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Rutin Attenuates Carfilzomib-Induced Cardiotoxicity Through Inhibition of NF-κB, Hypertrophic Gene Expression and Oxidative Stress

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Abstract Carfilzomib is a proteasome inhibitor, commonly used in multiple myeloma, but its clinical use may be limited due to cardiotoxicity. This study was aimed to evaluate the influence of rutin in carfilzomib-induced cardiotoxicity in rats. Wistar albino male rats weighing 200–250 g (approximately 10 weeks old) were taken for this study. Animals were divided into four groups of six animals each. Group 1 served as normal control (NC), received normal saline; group 2 animals received carfilzomib (dissolved in 1 % DMSO) alone; group 3 animals received rutin (20 mg/kg) + carfilzomib; and group 4 animals received rutin (40 mg/kg) + carfilzomib. Hematological changes, biochemical changes, oxidative stress, hypertrophic gene expression, apoptotic gene expression, NFκB and IκB-α protein expression and histopathological evaluation were done to confirm the finding of carfilzomib-induced cardiotoxicity. Treatment with rutin decreased the carfilzomib-induced changes in cardiac enzymes such as lactate dehydrogenase, creatine kinase (CK) and CK-MB. For the assessment of cardiotoxicity, we further evaluated cardiac hypertrophic gene and apoptotic gene expression such as α-MHC, β-MHC and BNP and NF-κB and p53 gene expression, respectively, using RT-PCR. Western blot analysis showed that rutin treatment prevented the activation of NF-κB by increasing the expression of IκB-α. Rutin also attenuated the effects of carfilzomib on oxidant-antioxidant including malondialdehyde and reduced glutathione. Histopathological study clearly confirmed that rutin attenuated carfilzomib-induced cardiotoxicity in rats.

Keywords Carfilzomib · Rutin · Cardiotoxicity · Natriuretic peptide · Nuclear factor-kappa B · Apoptosis · Oxidative stress

Introduction

Carfilzomib (CFZ) is a potent second-generation inhibitor of proteasome, approved for the treatment of patients with multiple myeloma [1–3]. It acts synergistically with histone deacetylase inhibitors and forms an irreversible covalent bond to inhibit 20S proteasome's chymotrypsin-like activity [1, 2, 4, 5]. Inhibition of proteasome-mediated proteolysis results in a buildup of polyubiquitinated proteins, which interfere with intracellular protein homeostasis resulting in cell cycle arrest, apoptosis and inhibition of tumor growth [6]. New onset or worsening of preexisting heart failure with reduced left ventricular function and myocardial ischemia has been described with CFZ [7, 8]. Similarly, another study has reported heart failure, cardiac arrest, and myocardial infarction with CFZ [9]. However, the underlying mechanism of carfilzomib related cardiotoxicity remains unclear.

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Cardiomyopathies are a heterogeneous group of diseases, either are confined to the heart or a part of generalized systemic disorders associated with ventricular hypertrophy or dilatation, cardiovascular death and progressive heart failure-related disability [10–12]. Cardiomyopathies often lead to myocardium damage such as myocarditis and myocardial infarction which results an increased release of cardiac enzymes, such as lactate dehydrogenase (LDH), creatine kinase (CK), CK-MB fraction, alanine transaminase (ALT) and aspartate transaminase (AST) into the blood stream, and serve as diagnostic markers [12, 13]. The elevated levels of these enzymes are associated with direct myocardial endothelial damage and destruction of myocardial cells [14].

Myocardial hypertrophy and disorganization of cardiac myocytes are generally attributed to an imbalance in the expression of several hypertrophic genes such as myosin heavy chain (MHC) and natriuretic peptides [15]. Hearts that express more α-MHC (faster MHC motor protein) show more powerful contraction than β-MHC (slower MHC motor protein). In nonfailing human ventricular myocardium, significant extent of α-MHC mRNA is expressed which is decreased 15-fold in end-stage failing left ventricles [16], whereas β-MHC is primarily expressed in failing adult ventricle [17]. Generally, natriuretic peptides, particularly BNP, have shown their indicative role for cardiotoxicity in patients receiving cardiotoxic anticancer therapy [18]. Anomalous dilatation of the cardiac wall chamber, increased fluid volume or reduced elimination of peptides results an increase in BNP [19].

Nuclear factor-κappa B (NF-κB) is present in the cytosol as complex with its inhibitory factor, inhibitory kappa B (IκB). It has been reported that NF-κB transcription factor is usually activated by the formation of reactive oxygen species (ROS) or inflammatory cytokines in myocardial tissue and direct activation of apoptotic genes which resulted in proapoptotic effect [20]. NF-κB activation seems to play a key role in the pathogenesis of endothelial dysfunction, unstable angina pectoris, acute myocardial infarction and heart failure [21].

ROS contribute to cellular signaling, distressing almost all characteristics of cellular physiology including gene expression, propagation, immigration and cell death [22]. Tissue glutathione (GSH) depletion and cellular damaging effects of ROS (including nitric oxide and peroxynitrite) is one of the primary factors which permit lipid peroxidation [23, 24]. GSH reduces hydroperoxide and hydrogen peroxide by oxidizing GSH to GSSG and other mixed disulfides [25] and acts as defense mechanism against free radicals and other oxygen [26].

Rutin (3′,4′,5,7-tetrahydroxyflavone-3β-d-oside), known as vitamin P, is the most abundant bioflavonoids generally recognized as cardioprotective, anti-inflammatory,

anticancer, antibacterial and antioxidant agent. Rutin makes the blood thinner and improves the circulation by inhibiting the platelet aggregation and decreasing the capillary permeability [27]. Rutin is also effective to treat hemorrhoids, varicosis and microangiopathy as reported in the literature [28, 29]. It decreases body weight as well as the belly fat content by enhancing the consumption of fat and the lipogenic carbohydrate, fructose, and by preventing further absorption and deposition of fat, and hence causes cardiovascular remodeling [30, 31]. Therefore, the current study was designed to investigate the possible protective role of rutin against CFZ-induced cardiotoxicity in rats using biochemical markers of oxidative stress, cardiac function, hypertrophic gene expression and histopathological measures of cellular damage.

Materials and Methods

Animals

Wistar albino male rats weighing 200–250 g (approximately 10 weeks old) were provided for this study from Experimental Animal Care Center, College of Pharmacy at King Saud University. Animals were kept in ideal research laboratory conditions on standard pellet diet and water ad libitum all over the study duration. All research work was conducted as per the standard guidelines of the Animal Care and Use Committee at King Saud University.

Drugs and Chemicals

Rutin, heparin and CFZ were purchased from Sigma-Aldrich (St. Louis, USA). Biochemical parameters were done using kits (Dimension®, Siemens, USA). The primers used in the current study for gene expression were purchased from Integrated DNA Technologies (IDT, Coralville, USA). SYBR® Green PCR Master Mix and high-capacity cDNA Reverse Transcription kit were purchased from Applied Biosystems (Paisley, UK). TRIzol were purchased from Invetrogen® (California, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Antibodies against rat NF-κB and IκB-α proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All the other reagents used were of standard research grade.

Experimental Protocol

Twenty-four male Wistar albino rats were randomly divided into four groups of six rats each as follows: Group 1, normal control (NC), received normal saline for 16 days. Group 2, CFZ group, received six doses of CFZ (4 mg/kg,

intraperitoneally [i.p.]) [32]. Group 3 animals received CFZ (4 mg/kg, i.p.) and rutin (20 mg/kg, administered p.o.) for 16 days. Group 4 animals received CFZ (4 mg/kg, i.p.) and rutin (40 mg/kg, administered p.o.) for 16 days.

At the end of study, all the rats were sacrificed by decapitation under ether anesthesia, as per the protocol. Whole blood was collected and serum separation was done at 3000 g for 10 min. For biochemical and hematological analysis, samples were kept at −20 °C until analysis. The rats' hearts were harvested and washed in ice-cold buffer saline and divided into three segments used for the assessment of oxidative stress, histopathology and gene expression analysis.

Biochemical Analysis

Biochemical estimations of LDH, CK, CK-MB were conducted using autoanalyzer (Dimension® RXL MAX™, Siemens, USA).

Extraction of RNA and Synthesis of cDNA

All extraction and synthesis processes were executed on crushed ice using ice-cold reagents. The total cellular RNA from rat's heart tissue was extracted from homogenates using TRIzol reagent (Invitrogen®, Carslbad, California, USA) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (>2.0). High-Capacity cDNA reverse transcription was utilized to synthesize first strand cDNA, according to the manufacturer's instructions. Briefly, 1.5 μg of RNA from each sample was added to a mixture of 2.0 μl of 10× reverse transcriptase buffer, 0.8 μl of 25× dNTP mix (100 mM), 2.0 μl of 10× reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase and 3.2 μl of nuclease-free water. The final reaction mixture was kept at 25 °C for 10 min and then heated to 37 °C for 120 min, followed by 85 °C for 5 min, and finally cooled to 4 °C.

Quantification of mRNA Expression in Heart Tissue via RT-PCR

Quantitative analysis of specific gene mRNA expression was performed via real-time PCR by subjecting the resulting cDNA obtained from the above preparation methods to PCR amplification using 96-well optical reaction plates in the ABI fast 7500 System (Applied Bio systems®). The 25-μl reaction mixture contained 0.1 μl of 10 μM forward primer and 0.1 μl of 10 μM reverse primer, 12.5 μl of SYBR Green Universal Master mix, 11.05 μl of nuclease-free water and 1.25 μl of cDNA

sample. The primers used in these assays were purchased from Integrated DNA technologies (IDT, Coralville, USA), selected from PubMed and other databases, and are listed in Table 1. The fold change in the level of target mRNA between control and treated animals was corrected by the level of β-actin. Assay controls were incorporated onto the same plate, namely no-template controls to test for the contamination of any assay reagents. The RT-PCR data were analyzed using the relative gene expression (i.e.,$\Delta\Delta$ CT) method as described in Applied Biosystems User Bulletin No. 2. Briefly, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (β-actin) and relative to a calibrator.

Protein Extraction and Western Blot Analysis

Cardiac tissues protein was extracted as described previously [33]. The modified method of Lowry et al. [34] was employed for determination of protein concentrations using bovine serum albumin (BSA) as a standard. Western blot analysis was conducted as described previously [33, 35]. Briefly, protein (30–40 μg) from each treatment group was separated in 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4 °C before further incubated for 24 h with primary antibodies against target proteins followed by peroxidase-conjugated secondary antibodies for 2 h at room temperature. The bands were visualized and then quantified by C-DiGit® Blot Scanner, LI-COR Biosciences (Lincoln, Nebraska, USA) using the enhanced chemiluminescence method according to the manufacturer's instructions, Merck Millipore (Billerica, MA, USA).

Determination of Lipid Peroxides, Measured as Malondialdehyde (MDA)

MDA, a product of membrane lipids peroxidation, was measured in cardiac tissue by modified method of Okhawa [36]. The MDA levels obtained were expressed as nmoles of MDA/mg protein. Lowry method was employed for the estimation of total tissue proteins [34].

Determination of Reduced Glutathione (GSH)

Content of GSH in cardiac tissue was estimated by the method of Sedlak and Lindsay [37]. The optical density of reaction mixture was recorded within 5 min at 412 nm (UV spectrophotometer), after addition of dithiobis-2-nitrobenzoic acid against blank.

Table 1 Rat primers sequence used for RT-PCR

Gene	Forward primer	Reverse primer
β-MHC	ATCAAGGGAAAGCAGGAAGC	CCTTGTCTACAGGTGCATCA
α-MHC	TCCTTTATCGGTATGGAGTCTG	TGATCTTGATCTTCATGGTGCT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
p53	ACAGCGTGGTGGTACCGTAT	GGAGCTGTTGCACATGTACT
NF-κB	ACCCCTTTCAAGTTCCCATAGA	ACCTCAATGTCTTCTTTCTGCAC
β-actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA

Histopathological Studies

Heart tissue was harvested from the rats and fixed in 10 % buffer formalin solution. A thin section of 3–4 μm thickness in paraffin was prepared and stained with hematoxylin and eosin (H&E). The stained sections were seen under light microscopy for histopathological examination [38].

Statistical Analysis

All results are presented as mean + SEM. Comparisons among different groups were analyzed by analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparisons test to find significance between groups. Values were considered statistically significant when $p < 0.05$. Statistical analysis was carried out using Graph pad prism 3.0 (La Jolla, CA).

Results

Effects of Rutin and CFZ on Hematological Changes

In this study, treatment of rats with CFZ resulted in hematological changes as evidenced by a statistically significant reduction in red blood cells (RBCs) count and white blood cells (WBCs) count and Hb and HCT % concentration as compared to NC group. Treatment with rutin significantly ($p < 0.05$) reversed CFZ-induced changes and resulted an increase in RBCs, WBCs, Hb and HCT % concentration (Figs. 1, 2).

Effects of Rutin and CFZ on Cardiac Markers

Treatment of rats with CFZ resulted in cardiac damage as showed by significant ($p < 0.05$) increased release of cardiac enzymes such as LDH, CK and CK-MB as compared to NC group which were significantly ($p < 0.05$) reversed by rutin treatment (Fig. 3).

Effects of Rutin and CFZ on α-MHC, β-MHC and BNP mRNA Expression

We further evaluated the effect of rutin against CFZ-induced cardiotoxicity by measuring the mRNA levels of α-MHC, β-MHC and BNP in the heart tissue. Our results showed that induction of cardiotoxicity by CFZ significantly down-regulated α-MHC mRNA expression while up-regulated β-MHC and BNP mRNA expression as compared to NC group (Fig. 4). In contrast, pre-treatment of rats with rutin 20 and 40 mg/kg resulted in a marked up-regulation of α-MHC mRNA expression and down-
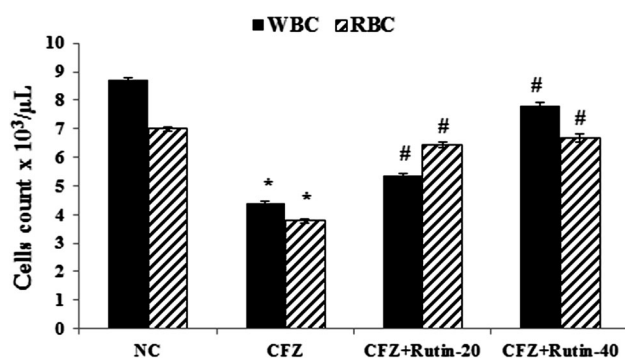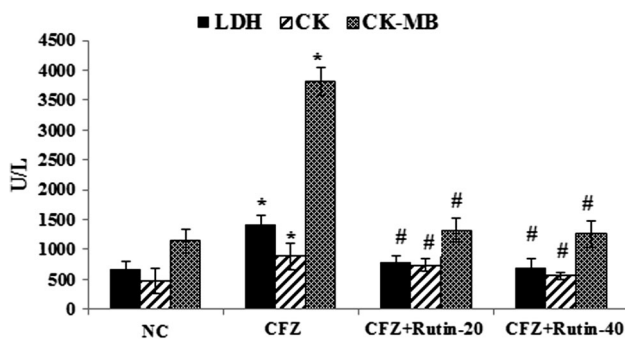

Fig. 1 Effects of rutin on CFZ-induced red blood cells (RBCs) and white blood cells count (WBCs) of different experimental groups. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with the normal control (NC) group; #$p < 0.05$ compared with the CFZ group
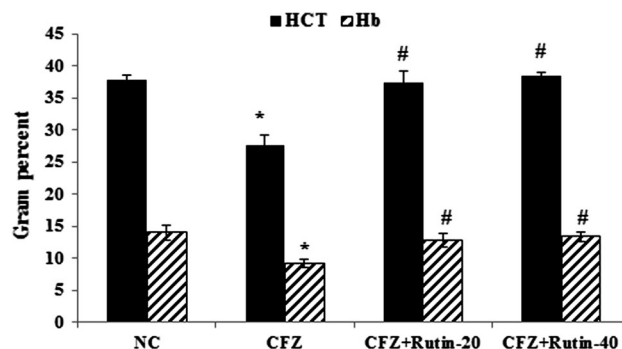
Fig. 2 Effects of rutin on CFZ-induced changes in hemoglobin and hematocrit value in whole blood of different experimental groups. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with the normal control (NC) group; #$p < 0.05$ compared with the CFZ group
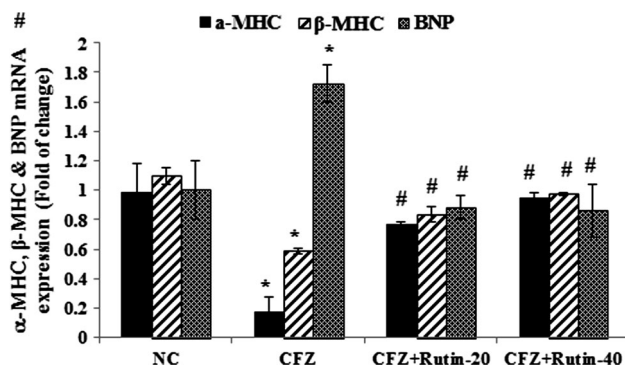
Fig. 3 Effects of rutin on CFZ-induced changes in cardiac enzymes of different experimental groups. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with the normal control (NC) group; #$p < 0.05$ compared with the CFZ group
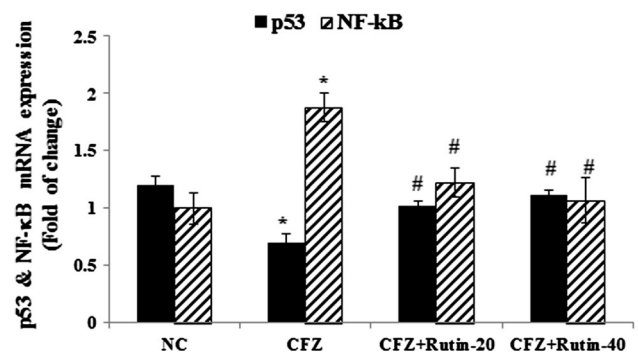

Fig. 5 Effects of rutin on CFZ-induced changes in mRNA expression of NF-κB and p53. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with control group; #$p < 0.05$ compared with the CFZ group

regulation of β-MHC and BNP mRNA expression (Fig. 4). The present study demonstrated that rats treated with rutin resulted in a significant reduction in the mRNA expression of hypertrophic gene.

Effects of Rutin and CFZ on NF-κB and p53 mRNA Expression

We further evaluated the effect of rutin against CFZ-induced cardiotoxicity by measuring mRNA levels of NF-κB and p53 in the heart tissue. Induction of cardiotoxicity by CFZ significantly up-regulated NF-κB mRNA expression while down-regulated p53 mRNA expression as compared to NC group (Fig. 5). In contrast, pre-treatment of rats with rutin at 20 and 40 mg/kg resulted in a marked down-regulation of NF-κB mRNA expression whereas up-regulation of p53 mRNA expression (Fig. 5).

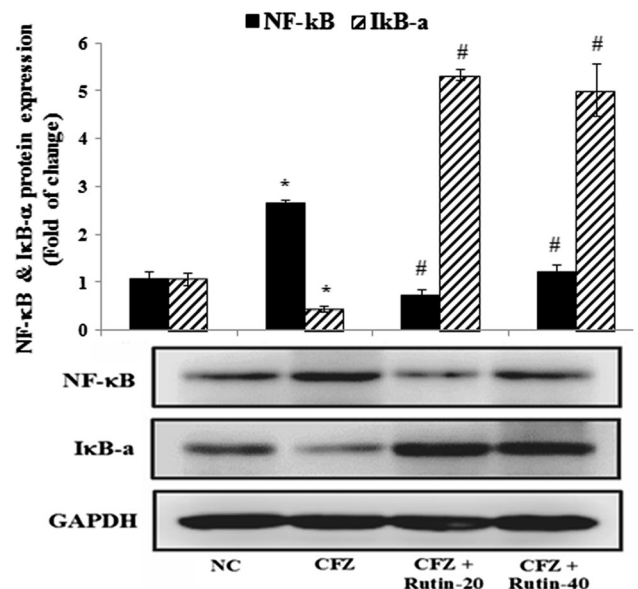## Effect of Rutin and CFZ on NF-κB p65 and IκB-α Protein Expression

To further explore the role of NF-κB for cardioprotective effect of rutin, for this purpose, the protein expression of NF-κB and its inhibitory protein IκB-α were determined by Western blot analysis. Figure 6 shows that CFZ significantly increases NF-κB protein by 2.5-fold which was associated with a significant decrease in its inhibitory protein IκB-α by approximately 60 % as compared to NC group. Importantly, treatment of rats with rutin restored the changes in protein expression of NF-κB and IκB-α, in that

Fig. 4 Effects of rutin on CFZ-induced changes in mRNA expression of α-MHC, β-MHC and BNP. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with control group; #$p < 0.05$ compared with the CFZ group

Fig. 6 Effects of rutin on CFZ-induced changes in protein expression of NF-κB and IκB-α in rats cardiac tissues. Data are presented as mean ± SEM ($n = 6$). Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with control group; #$p < 0.05$ compared with the CFZ group

rutin treatment prevented the activation of NF-κB by increasing the expression of IκB-α (Fig. 6).

Effects of Rutin and CFZ on Oxidative Stress Markers

The results are summarized in Fig. 7. Treatments of rats with CFZ resulted in a significant ($p < 0.05$) increase in heart MDA contents and decrease in cardiac GSH level compared to the NC group. A significant reversal in CFZ-induced increase in cardiac MDA levels and decrease in cardiac GSH level were observed with rutin treatment (Fig. 7).

Effects of Rutin and CFZ on Histopathological Changes in Heart

Normal morphological structures of cardiac tissue were observed in the control group (Fig. 8). However, three cycle treatment with CFZ showed myocardial degeneration and broken myocardial fibers with cytoplasmic vacuoles. Clusters of hypochromatic cells having pyknotic nuclei and inflammatory cells infiltration were the most significant change all over the heart (Fig. 8). Treatment with rutin reversed CFZ-induced cardiac damage as evidenced by normalization of shape and size of cardiac muscle fibers (Fig. 8).

Discussion

Cardiotoxicity is a major limiting factor in anticancer therapy such as CFZ, and the risk is even greater if there is a known history of heart disease [39, 40]. Rutin is the most abundant bioflavonoids having anti-inflammatory, anti-cancer, antibacterial, cardioprotective and antioxidant

Fig. 7 Effects of rutin on CFZ-induced changes in MDA and GSH levels assessed in heart tissue. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer posttest. *$p < 0.05$ compared with control group; #$p < 0.05$ compared with the CFZ group

activity. Rutin makes the blood thinner and improve the circulation by inhibiting the platelet aggregation and decreasing the capillary permeability [27]. However, the cardioprotective role of rutin against CFZ-induced cardiotoxicity has not been investigated before. Thus, the present study was conducted to explore the role of rutin against CFZ-induced cardiotoxicity through inhibition of NF-κB, hypertrophic gene expression and oxidative stress using rats model.

In the present study, treatment of rats with CFZ resulted in a significant decrease in RBCs, WBC, HCT and Hb % concentration. This reduction in the HTC and Hb % concentration may be related to abnormal decrease in total amount of body iron. Al-Shabanah et al. [41] reported that a single dose of doxorubicin resulted in significant reduction in RBCs and WBCs, count as well as HTC and Hb % concentration. Our results are in agreement with earlier reports. Similarly, Piura and Rabinovich reported that combination of doxorubicin and ifosfamide [42] causes hematological toxicity such as leukopenia, neutropenia, thrombocytopenia and anemia in patients with advanced/recurrent uterine sarcoma.

The changes in biochemical parameters in response to CFZ was assessed with significant increase in serum levels of cardiac enzymes such as LDH, CK, CK-MB compared to NC group, which are important parameters for the assessment of cardiotoxicity [43, 44]. However, treatment with rutin ameliorated CFZ-induced changes in these enzyme activities. The above reports are in agreement with previous studies [38, 45, 46].

Another evidence that supports the CFZ-induced cardiotoxicity is down-regulation of α-MHC mRNA expression, up-regulation of β-MHC and BNP mRNA expression, indicating cardiac hypertrophy. Imbalance in the expression of hypertrophic genes such as α-MHC, β-MHC and natriuretic peptides indicates myocardial hypertrophy and disorganization of myocytes [15]. In agreement with our results, it has been previously reported that sunitinib, a tyrosine kinase inhibitor, induced cardiotoxicity by β-MHC and α-MHC at both mRNA expression and protein expression levels in vitro [47] and in vivo using H9c2 cells [47, 48].

Previous studies have reported that as hypertrophy worsens to heart failure, apoptotic genes, such as p53 will be overexpressed suggest that p53 may influence cardiotoxicity. P53 is an important cellular signaling molecule that plays a significant role in apoptosis, genomic stability and inhibition of angiogenesis [49]. On the other hand, activation of NF-κB is known to inhibit apoptosis [50–52]. Thus, to explore the involvement of p53 and NF-κB, we measured the expression of these gene at the mRNA levels. In this study, mRNA expression levels of NF-κB were significantly increased, while p53 mRNA levels were significantly decreased in CFZ treated groups which were reversed by
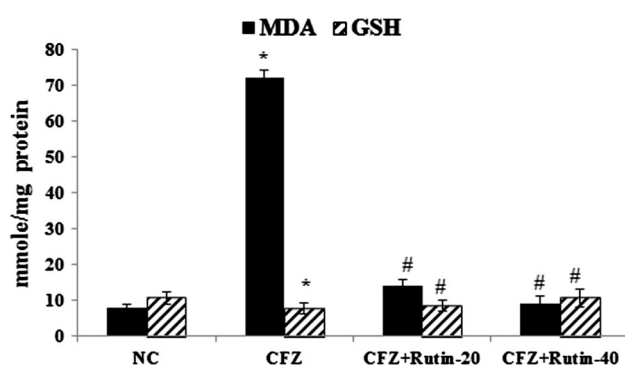
Fig. 8 Effects of rutin on CFZ-induced changes in heart histopathology of different experimental groups. **a** NC; **b** carfilzomib; **c** carfilzomib + rutin 20 mg/kg; and **d** carfilzomib + rutin 40 mg/kg. Staining of the thin section of heart tissues is done by hematoxylin and eosin method. ($n = 6$ per group; magnification $= 20\times$)
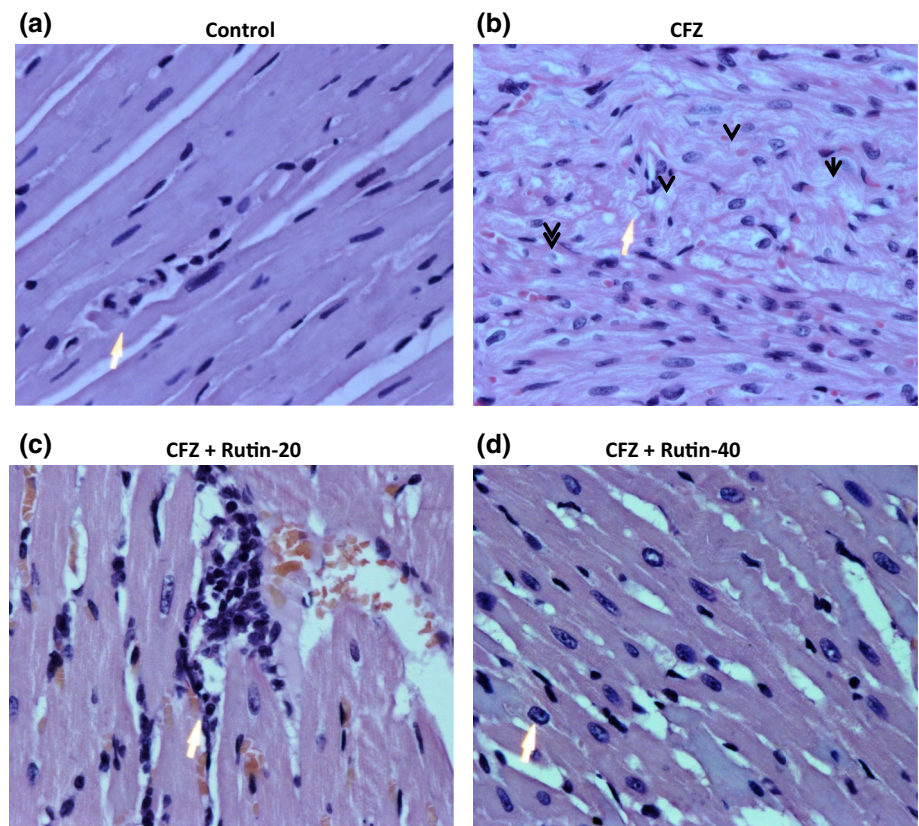
treatment with rutin. Thus, these results give evidence to believe that NF-κB signaling pathways would be an effective strategy to restore CFZ-induced cardiotoxicity.

NF-κB is involved in the expression of many genes associated with inflammation, cell injury and stress, and plays an important role in the regulation of cell survival and death. Activation of NF-κB appears to play a significant role in the pathophysiology of endothelial dysfunction, unstable angina pectoris, acute myocardial infarction and heart failure [21]. NF-κB consists of p50 and p65 heterodimer retained in the cytoplasm by inhibitory proteins called IκBs. In response to various stimuli, IκB kinase (IKK) is activated, leading to IκBα phosphorylation, ubiquitination and degradation by the proteasome. The dissociated p50–p65 complex then translocates to the nucleus, binds to its consensus sequence within the promoter of NF-κB target genes, and regulates gene transcription [53, 54]. In the current study, mRNA and protein expression levels of NF-κB were significantly increased in response to CFZ, which was restored by rutin treatment. Mechanistically, this effect was mediated through increasing IκBα protein levels. These observations were in agreement with previous studies showed that cellular NF-κB activity is regulated by IκB kinase (IKK)-mediated phosphorylation and degradation of the cytoplasmic inhibitor protein IκBα [54, 55].

Tissue GSH depletion and cellular damaging effects of ROS is one of the primary factors which permit lipid peroxidation [23, 24]. GSH also reduces hydrogen peroxide and hydroperoxide by oxidizing GSH to GSSG and other mixed disulfides [25] and acts as defense mechanism against free radicals and other oxygen [26]. Therefore, we estimated MDA and reduced glutathione in cardiac tissue as a measure of oxidative stress. In our research, we observed a statistically significant increase in MDA content and decrease in GSH level in cardiac tissue after CFZ administrations which were reversed by rutin treatment. The elevated MDA content may be attenuated to over production of ROS (superoxide radicals, hydrogen peroxide and hydroxyl radicals) and decreased GSH content may be due to scavenging of ROS as GSH in a major intracellular redox buffer and has ability to detoxify various ROS through direct interaction.

Histopathological examination of cardiac tissues showed myocardial degeneration and broken myocardial fibers with cytoplasmic vacuoles in toxic group. Clusters of hypochromatic cells with pyknotic nuclei and inflammatory cell infiltrate were the most significant change all over the heart. Similar histopathological and ultrastructural changes associated with doxorubicin-related cardiotoxicity have been reported earlier [38, 45, 50]. Carfilzomib-induced histopathological changes were reversed by

treatment with rutin. There was restoration of myofibril architecture after treatment with rutin. In conclusion, the current study provides a strong evidence that rutin has protective effects against CFZ-induced cardiotoxicity through modulation of cardiac hypertrophic gene and NF-kB pathway.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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