OXFORD Toxicology Research

Toxicology Research, 2022, 1–13
https://doi.org/10.1093/toxres/tfac023
Paper

Synergistic impacts of Montelukast and Klotho against doxorubicin-induced cardiac toxicity in Rats

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Doxorubicin (DOX) is a powerful antitumor agent with a well-known cardiaotoxic side effects. In the current study, the ameliorative combined impacts of montelukast (Mont) and Klotho against doxorubicin-induced cardiac toxicity were examined. Fifty-six adult male rats (2 months age and weighting 150–200 g) were grouped into 7 groups (8 rats per group). Animals received doxorubicin alone or in combination with either Mont or Klotho. After 2 weeks of treatments, serum samples were examined to assess the changes in cardiac activity biomarkers such as LDH, CK-MB, cardiac troponin-I (cTn-I), and heart fatty acid binding protein (H-FABP). Serum changes of IL-6, inducible nitric oxide synthase (iNOS), and caspase-3 levels were assayed. The oxidative stress biomarkers such as total antioxidant capacity (TAC) and inflammatory (rat IL-1β and rat TNF-α,) and anti-inflammatory (rat IL-10) cytokines were examined. Heart histology and transforming growth factor-β1 (TGF-β1) immunoreactivity were measured. DOX induced cardiomyopathy, which was reflected by the increases in all examined cardiac parameters. Real-time PCR confirmed that DOX upregulated the expression of TNF-α and IL-1β and decreased the expression of IL-10. Moreover, DOX showed marked elevation in the ST segment T wave complex, causing profound tachycardia. Heart histology assessments showed cardiac cell necrosis, inflammatory cell infiltration, interstitial congestion, and increased TGF-β1 immunoreactivity. Montelukast and Klotho administration ameliorated all the altered parameters when administered alone or in combination to DOX-intoxicated rats. Klotho was more effective compared with montelukast in terms of reductions in heart rate, ST segment T wave complex elevation, cardiac enzymes (lactate dehydrogenase; LDH, creatine kinase-MB; CK-MB, cardiac troponin I; cTn-I, heart fatty acid binding protein; H-FABP) cardiac histology, and caspase-3 levels and increases in TAC activity. Montelukast was more effective in reducing serum levels of IL6 and iNOS, expression of TNF-α and IL-1β, and the upregulation of IL-10 expression. The co-administration of both drugs led to significantly more synergistic results in terms of reducing cardiac toxicity. In conclusion, montelukast and Klotho either alone or in combination were confirmed to be effective in suppressing DOX-induced cardiac toxicity in rats.

Key words: cardiac biomarkers.; gene expression; Klotho; montelukast; doxorubicin; cardiotoxicity.

1. Introduction

Doxorubicin (DOX) is an effective antitumor agent. It has been proven to be potent in the treatment of solid and hematological malignancies.[1] The development of cardiomyopathy, however, limits its usage. This serious side effect, once developed, results in a poor prognosis and is frequently fatal.[2] The mechanisms of therapeutic antitumor outcomes of DOX on tumor cells differ from that associated with the development of cardiotoxicity. Nevertheless, they share a common factor, namely increased liberation of reactive oxygen species (ROS) markers, which may induce mitochondrial damage, apoptosis, and impairment of gene function of both tumor cells and cardiomyocytes.[3]

The production of ROS and superoxide anion-free radicals exacerbates cardiac dysfunction and DNA damage induced by DOX treatment.[4] Multiple proinflammatory mediators, such as cytokines and fibrosis-associated markers and their downstream apoptotic pathways, are elevated as a result of this cardiac dysfunction.[5] Furthermore, DOX directly and indirectly initiates apoptotic processes and stimulates the last stages of programmed cell death.[6] Several studies have shown that the harmful effects of DOX on the heart are due to ROS production and initiation of various inflammatory pathways and other inflammatory cytokines.[7]

Because DOX-induced cardiotoxicity is often irreversible, novel protective techniques that can block DOX-induced pathogenic processes and give protection against cardiotoxicity should be developed.[8,9] Some trials have been performed to search for safe medication to be given during the course of DOX chemotherapy. EDTA or dextrazoxazone, vitamin E, and vitamin C can be used as free radical scavengers to antagonize DOX cardiomyopathy.[10–12] Unfortunately, their beneficial effect is only manifested after long cumulative doses. Another trial performed to counteract the cardiotoxicity of DOX involved the suppression of cardiac remodeling

Received: January 2, 2022. **Revised:** February 24, 2022. **Accepted:** April 2, 2022

by angiotensin-converting enzyme inhibitors or the inhibition of cardiac apoptosis by alpha one adreno receptor blockade.[13]

Montelukast is a cysteinyl leukotrienes receptor antagonist[14] and is located mostly in the sarcoplasmic reticulum of cardiomyocytes. It has the potential to regulate ROS formation.[15] Leukotriene receptor antagonists have been shown to be effective in several inflammatory models such as experimental gastric mucosal ulceration,[16] methotrexate-induced renal and liver damages,[17,18] may ameliorate the pathognomonic chronic inflammatory changes in DOX-induced cardiomyopathy. Montelukast is a medication that is used to prevent asthma symptoms. When asthma is mild, it is usually recommended to keep it from growing worse.[19] It can also help patients with asthma who experience breathing problems when they exercise (exercise-induced asthma), as well as seasonal allergies including sneezing, itching, and runny nose (allergic rhinitis).[20]

Klotho was first described as a single-pass transmembrane protein that regulates age-related events in the renal tubular epithelium,[21] bounded to the cell membrane, or released into extracellular space and then found in the blood, urine, or cerebrospinal fluid. The main function of Klotho is the regulation of fibroblast growth factor signaling and ion homeostasis.[22] Klotho acts as a cardioprotective agent due to its role in the activation of phosphate homeostasis and reducing oxidative stress.[23,24] Moreover, it has anticancer and antiaging effects through the regulation of vitamin D function.[25] Klotho regulates cellular calcium metabolism; therefore, may be effective in treating cardiac toxicity.[26] Previous studies documented that defects in Klotho gene expression in mice accelerated cardiac hypertrophy[27] and remodeling as well as vascular calcification.[28]

Therefore, the current study aimed to test the ameliorative effects of montelukast and Klotho as prophylactic drugs against doxorubicin-induced cardiac toxicity either alone or in combination.

2. Materials and methods

2.1. Medications and chemicals

Klotho was bought from R&D Systems (Minneapolis, MN, USA). Montelukast was purchased from Sigma-Aldrich. Doxorubicin HCL was purchased from Sigma Aldrich Co, Memphis USA. All chemicals were pure powder and of pure molecular grade. They were dissolved in saline before use on experimental animals. SYBR Green PCR Master Mix, Oligo dT primers, and Qiazol were imported from QIAGEN (Valencia, CA, USA).

2.2. Animals

Fifty-six male adult Sprague–Dawley rats were bought from Experimental Animal Breeding Farm (Helwan-Cairo, Egypt). Animals weighed 150–200 g and were 2 months of age. Eight rats were housed per cage at room temperature in a well-ventilated area at the Faculty of Medicine, Department of Pharmacology, Benha University. The current study got an ethical approve of both Taif and Benha University, under number TURSP-2020-09. They had free access to water and standard food. Animals were handled manually for 30 min for 7 consecutive days to ensure full acclimatization and to avoid treatment and administration stress.

2.3. Animal grouping

Rats were allocated into 7 groups of eight rats per group, as follows:

Group I: Normal control: normal rats received saline.

Group II: Montelukast-administered group (Mont): rats received orally Mont in a dose of 20 mg/kg daily for 2 weeks.[26,29]

Group III: Klotho-administered group (Klotho): rats received intraperitoneal injections of Klotho (0.01 mg/kg, in 100-μL saline, every 2 days for 2 weeks).[5,9]

Group IV: Doxorubicin group (DOX): rats received doxorubicin HCL (2.5-mg/kg intraperitoneally (i.p) 3 times a week for 2 weeks (cumulative dose is 15 mg/kg).[30]

Group V: Montelukast-treated doxorubicin group (Mont + DOX): rats received montelukast daily for 2 weeks as in group II, and doxorubicin 3 times a week for 2 weeks as in group IV.

Group VI: Klotho-treated doxorubicin group (Klotho + DOX): rats received Klotho every 2 days for 2 weeks as in group III, and doxorubicin 3 times a week for 2 weeks as in group IV.

Group VII: Klotho + Mont-treated doxorubicin group (Mont + Klotho + DOX): rats received montelukast as in group II, Klotho as in group III, and doxorubicin as in group IV in same pattern.

At the end of the experiment, rats were anaesthetized with urethane (1.5–1.75 gm/kg BW). The rats were set on their backs after total anesthesia. Needle electrodes were used to capture ECGs. The 4 limb electrodes were attached to the rats' 4 limbs and the recordings were made at a rate of 25 m/min using the standard lead II.[31,32] After completion of the ECG recording, blood samples were taken by direct heart puncture[33] then decapitated. No mortalities were reported during the course of experiment. Serum was extracted from clotted blood after centrifugation at 3,000 rpm for 10 min and stored at 4 °C for future serum cardiac measurements. Heart samples were taken and rapidly washed with ice cold saline. The heart of each animal was divided into 5-mm transverse slices containing portions of both ventricles and the interventricular septum. Small pieces of the left ventricle measuring about 100 mg were homogenized in an ice-cooled glass homogenizer with 1 mL of phosphate-buffered saline (PBS; 50 mM, pH = 7.4), then centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was assayed for determination of total proteins using Bradford assay.[34] The supernatant was kept at –80 °C for measurement of the total antioxidant capacity (TAC). Cardiac slices were preserved in 10% formalin for histopathological and immunohistochemical

examination. Small pieces of heart tissue measuring about 50 mg were inserted in Qiazol for real-time PCR.

2.4. Biochemical measurements of cardiac enzymes biomarkers

Serum levels of creatine phosphokinase (CK-MB) were determined using a CPK ELISA kit (Cat. No. C3755-3.5KU). Serum levels of cardiac troponin-I (cTn-I) were determined using a cTn-I ELISA kit (cTnI; ab200016). In parallel, serum levels of lactate dehydrogenase (LDH) were determined using an LDH ELISA kit (Cat. No. 601170) according to the manufacturer's instructions. All kits were imported from Abcam Co., USA. For heart-specific fatty acid binding protein (H-FABP) measurements, ELISA kits (CSB-E16184r) were used. The absorbance was measured at 450 nm to measure H-FABP in the spectrophotometer. Plotting the absorbance (linear) vs. the relevant concentrations of the rat H-FABP standards yields a standard curve.

2.5. Measurements of TAC in cardiac muscle tissue homogenate

Cardiac muscle (500 mg) was homogenized in ice cold buffer in 1-mM EDTA and 1-mL/L Triton X-100 for TAC. The homogenized samples were centrifuged at 6000 × *g* for 8 min at 4 °C. The supernatant was stored at −20 °C. The TAC of the hearts was measured as discussed before.[35] TAC was expressed as mmol/mg protein of cardiac tissue.

2.6. Determination of serum levels of IL-6, inducible nitric oxide synthase level and caspase-3

Based on a recent study,[36] IL-6 levels in the serum were assayed using a solid-phase ELISA using a rat IL-6 kit (Cat # ELR-IL6-1, Ray Biotech, Norcross, USA) and a Microtiter plate reader at 450 nm. The concentration of inducible nitric oxide (iNOS) in the serum was determined using commercially available ELISA kits (Multi Sciences Biotech, China) as directed by the manufacturer. At a wavelength of 450 nm, the samples or standards were examined using an ELISA plate reader. As previously described,[37,38] the quantitative assessment of caspase-3 levels was performed using a caspase-3 colorimetric assay kit (Catalog no. 907-013).

2.7. Measurement of related mRNA expression in cardiac tissue by quantitative real-time polymerase chain reaction

Using a 7500 fast real-time PCR system, the quantification of interleukin-1 (IL-1), tumor necrosis factor (TNF-α), and interleukin-10 (IL-10) at the mRNA level was investigated (Applied Biosystems, CA). Total RNA was extracted from cardiac tissue (6 samples per group) using TRIzol (Thermo Fisher Scientific, USA), as described in the manufacturer's instructions. One hundred milligrams of cardiac tissues were put in a micro-centrifuge tube with 0.750 mL Trizol reagent, then homogenized with a Rotor Tissue Ruptor (Qiagen, GmbH, Germany). The amount and purity of the RNA were determined using Bio-Rad spectrophotometer (BMG Lab Tec, GmbH, Germany). Following the manufacturer's instructions, complementary DNA was generated using a 2X Reverse Transcriptase Master Mix (Applied Biosystems, USA). Here, quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 20-μL reaction mixture containing 10 μL of SYBR Green qPCR Master Mix (TOPreal ™ qPCR 2X PreMIX), 1 μL of 1 μg cDNA, 1 μM of each forward and reverse primer (Table 1), and 20 μL of nuclease-free water. Additionally, qRT-PCR was conducted by heating at 95 °C for 10 min, then followed by 40 cycles at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Primer-3 software was used to construct the PCR primers, which were then produced by Invitrogen in the United States. Table 1 shows the primer sequences (5′-3′) used in this study. The 2Ct computation was used to determine relative changes in gene expression, with Ct revealing changes in Ct in target genes compared with Ct values of the housekeeping gene (beta actin gene).[39]

Table 1. Oligonucleotide sequences of primers used in RT-qPCR.

Gene name	Primer Sequence (5′-3′)
TNF-α	F: 5'-AAATGGGCTCCCTCTCATCAGTTC- 3′ R:5'-TCCGCTTGGTGGTTTGCTACGAC-3′
IL-1β	F:5'-CCAGGATGAGGACCCAAGCA-3′ R: 5'-TCCCGACCATTGCTGTTTCC-3
IL 10	F: 5'-TGCCAAGCCTTGTCAGAAATGATCAAG- 3′ R:5′- GTATCCAGAGGGTCTTCAGCTTCTCTC-3′
β-actin	F: 5'- AAGTGTGACGTTGACATCCG-3′ R: 5′- TCTGCATCCTGTCAGCAATG- 3′

2.8. Cardiac histopathology and immunohistochemical immunoreactivity

Transverse sections from previously formalin-preserved cardiac slices containing the ventricles and interventricular septum samples were obtained, fixed in a 1:1 mixture of methanol and ethanol, paraffin-waxed, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). A representative slide/organ/rat was selected, and then a 6 fixed size nonoverlapped randomly chosen 40× images were snapshotted. The sections from each animal were examined for the presence of myocardial degeneration, inflammatory cell infiltration, and interstitial hemorrhage. They were graded as 0 (absent), 2 (mild), 3 (moderate), or 4 (severe).[40] Sections were stained with primary antibody against TGF-1 (ab92486, 1:200) acquired from Abcam, incubated at 4 °C overnight, and then stained with secondary antibody at room temperature for 30 min for TGF-β1 immunohistochemistry. Next, the sections were rinsed in PBS and treated for 5 min at room temperature with 3, 3′-diaminobenzidine (Gene Tech, Shanghai, China). The entire technique has already been documented elsewhere.[41]

2.9. Statistical analysis

SPSS version 16 software was used to tabulate and analyze the collected data (SpssInc, Chicago, ILL Company).

Table 2. Effect of administration of montelukast and or koltho in a rat model of DOX-induced cardio toxicity on average (Mean ± SD) Heart rate (b/min), ST segment T wave complex elevation (mm).

Animals grouping	Heart rate (b/min)	ST segment T wave complex elevation (mm)
Group I: Normal control	313.64 ± 7.5	0
Group II: Montelukast (Mont)	310.55 ± 10.3	0
Group III: Klotho	312.47 ± 9.8	0
Group IV: Doxorubicin (DOX)	670.12 ± 19.6[a]	15.3 ± 0.19[a]
Group V: DOX + Mont	578.45 ± 17.11[b]	9.9 ± 2.3[b]
Group VI: DOX + Klotho	480.37 ± 12.2[c]	5.8 ± 1.2[c]
Group VII: DOX + Mont + Klotho	401.43 ± 12[d]	3.9 ± 0.2[d.]

The letters express the significance levels relative to animal's grouping. Values with different letters are statistically significant at $P > 0.05$. Values are means ± SD for 8 different rats per each treatment.

The means and standard deviations (SD) for all examined biomarkers and SEMs for examined genes were used to represent the data. The data were tested for normality using the Shapiro–Wilk test assuming a normality level of $P < 0.05$. The one-way analysis of variance (ANOVA) test was used to look for differences between normally distributed data. To find significant pairings, a significant ANOVA test was followed by post hoc multiple comparisons using Bonferroni testing. The accepted level of significance in this work was stated at 0.05 ($P < 0.05$ was considered significant).

3. Results

3.1. Protective effects of montelukast and Klotho against DOX-induced cardiac changes in heart beat and ST segment T wave complex elevation

Intraperitoneal injection of doxorubicin 2.5 mg/kg thrice a week for 2 weeks resulted in alterations of all tested indices of cardiac parameters in the form of marked increases in heart rate and ST segment T wave complex height (Table 2). Administration of Mont or Klotho alone significantly normalized the increases in heart beat and ST segment T wave complex height (Table 2).

3.2 Protective effects of Montelukast and Klotho against DOX-induced changes on cardiac biomarkers

The cardiac cell necrosis parameters LDH, CK-MB, cTn-I, and H-FABP were elevated ($P < 0.05$) after DOX injection to rats (Fig. 1). In parallel, serum levels of the inflammatory cytokine IL-6, iNOS, and apoptotic mediator caspase-3 were significantly elevated ($P < 0.05$) in DOX-injected rats compared with normal control group (Table 3). Pre-administration of the tested drugs Mont and Klotho ameliorated the increases in all examined parameters in serum and cardiac homogenate. Of interest, the co-administration of Mont and Klotho together showed synergistic effects upon most altered parameters.

3.3. Protective quantitative effects of montelukast and Klotho against DOX-induced changes in cardiac inflammatory cytokines and IL-10 expression

Real-time PCR confirmed that the expression levels of TNF-α and IL-1 β were upregulated ($P < 0.05$) in the DOX-administered rats (Fig. 2a and b). The expression patterns of inflammation mediators were mostly restored when Mont and Klotho were administered either alone or in combination. Unlike the inflammatory mediator results, the anti-inflammatory cytokine IL-10 mRNA was downregulated ($P < 0.05$) in DOX-injected rats. Mont and Klotho upregulated IL-10 expression, which was decreased in DOX-injected rats alone. Combined administration of Mont and Klotho upregulated and significantly ($P < 0.05$) restored IL-10 expression compared with DOX-injected rats receiving either Mont or Klotho alone (Fig. 2c).

3.4. Protective effects of montelukast and Klotho against DOX-induced cardiac muscle histology

The examined drugs did not cause any changes in cardiac muscle histology in control, Mont- or Klotho-administered animals. Control, Mont, and Klotho rats showed normal cardiomyocyte cytoplasm and centrally located oval nuclei histology (Fig. 3a–c). DOX-injected rats showed marked degeneration of cardiomyocytes, interstitial inflammation, and interstitial congestion (Fig. 3d). In DOX-injected rats receiving Mont or Klotho alone or in combination, mild improvement of myocardium degeneration (red arrow) was observed, with moderate improvements in interstitial inflammation (yellow arrow) and interstitial congestion (green arrow) (Fig. 3e–g). The degree of lesion scoring in the cardiac muscle is presented in Fig. 3h. The degree of cardiomyopathy scoring was high in DOX-injected rats and then decreased when Mont or Klotho was administered alone or in combination to DOX-receiving rats (Fig. 3h). Combined administration of Mont and Klotho showed more significant synergistic ameliorative effects.

3.5. Protective effects of Mont and Klotho on changes in TGF-β1 immunoreactivity

For the expression of TGF-β1 in the cardiac tissue (Fig. 4a–c), these 3 groups revealed normal cytoplasmic expression of TGF-β1 (brown color). DOX-intoxicated rats showed marked increases in TGF-β1 expression in the form of multiple scattered foci of strong immune positivity (deep brown cytoplasmic stained) (Fig. 4d). Monotherapy by Mont or Klotho to DOX-injected rats resulted in moderate expression of TGF-β1 (Fig. 4e and f).

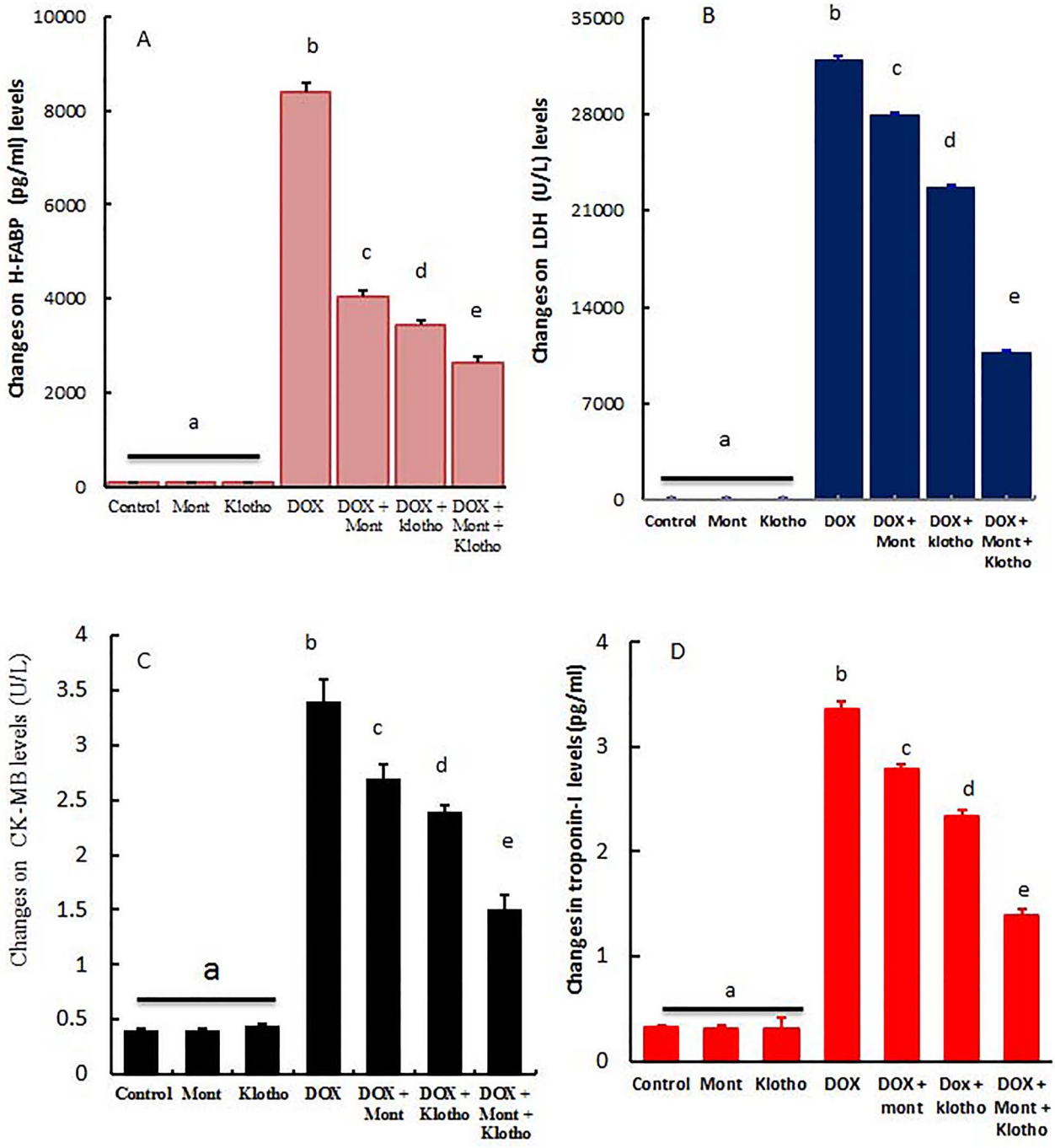

Fig. 1. Montelukast and Klotho ameliorate serum changes in A) H-FABP, B) LDH, C) CK-MB, and D) troponin-I in DOX-intoxicated cardiac toxicity. Data are expressed as means ± SD, ($n = 6$). ANOVA test was followed by post hoc multiple comparisons test. Values with different letters are significant at $P < 0.05$.

Combined therapy of Mont and Klotho to DOX-receiving rats resulted in minimal expression of TGF-β1 (faint brown cytoplasmic stained) (Fig. 4g).

4. Discussion

The current study confirmed the protective effects of montelukast and Klotho either alone or in combination against doxorubicin-induced cardiac toxicity. Both drugs induced their effects through the restoration of elevated cardiac parameters, regulating the expression of inflammatory and anti-inflammatory cytokines and normalizing the alterations in cardiac histopathology and fibrosis-associated gene expression (TGF-β1).

DOX is an antineoplastic and broad-spectrum antibiotic used to treat hematological and solid malignancies. The incidence of dose-dependent toxicity to different organs such as the heart has limited the use of DOX.[42] The mechanism of DOX-induced cardiotoxicity is not fully known—the most often postulated

Table 3. Protective effect Montelukast and Klotho against DOX-induced cardiaomyopathy.

	TAC (mmol/mg protein)	IL-6 (pg/mL)	iNOS (IU/mL)	Caspase-3 (Pg/mL)
Group I: normal control	97.81 ± 0.01^{a}	17 ± 6.3^{a}	21.67 ± 3.01^{a}	0.66 ± 0.02^{a}
Group II: Mont	94.85 ± 0.14^{a}	14 ± 5.1^{a}	23.83 ± 3.81^{a}	0.71 ± 0.04^{a}
Group III: Klotho	91.79 ± 0.17^{a}	16 ± 5.9^{a}	22 ± 3.89^{a}	0.63 ± 0.07^{a}
Group IV: DOX	28.11 ± 0.23^{b}	72 ± 8.1^{b}	107.14 ± 5.2^{b}	11.1 ± 1.4^{b}
Group V: DOX+ Mont	51.46 ± 0.41^{c}	37 ± 3.4^{c}	53.33 ± 3.93^{c}	7.9 ± 2.3^{c}
Group VI: DOX + Klotho	71.33 ± 0.21^{d}	48 ± 2.1^{d}	71.67 ± 2.16^{d}	5.2 ± 1.2^{d}
Group VII: DOX + Mont + Klotho	85.11 ± 0.2^{e}	27 ± 1.8^{e}	34.17 ± 3.31^{e}	$\pm 0.6^{e.}$

Presented values are means ± SD for 8 different rats per group. Values with different letters are statistically significant at $P > 0.05$.

processes are oxidative stress, intracellular calcium dysregulation, inflammation, and cardiomyocyte death.[43] The problem with managing its cardiotoxicity is finding an agent that has cardioprotective properties while maintaining anticancer efficacy. In this study, rats were given a cumulative dosage of DOX (15 mg/kg BW) for 2 weeks to imitate chronic cardiotoxicity, as reported in previous clinical treatments.[9,44] Previous studies proved that Mont is nephron-protective and hepatoprotective in methotrexate-induced organ toxicity.[17,18] Klotho has been studied extensively since its discovery to identify its potential role in treatment of oxidative stress, inflammation, fibrosis, and apoptosis.[24,45,46] As a result, we set out to investigate the cardioprotective properties of Mont and Klotho, focusing on their potential combined use to control oxidative stress, inflammatory response, and apoptosis. Both normalized the significant increase in the ST segment T wave complex, as well as severe tachycardia occurred by DOX due to a delay in ventricular repolarization. This was consistent with earlier research results.[47,48]

Furthermore, the DOX-induced cardiotoxicity in rats was characterized by significant increases in the serum levels of LDH, CK-MB, troponin-I, and H-FABP,[49–51] as well as massive changes in the cardiac tissue degeneration and fragmentation of the cytoplasm and nuclei.[5,50] Both Mont and Klotho either alone or combined decreased cardiotoxicity caused by DOX treatment, as indicated by substantial reductions in LDH, CK-MB, cTn-I, and H-FABP levels, as well as a decrease in myocardial degeneration, as seen in the histological results and validated by us and others.[14,52] Furthermore, combining Mont and Klotho resulted in greater synergistic decreases in cardiac enzymes and an improvement in cardiac architecture, as seen by a reduction in DOX-induced cardiac damage.

H-FABP is an excellent cardiac marker.[53] The marked increase in H-FABP levels may reflect cardiomyocyte necrosis with the release of cell membrane proteins.[54] Furthermore**,** the histopathological changes were accompanied by marked states of oxidative stress manifested by significant decreases in the TAC of cardiac tissue in DOX-administered rats. Combination therapy with Mont and Klotho showed more significant restorative effects in TAC compared with their separate monotherapy effect as stated here and by others.[52,55]

As known, ROS promotes lipid peroxidation and the depletion of antioxidant enzymes.[56] Because cardiac tissue requires a lot of energy, it contains a lot of mitochondria, making it more vulnerable to DOX poisoning.[8] Their inner membrane contains cardiolipin, an anionic phospholipid with a high binding affinity for cationic DOX.[8] DOX is converted to a semiquinone radical in mitochondria, which reacts with molecular oxygen to create a superoxide anion and an additional ROS.[8] Moreover, the DOX metabolite (doxorubosol) via inhibition of iron regulatory protein increases intracellular free iron, which induces free radical toxicity.[57] Previous research has shown that DOX causes the depletion of antioxidant molecules such as GSH and the fatigue of cardiac antioxidant enzymes such as CAT and SOD.[58,59] Because free radicals are the primary cause of DOX-induced cardiotoxicity and as oxidative stress is a key component of cardiac toxicity, antioxidant substances have long been recognized as potential preventive and therapeutic agents.[60] Current findings confirmed the impact of Mont or Klotho alone or in combination in alleviating doxorubicin associated cardiac stress.

Increased free radical production and lipid peroxidation promote the inflammatory response in heart tissue.[61] Cell survival, inflammation, and immunological responses are all affected by the transcription factor NF-kB. The transcription of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) is enhanced by NF-kB, which controls inflammatory responses.[14,50,60] These cytokines cause leukocyte infiltration into the myocardium, which worsens the inflammatory damage.[62] Such increase in NF-B, TNF-α, IL-1, and IL-6 production in cardiac tissues can consistently promote cardiomyocyte death.[5] Therefore, administration of Mont or Klotho alone or in combination normalized such deviation in IL-1 and TNF-α production to control cardiac inflammation.

The overexpression of iNOS caused by inflammation is responsible for the increase in cardiac NO levels caused by DOX. By reacting with the free radical superoxide anion, high levels of NO produce peroxynitrite, which causes cardiac oxidative damage, apoptosis, and lipid

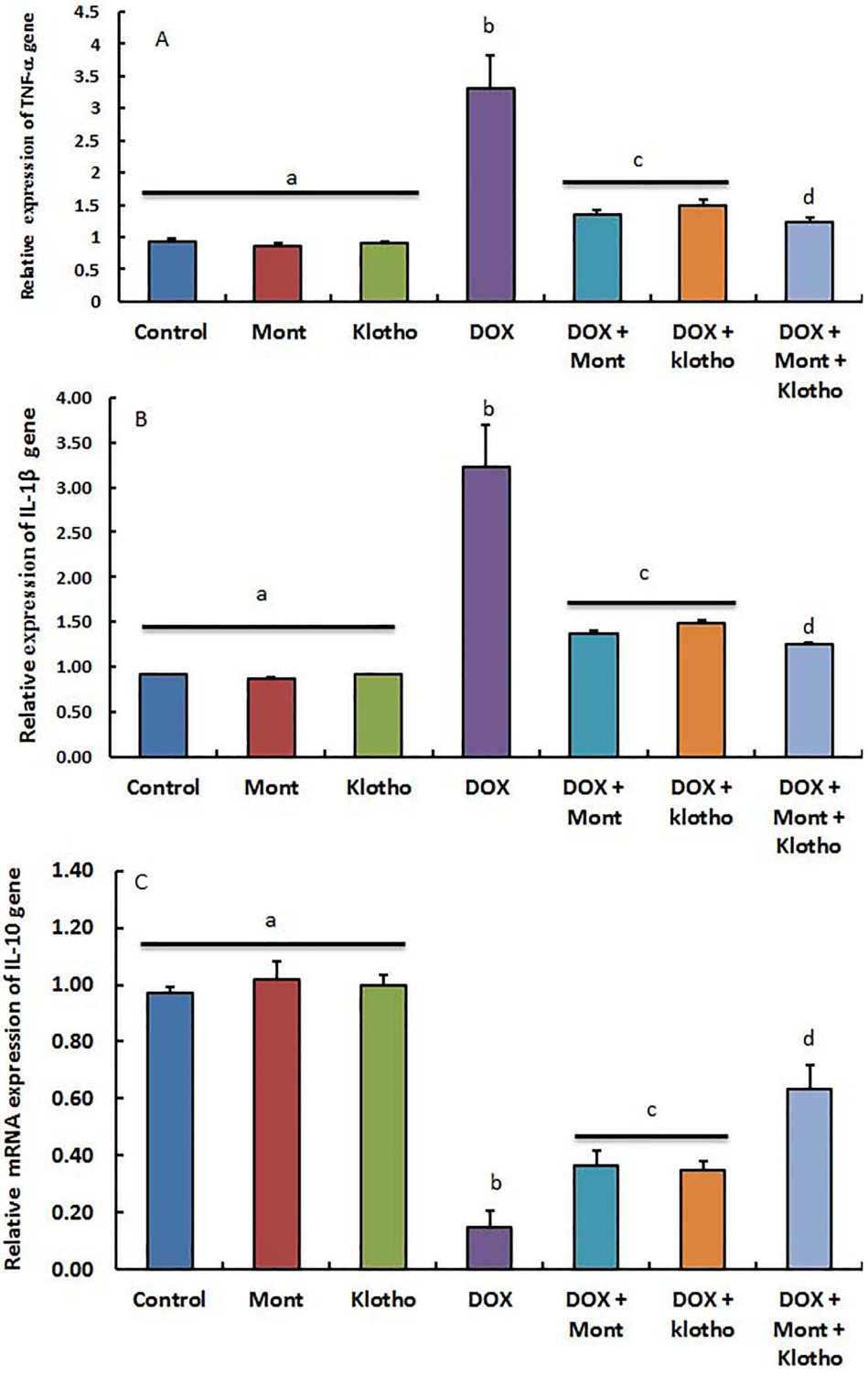

Fig. 2. Quantification of A) TNF-α, B) IL-1β, and C) IL-10 expression in rat cardiac tissue. Values expressed as means ± SEM ($n = 6$). ANOVA test was followed by post hoc multiple comparisons test. Values with different letters are significant at $P < 0.05$.

peroxidation.[63] Based on our findings, DOX-administered group resulted in considerable increases in mRNA gene expression of proinflammatory cytokines such as TNF-α and IL-1 β, serum levels of IL-6 and iNOS, as well as downregulation of IL-10 mRNA. Montelukast alone or combined with Klotho resulted in decrease in iNOS levels and TNF-α and IL-1β mRNA. Moreover, there was a significant upregulation in IL-10 mRNA. Such findings were confirmed in partial similar studies.[14,64] Clearly, the combination of Mont and Klotho resulted in more potent anti-inflammatory effects than that with Mont or Klotho alone.

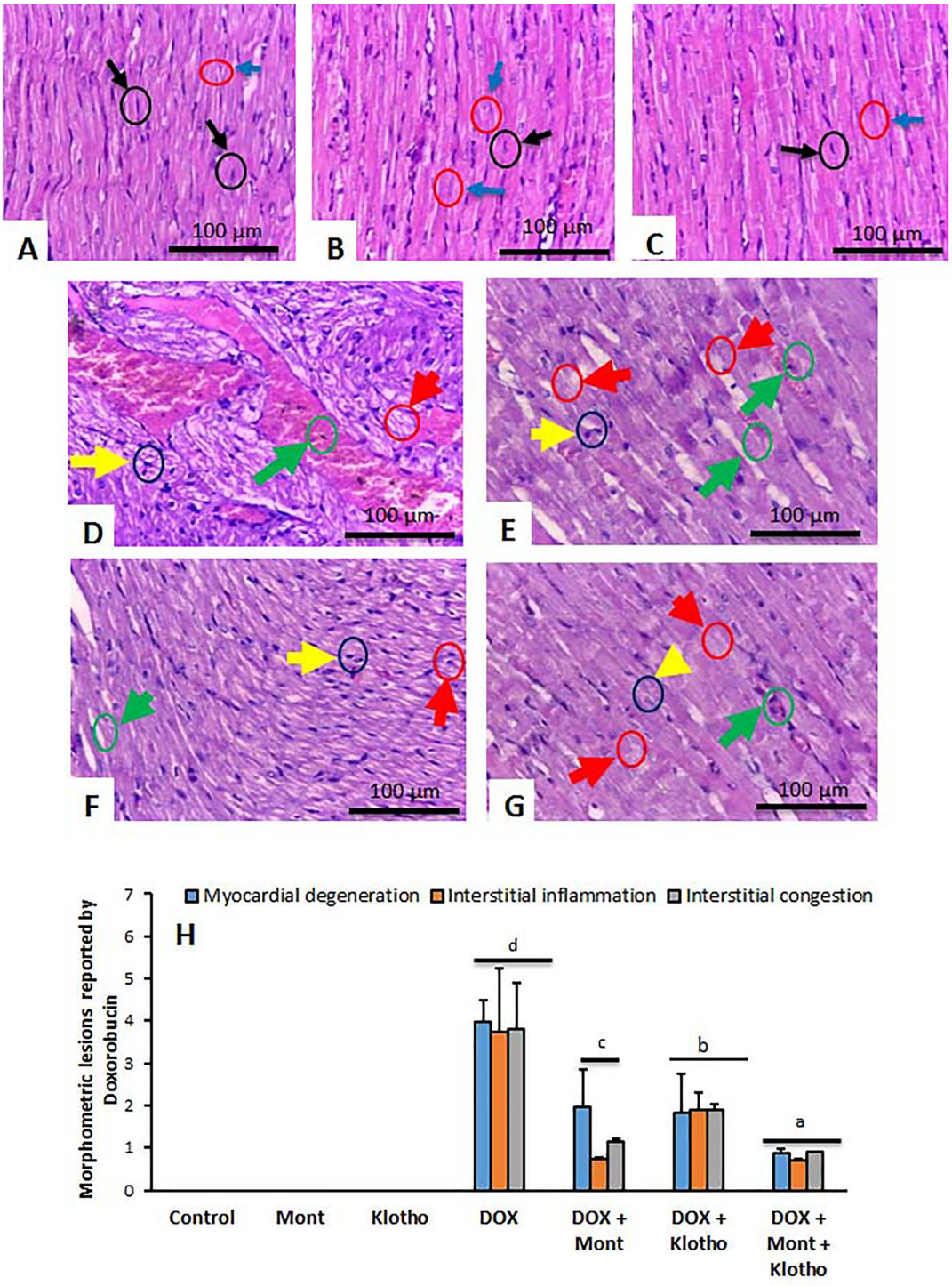

Fig. 3. Photomicrography of histopathological changes of rat cardiac tissues (H&E, ×400). Rats from the A) control, B) montelukast-treated group, and C) Klotho-treated group showed normal histology of cardiomyocyte cytoplasm (blue arrow) and centrally located oval nuclei (black arrow). D) Represents DOX-injected rats showing marked degeneration of cardiomyocytes (red arrow), interstitial inflammation (yellow arrow), and interstitial congestion (green arrow). E) Represents Mont + DOX-treated rats showing mild improvements in myocardium degeneration (red arrow) with moderate improvement in interstitial inflammation (yellow arrow) and interstitial congestion (green arrow). F) Represents Klotho + DOX-treated rats showing moderate improvements in myocardium degeneration (red arrow), interstitial inflammation (yellow arrow), and interstitial congestion (green arrow), although the letter 2 parameters were of a lesser degree than Mont + DOX-treated rats. G) Represents Mont + Klotho + DOX-treated rats showing marked improvements in myocardium degeneration (red arrow), interstitial inflammation (yellow arrow), and interstitial congestion (green arrow). In Fig. 3H, Values are mean ± SE for 6 slides/group. Values with different letters are statistically significant at $P > 0.05$.

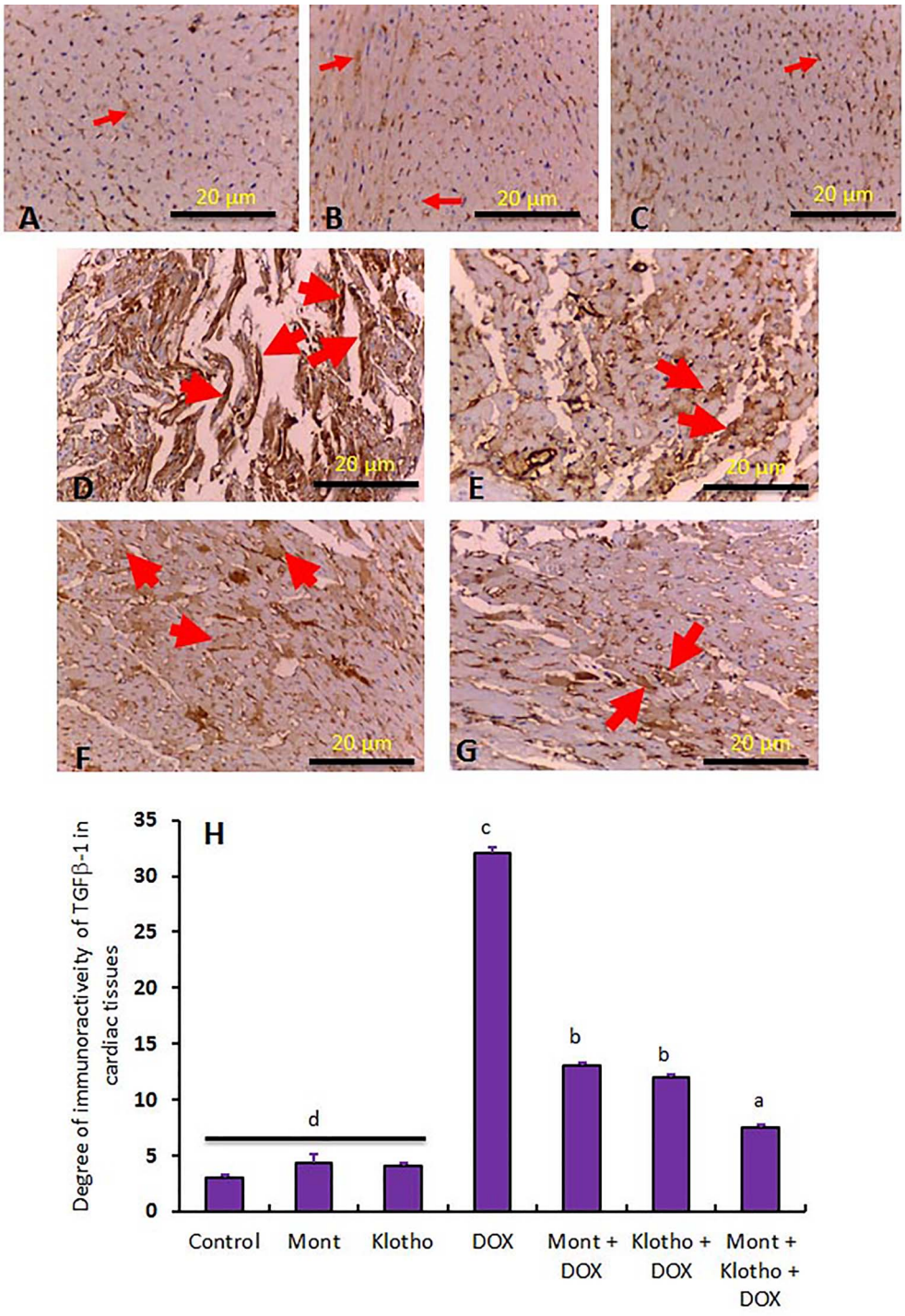

Fig. 4. Immunohistochemical staining of transforming growth factor-β1 (TGF-β1) in the experimental rats' cardiac cells (IHC, ×400). A–C) Represent control rats showing normal expression of TGF-β1 (brown color). D) Represents DOX-intoxicated rats showing marked increases in TGF-β1 expression in the form of multiple scattered foci of strong immune positivity. E) Represents Mont + DOX-treated rats and F) represents Klotho + DOX-treated rats showing moderate TGF-β1 expression. G) Represents Mont + Klotho + DOX-treated rats showing minimal TGF-β1 expression in the form of marked decreases in immune reactivity compared with e and f.

One of the possible cardioprotective mechanisms of Mont may due to its inhibitory effect on leukotriene-mediated sarcoplasmic reticulum oxidative stress.[65,66] Previous human studies have shown that montelukast contributes to the inhibition of inflammation by increasing IL-10 levels.[67,68] Mont inhibits NF-kB translocation and decreases TNF-induced oxidative damage.[69] Many proinflammatory cytokines, such as TNF-α, are inhibited by IL-10, which is also known as a cytokine production inhibitory factor. Klotho upregulated the

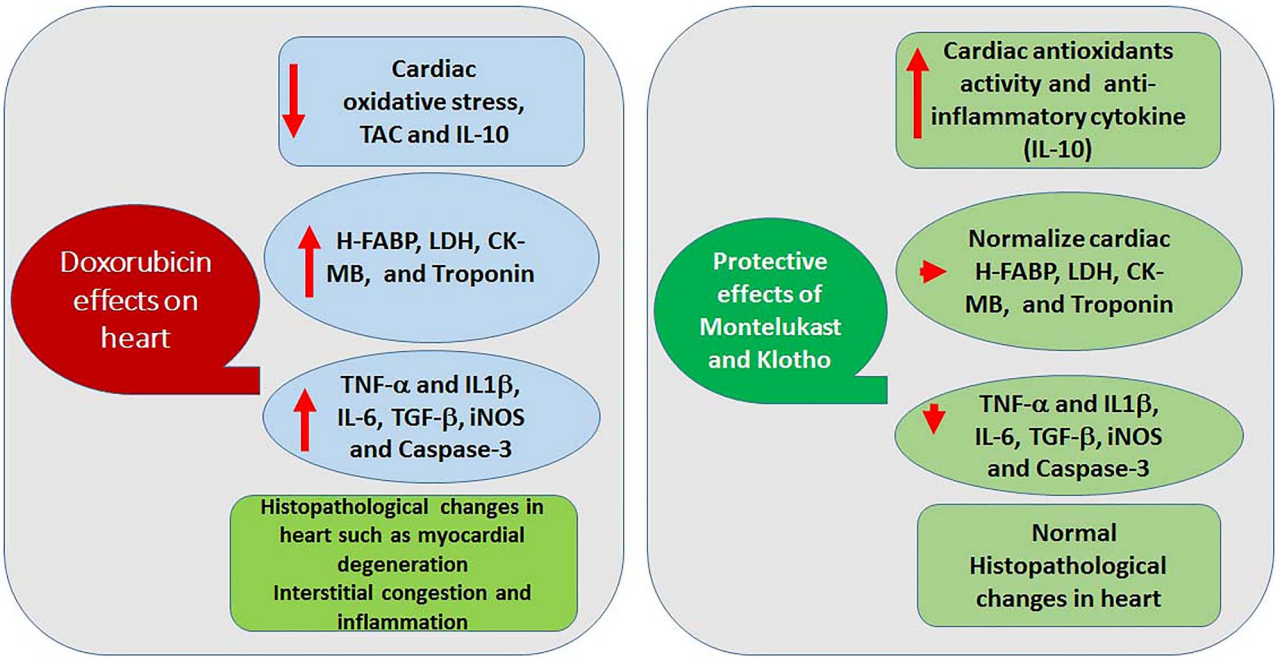

Fig. 5. Representative impacts of montelukast and Klotho against cardiac toxicity induced by doxorubicin.

secretion of IL-10[70] through the activation of the JAK2/STAT3 signaling axis and inhibition of NF-kB may be responsible for IL-10-induced TNF-α inhibition.[70,71]

Growing evidences has revealed that apoptosis of cardiomyocytes is an essential cause of DOX-induced cardiotoxicity.[72] Bcl-2 family members, which include proapoptotic (Bax, caspase-3) and antiapoptotic (Bcl-2) proteins, are the most important regulators of apoptosis.[73] Our data showed significant increases in the cleaved caspase-3 levels in DOX-injected rats, in agreement with previous studies.[14,49] Monotherapy with Mont or Klotho exhibited reductions in cleaved caspase-3 levels, while combined therapy provoked more significant reductions, confirming that Mont and Klotho have antiapoptotic effects.[74] Previous studies documented antiapoptotic effects of Klotho in DOX-induced cardiotoxicity,[24] which were associated with the activation of MAPKs.[75]

Fibrosis is the body's natural response to DOX-induced cardiotoxicity.[76] TGF-β1 regulates inflammatory and growth factor signaling pathways, which are involved in DOX-induced cardiac fibrosis. Increased oxidative stress, followed by antioxidant depletion and lipid peroxidation, causes tissue inflammation and necrosis, in addition to promoting tissue fibrogenesis.[56] Another important regulator of collagen formation in DOX-induced cardiomyopathy is TGF-β1. TGF-β1, a profibrogenic cytokine generated by cardiac myofibroblasts, causing cardiomyocyte hypertrophy, death, and fibrosis.[77] In this study, the immunohistochemical staining of myocardial tissues revealed marked increases in the TGF-β1 expression in cardiac tissues in DOX-injected rats.[76] However, administration of Mont or Klotho alone resulted in a moderate reduction in TGF-β1 expression, while combined therapy resulted in a marked reduction in TGF-β1 expression. It has been shown that Mont attenuates hepatic TGF-β1 in hepatic fibrosis.[78] In parallel, Klotho suppresses pro-fibrotic TGF-β1 signaling and organs fibrosis.[45,69] Therefore, combination therapy has more potential to inhibit DOX-induced myocardial damage and fibrosis development.

5. Conclusions

Combined administration of montelukast and Klotho was proven to be effective in suppressing DOX-induced cardiotoxicity in rats than do alone. They ameliorated the cardiotoxicity induced by DOX through the partial restoration of the elevated cardiac biomarkers, downregulating the expression of inflammatory cytokines and fibrosis- and apoptosis-associated proteins, upregulating the mRNA expression of IL-10 and increasing the antioxidant activity of affected rats. The collective synergistic impacts of montelukast and Klotho against doxorubicin-induced cardiac dysfunction are illustrated on Fig. 5.

Acknowledgments

The authors are thankful to the technical and administrative staff at the Departments of Pharmacology, Pathology and Clinical Pathology, Faculty of Medicine, Benha University, Egypt, for their support during this study.

Authors' contributions

All authors contributed equally to this study.

Funding

This study was supported by Taif University Researchers Supporting Project (TURSP-2020/09), Taif University, Taif, Saudi Arabia.

Conflict of Interest statement

No conflicts of interest regarding the current data were reported.

Ethical approval

The techniques and protocols used in this study were created in compliance with the Institutional Animal Care and Ethical Committee of Taif University, Saudi Arabia's criteria for animal welfare and the use of animals for project TURSP-2020-09.

Data availability

Data are available up on request.

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