity of the staining [26]. The percentage of area expressing 8-OHDG, 4-HNE, and DT was estimated by measuring the stained areas of each section and calculating an average using imaging software called Image J. The person analyzing the images was not aware of the treatments given to the animals. The individual examining the images was blinded to the treatments administered to the animals.

Statistical analysis

The data collected from the experiments were analyzed using the SPSS STATISTIC software version 23, and the results were presented as mean ± standard deviation (mean ± SD). The ELISA and RT-PCR results were subjected to a one-way ANOVA followed by post hoc Tukey's test, while the histopathological and immunohistochemical evaluations were analyzed using the Kruskal-Walls test followed by post hoc Mann-Whitney U test. A *p*-value of less than 0,001 was considered statistically significant.

Results

Cardiac biomarkers

The concentrations of serum cardiac biomarkers, including CK-MB, CK, and LDH, were assessed to ascertain the occurrence of DX-induced cardiotoxicity and to evaluate any potential protective effects conferred by ARB. In the DX group, there was a significant increase in CK-MB, CK, and LDH ($p < 0.001$) compared to the normal control group. However, in the treatment group, there was a

Table 1 Sequence list of the primers used for RT-PCR

Genes	Forward Sequence (5′-3′)	Reverse Sequence (3′-5′)
TNF-α	CACACGAGACGCTGAAGTAG	AACAGTCTGGGAAGCTCTGA
IL-1β	TGCTGTGTGATTGCAGACAA	GTACAGCGTTCCCAGTCATC
Caspase-3	TGGACAACAACGAAACCTCC	CCAGGCACAGTATCTCTTGC

Tnf-α, tumor necrosis factor alpha; IL-1β: interleukin 1β

significant decrease in CK-MB and CK compared to the DX group. Additionally, ARB significantly reduced CK-MB ($p < 0.001$) compared to the DX group. The increase in CK-MB, CK, and LDH levels indicate damage to the cardiomyocytes. These findings showed that DX induced cardiotoxicity in rats, while treatment with ARB protected against it by reducing CK-MB (Fig. 1A), CK (Fig. 1B) and LDH (Fig. 1C) levels.

Reduced oxidative damage, inflammation and apoptosis in cardiac tissues with ARB treatment against DX-induced injury

We found that giving DX to rats markedly decreased the activity of enzymes like SOD (Fig. 2A) and CAT (Fig. 2B) compared to control rats. However, when the rats were given ARB before DX, marked improvement was seen in the activity of these enzymes, compared to rats that were only given DX (shown in Fig. 2). In addition, giving DX to rats caused an elevation in the amount of MDA (Fig. 2C), a biomarker of oxidative stress, in their heart tissues, and a decrease in the activity of CAT and SOD enzymes in their hearts, compared to control rats. But when rats were given ARB before DX, there was a significant reduction in MDA levels and an increase in the activity of these enzymes in their heart tissues, compared to rats that were only given DX (Fig. 2).

DX treated rats showed marked increase in mRNA expressions of TNF-α (Fig. 3A) and IL-1β (Fig. 3B) in the cardiac tissues compared to the control group. Despite that, in the ARB group, the expression levels of TNF-α and IL-1β were reduced compared to the DX group (Fig. 3). Furthermore, caspase-3 levels in the cardiac tissues were markedly elevated in the DX group compared to the control group. ARB treatment resulted in a significant reduction in caspase-3 levels (Fig. 3C).

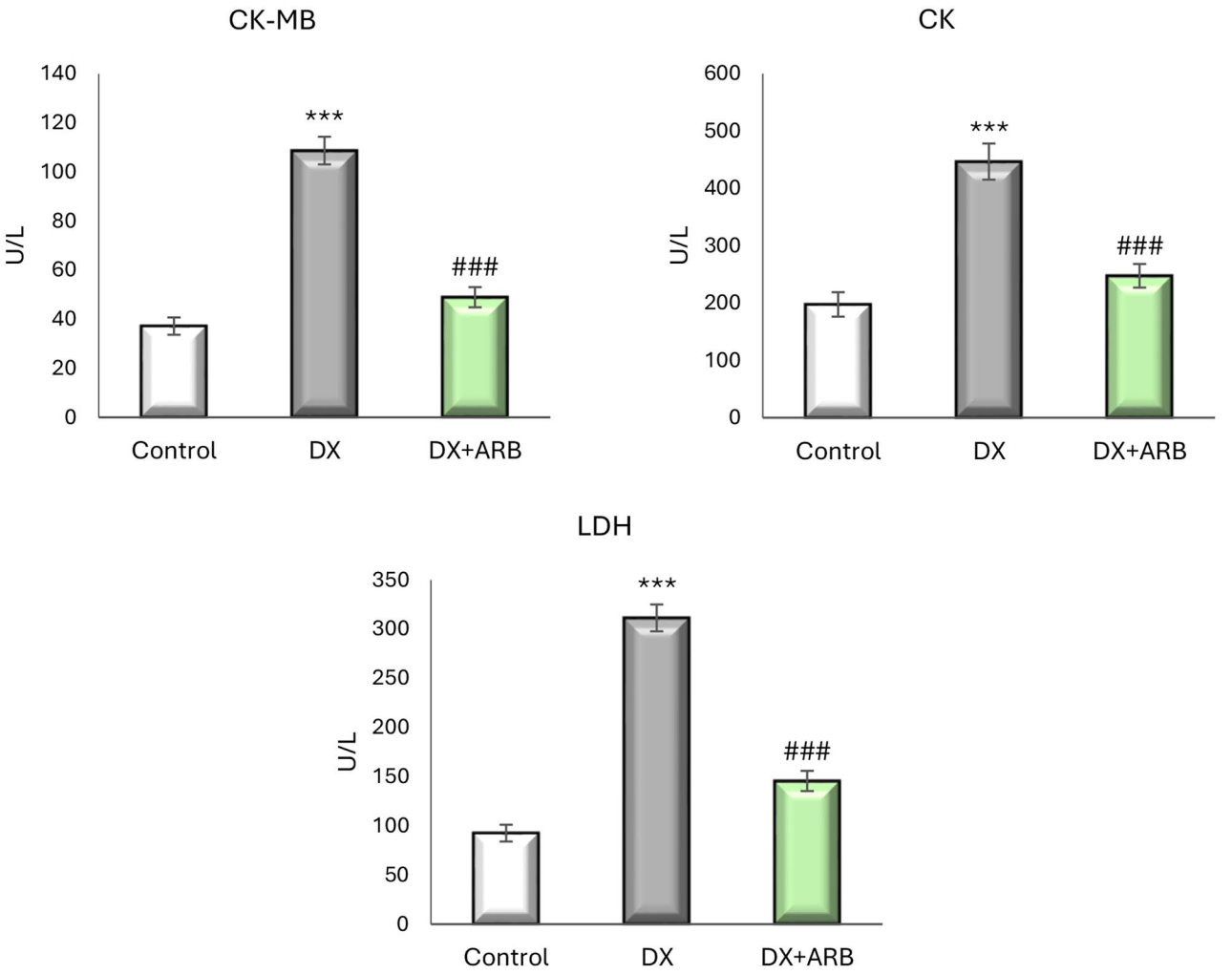

Fig. 1 Serum levels of cardiac markers in control and treatment groups. *** $p < 0{,}001$ vs. control group, ### $p < 0{,}001$ vs. DX group

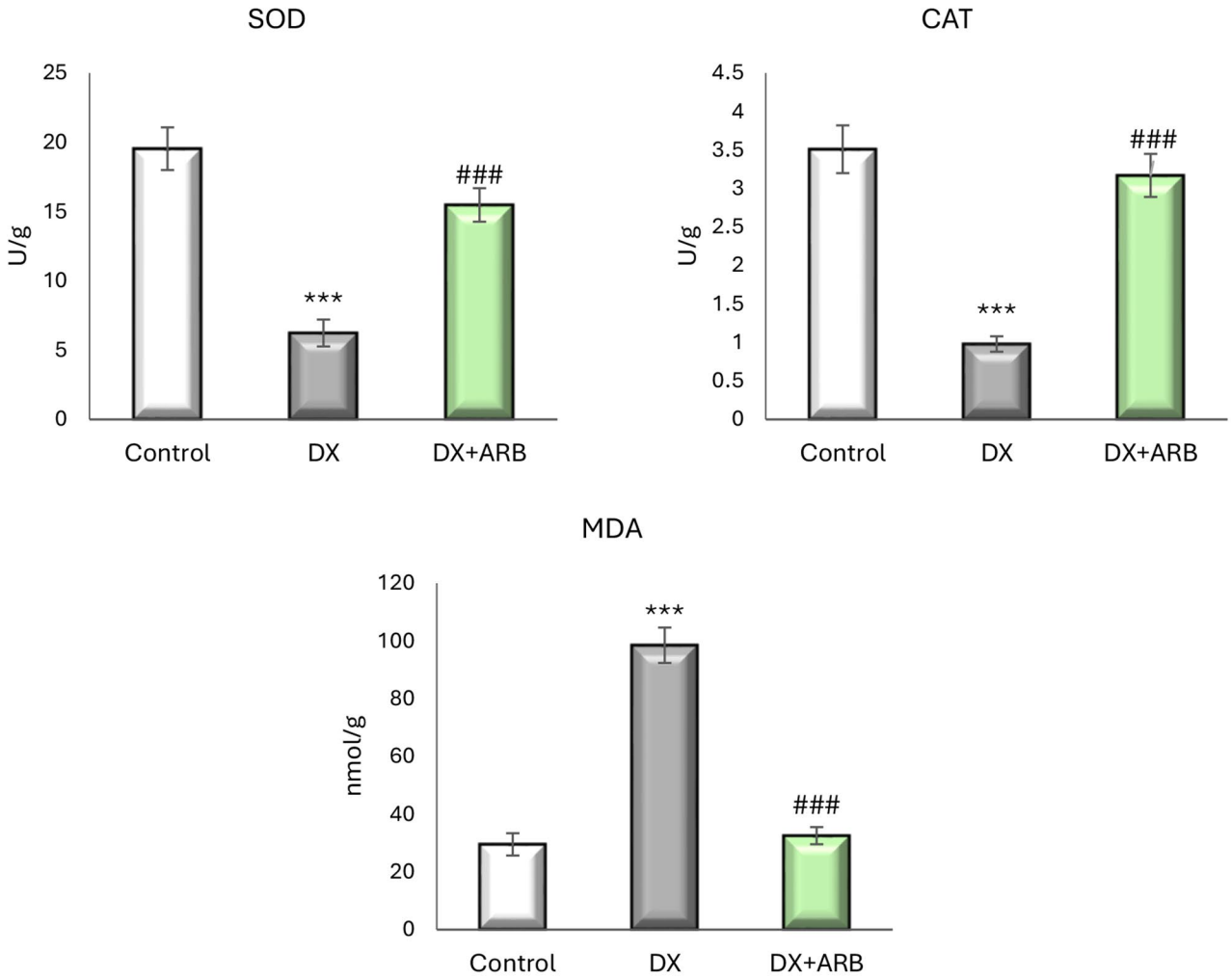

Fig. 2 Tissue levels of oxidative stress markers in control and treatment groups. *** $p < 0{,}001$ vs. control group, ### $p < 0{,}001$ vs. DX group

Histopathological analysis

The slides of heart muscle tissue from different groups of rats were examined. The control group had healthy muscle tissue with visible muscle striations and central nuclei. However, the groups treated with DX had damaged tissue with loss of striations and vascular congestion. The group treated with ARB showed a significant improvement in the tissue, with no notable damage or necrosis. These findings suggest that ARB may have a protective effect on the heart tissue (Fig. 4).

Immunohistochemical analysis

The analysis showed that 8-OHDG, 4-HNE, and DT expression was significantly higher in the DX group in comparison with the control group. However, the ARB pretreatment group had significantly lower 8-OHDG, 4-HNE, and DT immunoreactivity score in comparison with the DX group (Fig. 5). The control group showed negative expression (Fig. 5).

Discussion

The detrimental effects of DX-induced cardiotoxicity are well established and associated with the initiation of inflammation [8] and an overexpression of reactive oxygen species with DX [28, 29]. The myocardium is particularly susceptible to DX related oxidative stress due to decreased activity of antioxidant enzymes in the myocardium [30]. Furthermore, the cardiac muscle contains cardiolipin-rich mitochondria that have a high affinity for DX, leading to its accumulation in the cardiac mitochondria. This accumulation impairs the respiratory chain and triggers apoptotic death [31]. Our study's biochemical, molecular,

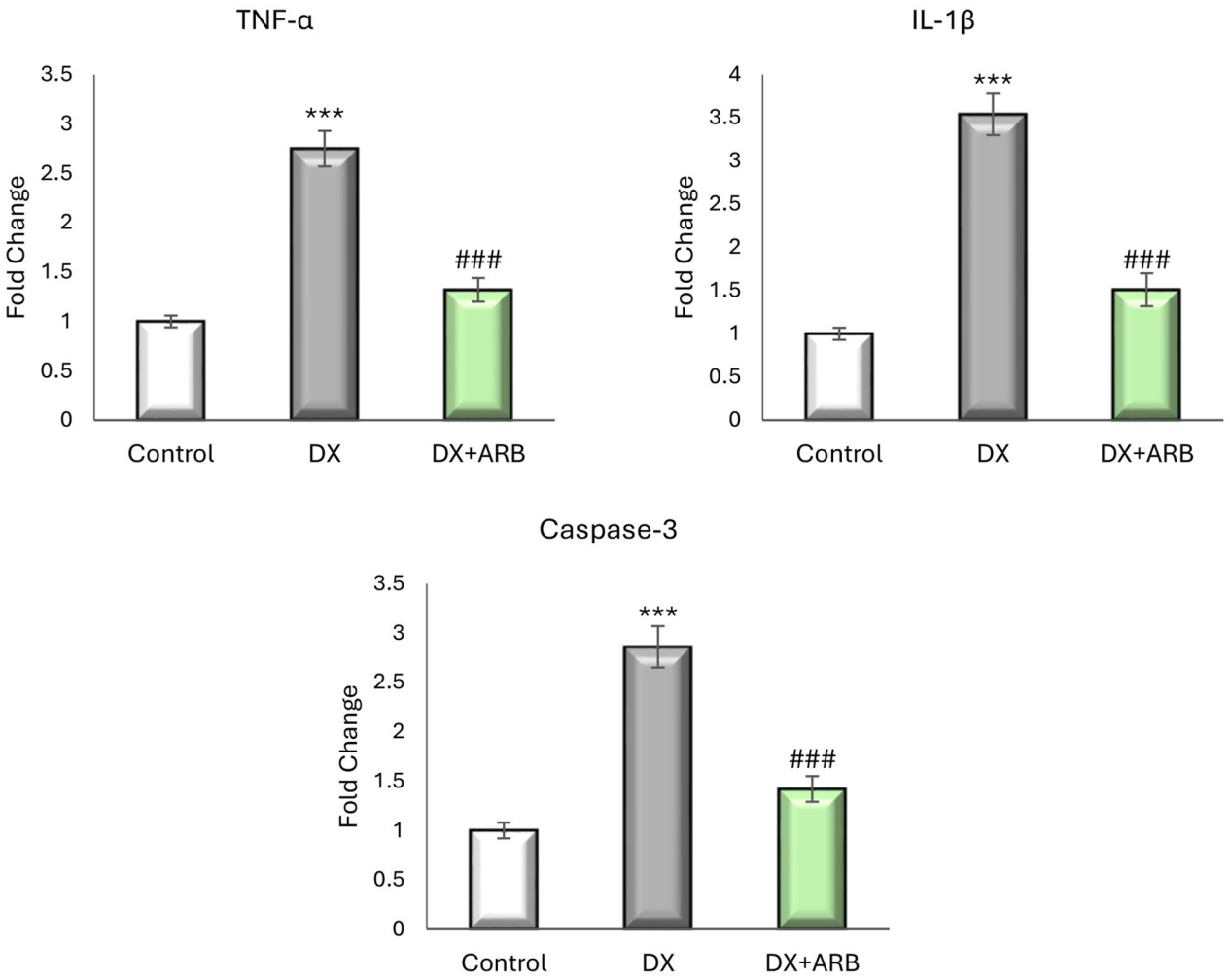

Fig. 3 Tissue mRNA fold changes of inflammation and apoptotic markers in control and treatment groups. *** $p < 0{,}001$ vs. control group, ### $p < 0{,}001$ vs. DX group

histopathological, and immunohistochemical analyses provided evidence of oxidative damage, apoptosis, and inflammation in an animal model of DX-induced cardiotoxicity, in line with previous studies.

Our current study provides compelling evidence that DX administration in rats results in a notable increase in the levels of serum cardiac injury markers such as CK, CK-MB, and LDH, thereby indicating significant cardiac damage. Interestingly, pretreatment with ARB was found to significantly decrease the levels of these injury markers, pointing towards its potential as a cardioprotective agent. Importantly, our results are consistent with earlier findings, which demonstrated that ARB treatment led to a decrease in the levels of CK-MB and LDH in rodent models of septic cardiomyopathy and isoproterenol-induced cardiac hypertrophy [18, 32]. These findings highlight the potential of ARB as a therapeutic agent for the prevention and management of DX-induced cardiotoxicity.

Results of the current study showed a significant decrease in cardiac SOD and CAT activities, important antioxidant enzymes that break down superoxide anions and hydrogen peroxide, respectively, following DX administration in rats. These results were consistent with other studies that also found DX caused a reduction in antioxidant mechanisms [33, 34]. We also found a significant increase in cardiac tissue levels of MDA and 8-OHdG in the DX group, indicating DX caused an increase in lipid peroxidation and DNA damage [35, 36]. 4-HNE is formed as a result of oxidative stress and is a product of lipid peroxidation. It has been shown to be highly reactive and can form adducts with proteins and DNA. Studies have suggested that 4-HNE plays a role in the pathogenesis of several diseases, including Alzheimer's disease, Parkinson's disease, and atherosclerosis [37]. The

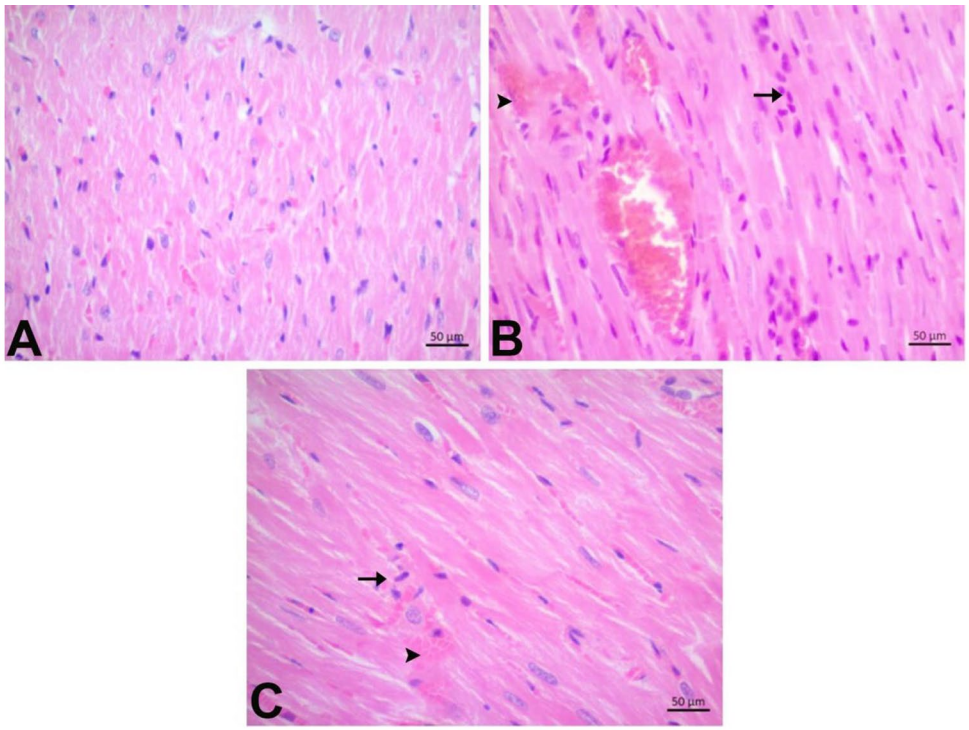

Fig. 4 Histopathological microphotograph of control and treatment groups stained with H&E. (**A**) Control group: Normal architecture of myocardium, (**B**) DX treated group: Severe mononuclear cell infiltrates (arrow) and severe hemorrhage (arrowhead), (**C**) DX + ARB treated group: Mild mononuclear cell infiltrates (arrowhead) and mild hemorrhage (arrowhead)

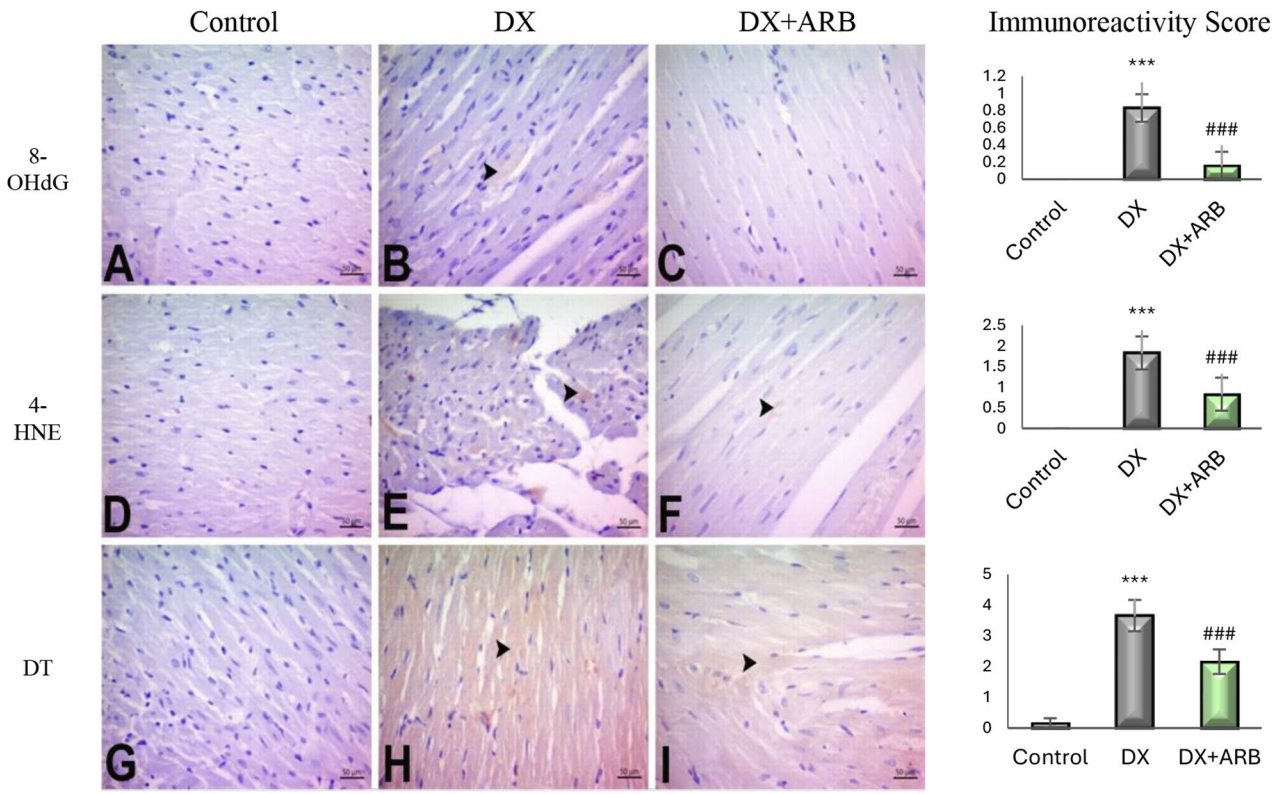

Fig. 5 Immunohistochemical analysis of 8-OHdG, 4-HNE and DT in control and treatment groups. *** $p < 0{,}001$ vs. control group, ### $p < 0{,}001$ vs. DX group

measurement of 4-HNE levels has been used as a marker of oxidative stress in various biological systems, including in animal models. Its accumulation has been associated with the activation of signaling pathways involved in apoptosis and inflammation. 3,3'-dityrosine has emerged as a key marker of protein oxidation due to its specific formation from the reaction of tyrosine residues with reactive oxygen species. The tyrosine radical, which is generated by the attack of various reactive oxygen species such as peroxynitrite and hydroxyl radicals, undergoes cross-linking with neighboring tyrosine residues to produce 3,3'-dityrosine [7]. These findings and previous studies suggest that DX causes oxidative damage in cardiac tissue by inhibiting antioxidant mechanisms.

Previous studies have shown that pretreatment with ARB can mitigate the DX-induced lipid peroxidation [38], and significantly reduce the levels of antioxidant enzymes. Hence, it is hypothesized that ARB could efficiently scavenge the uncontrolled production of reactive oxygen species caused by DX and safeguard the myocardium from DX-induced damage. The current results are supported by earlier findings that have highlighted the antioxidant properties of ARB both in vitro and in vivo [16, 39].

The observed association between DX-induced cardiotoxicity and inflammation is well-documented, primarily attributed to the release of pro-inflammatory cytokines [40]. Notably, two pivotal cytokines, TNF-α and IL-1β, have been consistently shown to exhibit elevated levels in response to cardiac injury induced by DX [41]. In this context, ARB, characterized by its notable anti-inflammatory properties, emerges as a compelling candidate for intervention. Studies across various experimental models have consistently demonstrated ARB's ability to mitigate the levels of TNF-α and IL-1β, as also evidenced by the results of the present investigation [42, 43]. These findings collectively suggest a promising role for ARB as a therapeutic avenue in both preventing and managing DX-induced cardiotoxicity through its potent anti-inflammatory effects. By modulating the inflammatory response, ARB holds potential not only in attenuating the adverse effects of DX on the heart but also in preserving cardiac function during chemotherapy. Furthermore, the extensive body of evidence supporting ARB's anti-inflammatory properties underscores its broader utility beyond DX-induced cardiotoxicity. Its efficacy as an adjunctive therapy in chemotherapy-induced cardiotoxicity is particularly noteworthy, suggesting a multifaceted approach to mitigating cardiac complications associated with cancer treatment [42, 43].

The harmful effects of DX on the heart are not limited to oxidative stress and inflammation; it can also induce apoptosis, which is the programmed cell death mechanism that occurs naturally in cells. DX-induced apoptosis is mediated by the overproduction of caspase-3 enzyme. According to a previous study, DX has been shown to induce the release of cytochrome C into the cytoplasm and increase the expression of caspase-3 and caspase-9 in cardiomyocytes [44]. Furthermore, DX can disturb calcium homeostasis, resulting in cellular and mitochondrial calcium overload. This can disturb cellular metabolism, increase the production of free radicals, and initiate apoptosis. The opening likelihood of sarcoplasmic reticulum Ca channels increases with DX, while Na+–Ca2 + exchanger membrane proteins are inhibited [45]. In line with previous studies that showed the anti-apoptotic effects of ARB, our study also found that ARB reduces apoptosis by decreasing caspase-3 levels [46]. ARB can help protect the myocardium from DX-induced damage by reducing the expression of caspase-3 and preventing the occurrence of apoptosis. Our results are consistent with other reports that demonstrated the anti-apoptotic effect of ARB in vitro and in vivo [16, 39].

Conclusion

In the present study, our investigation demonstrated that ARB exerted significant ameliorative effects against DX-induced cardiotoxicity in animal subjects. The administration of ARB effectively attenuated DX-induced oxidative stress, inflammation, and apoptosis, thus indicating its potential as a protective agent against cardiac damage induced by DX. Furthermore, our histopathological analyses revealed noteworthy improvements in the myocardial tissue profile, reinforcing the cardioprotective properties of ARB. The findings from this preclinical study provide valuable evidence supporting the therapeutic potential of ARB as a promising intervention to mitigate DX-induced cardiotoxicity in cancer patients. Despite the encouraging outcomes presented in this study, it is essential to acknowledge the need for future clinical trials to validate the safety and efficacy of ARB in human subjects.

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Author contributions O.B., I.F.O., U.O. designed the study. O.B., I.F.O., U.O., C.B., M.S.E and B.M. carried out the experiments. O.B., I.F.O., U.O., A.H., H.S. and E.A. wrote the main manuscript. All authors provided critical feedback and helped shape the research.

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Data availability Data and materials are available from the authors upon request.

Declarations

Ethical approval All experiments were conducted in compliance with the "Guide for the Care and Use of Laboratory Animals" and the approval of the Ataturk University-Ethical Committee (2200370605).

Consent to participate All individuals have signed consent forms before sample acquisition. Information on potential publications is included in consent forms signed by all individuals.

Consent for publication The participants were informed about publishing the results and they all agreed.

Competing interests The authors declare no competing interests.

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