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Mediation of FOXO3 Gene on Momism of Quercetin against Isoproterenol Induced Cardiotoxicity in H9C2 Cardiomyoblasts

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ABSTRACT

Background: Due to over production of reactive oxygen species, oxidative stress forms one of the major reasons for progression of Cardiovascular Diseases (CVDs) and the associated cardiomyopathies. Thus, controlling these reactive radicals is one of the major management strategies for CVDs. Natural compounds and phytochemicals have already proven their potency in preventing oxidative stress and the molecular mechanism behind the action of most of these bioactive compounds is still an enigma.
Objectives: The present study aimed to assess the cardioprotective effects of quercetin on isoproterenol-induced toxicity in H9c2 cardiomyoblast cells.
Materials and Methods: H9C2 cells were treated with different concentrations of isoproterenol and quercetin and the cells viability was determined by Mitochondrial Tetrazolium (MTT) assay. The non-toxic concentration of quercetin was used to check its protective effect on the cells treated with 50 µM isoproterenol. Indeed, intracellular antioxidants, such as catalase, reduced glutathione (GSH), and lipid peroxidation, were determined spectrophotometrically in cell lysates. Moreover, mRNAs isolated from the treated groups were subjected to expression studies of FOXO3 and STAT3 genes by reverse transcriptase Polymerase Chain Reaction (PCR).
Results: According to the results of MTT assay, the effective concentration of isoproterenol for inducing toxicity in H9c2 cells was 50 µM and the protective concentration of quercetin was 5 µm. Treatment with quercetin elicited a protective effect by enhancing the antioxidant status of the cells. This was confirmed by the decrease in peroxidation of membrane lipids, increase in catalase induction, and increase in GSH reserve. Gene expression analysis of FOXO3 and STAT3 genes revealed that the protective effect was mediated through up regulation of FOXO3 transcription factor.
Conclusions: The study results signified the potential of quercetin in relieving oxidative stress and the associated cardiomyopathies.

1. Background

The active role of Reactive Oxygen Species (ROS) and the associated oxidative stress in progression of Myocardial Infarction (MI) is clearly evident (1). Cumulative effects of ROS-mediated fibroblast proliferation, collagen deposition, Matrix Metallo Protease (MMPs) – Tissue Inhibitors of MMPs (TIMPs) imbalance, and reperfusion injury aggravate the clinical status of MI (2). The lion's share of ROS at the infarct zone is contributed by infiltrating inflammatory cells, especially neutrophils. Involvements of ischemic mitochondrial electron transport, xanthene oxidase system, non-phagocytic NADPH oxidases, heme-oxygenase, and cytochrome P450 mono-oxygenase were also clearly unveiled to be other potent sources of oxidative stress in the infarcted myocardium (3). During myocardial ischemia, the native antioxidant machinery was reported to be insufficient for controlling the progression of ROS, which normally ends up in oxidative stress (4, 5).

Antioxidant defense components, such as ROS scavengers and enzymes and proteins bearing Antioxidant Response Elements (AREs) in their promoter, respond to oxidative stress to protect cells from deleterious effects. Oxidative

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stress also stimulates the expression of a battery of genes involved in the antioxidant mechanism most of which are mediated by Nrf-2 transcription factor (5). Similarly, FOXO3, a member of forkhead family of transcription factors, acts to prevent oxidative stress by upregulating the expression of the scavenging enzymes superoxide dismutase 2 (SOD2/MnSOD) and catalase. Furthermore, FOXO3 functions in modulating cell cycle arrest, DNA repair, and apoptosis by its phosphorylation/dephosphorylation mechanism (6). Being terminally differentiated, prevention of oxidative stress-induced apoptosis in cardiomyocytes after infarction is crucial for survival of the heart. Moreover, for restoration and sustenance of cardiac function at the infarct zone, the signals triggering cell proliferation, migration, maturation, and differentiation are activated. Signal Transducers and Activators of Transcription (STATs) and transcription factors act in coordination with JAK family of tyrosine kinases and elicit the expression of genes for restoration pathways (7).

Availability of exogenous antioxidants relieves oxidative stress either by upregulating the antioxidant signaling or by direct neutralization of ROS. Exogenous antioxidants are mainly composed of dietary bioactive phytochemicals. These molecules were reported to improve cardiovascular performance after infarction (8). Vitamins (mainly C and E), caroteinoids, flavonoids, polyphenols, terpenes, glycosides, etc. form the major group of plant-derived antioxidants, which are effective against induction and progression of ROS-mediated diseases, including cancer, MI, and diabetes (9). At their lower concentrations, these phytochemicals prevent the oxidation of biological molecules and membranes and minimize disease susceptibility (10).

Amongflavonoids,quercetin(3,5,7,3',4'-pentahydroxyflavone) has already proven to be effective in avoiding oxidative injury by scavenging ROS, chelating the toxic metal ions, and preventing the peroxidation of membrane lipids (11). Quercetin has been hailed to be the excellent ROS scavenger within the flavonoid family of antioxidants. The scavenging properties of quercetin have been attributed to the presence of catechol group within the structure. Several experimental data have confirmed that the efficiency of quercetin was more than six times greater than the synthetic antioxidant trolox (Vitamin E analog) and much superior to the natural antioxidant Vitamin C (12). The antioxidant activities of quercetin were also reported to contribute to its cardio-protective effects against several models of cardiomyopathy. These protective effects were reported to be mediated through the STAT3 pathway (13).

Even though several antioxidant defense and apoptotic pathways were come into picture through elucidation of the expression of specified sets of genes, the actual mechanism behind the antiradical action of quercetin is still an enigma.

2. Objectives

The present study aims to elucidate the effect of quercetin on eliciting the antioxidant status of isoproterenol-induced cardiotoxicity in H9c2 cardiomyoblast cells. Involvement of FOXO3 and STAT-3 pathway in mediating the protective effects is also evaluated.

3. Materials and Methods

3.1. Materials

Quercetin (98.0%), Ethidium Bromide (EB), and isoproterenol were purchased from Sigma Aldrich. Trypsin, Ethylenediaminetetraacetic Acid (EDTA), Mitochondrial Tetrazolium (MTT), penicillin, streptomycin, neutral red, and Foetal Bovine Serum (FBS) were bought from Invitrogen. H9c2 cell line was procured from NCCS, Pune, India. Other used chemicals were of standard analytical grade.

3.2. Cell Culture

H9c2 cardiomyoblast cells were grown in Dulbecco's modified Eagle's medium supplemented with FBS, penicillin, and streptomycin. After attaining confluence, the cells were subcultured by trypsinization (0.25% in 0.5mM EDTA) and were maintained at 5% CO2 at 37 ºC in a humidified atmosphere. The medium was changed according to standard cell culture protocols at regular intervals.

3.3. Dose Determination for Isoproterenol and Quercetin

The effective concentration of isoproterenol for inducing cardiotoxicity and the concentration of quercetin to elicit a protective effect were determined by evaluating the cells viability using MTT assay. Briefly, varying concentrations of isoproterenol (25, 50, 100, and 200 µM) and quercetin (5, 10, 20, 40, and 80 µm) were administered to a sub-confluent monolayer of H9c2 cells. The cells were then allowed to proliferate for 24 h. Then, the cells were washed with sterile PBS and MTT solution (5 mg/mL) was added and incubated at 37 ºC for 3 hours. After incubation, the cells were lysed with Dimethyl Sulfoxide (DMSO) to dissolve the formazan product and the absorbance was read at 540 nm. Based on the absorbance values, the percentage of the viable cells was calculated (14).

3.4. The Effects of Quercetin and Isoproterenol on Intracellular Enzymes and Markers

The dose-optimized concentration of isoproterenol (50 µM) and the non-cardiotoxic concentration of quercetin (5 µM) were used to study the effect of quercetin on intracellular enzymes and markers in H9c2 cardiomyoblast cells. After the treatment, the cells were lysed and the status of the antioxidant biomarkers was assessed.

3.4.1. Lipid Peroxidation

At first, 50 µL cell lysate was taken in a test tube. Then, 50 µL of 70% ethanol and 1 mL of 1% Thiobarbutaric Acid (TBA) were added and boiled for 30 min. After cooling to room temperature, 50 µL acetone was added, the mixture was centrifuged, and the absorbance was read at 535 nm using a spectrophotometer (15).

3.4.2. Reduced Glutathione

At first, 1 mL cell lysate was mixed with the reaction mixture containing 0.5 mL of 0.2 M phosphate buffer, 1.3 mL distilled water, and 0.2 mL of 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Then, the absorbance was read at 420 nm (16).

3.4.3. Catalase

At first, 3 mL of phosphate buffer was added to 0.04 mL

of enzyme solution (0.01 mM) (pH: 7). Afterwards, the absorbance was read at 260 nm every 15 seconds. It should be noted that H2O2 was taken as blank.

3.5. Analysis of the Effect of Quercetin-Induced Regulation of FOXO3 Gene on Isoproterenol-Treated H9c2 Cells

H9c2 cells were cultured under the above-mentioned conditions and were treated with isoproterenol (50 µM) and quercetin (5 µM). After that, total RNA was isolated using the RNA isolation kit (Thermo fisher scientific) according to the manufacturer's instructions. The isolated RNA was suspended in 50 µL TE buffer and used for gene expression analysis.

3.5.1. Reverse Transcriptase PCR

Firstly, 5 µL of RNA template (suspended in 50 µL of Di Ethyl Pyro Carbonate (DEPC) water, incubated at 65 ºC for 15 min) was mixed with 4 µL of the control reverse primer, incubated at 65 ºC for 10 min, and immediately chilled on ice. Polymerase Chain Reactions (PCRs) were set by adding the components in the following order: 45 µL RNA super mix, 2 µL of reverse and forward primers, and 4 µL of RNA template. The denaturation temperature was set at 94 ºC for 15 - 30 seconds, annealing was done at 55 ºC for 15 - 30 seconds, and extension was carried out at 72 ºC for 1 min. Overall, 30 cycles of amplification were carried out and the amplified products were run under 1% agarose gel electrophoresis system. The obtained bands were observed using gel doc imaging system (table 1).

4. Results

In order to study the protective effects of quercetin on isoproterenol-induced cardiotoxicity of H9c2 cardiomyoblast cells, the effective concentrations of both compounds were determined by MTT assay. Accordingly, the effective concentrations of isoproterenol and quercetin were 50 µm and 5 µm, respectively (Figure 1). These concentrations were used for evaluation of antioxidant biomarkers and gene expression.

4.1. The Effects of Quercetin and Isoproterenol on Intracellular Enzymes and Markers

4.1.1. Lipid Peroxidation

Generally, peroxidation of membrane polyunsaturated fatty acids generates Malondialdeyde (MDA) and its measurement has been used as an indicator of lipid peroxidation. In this study, the cells treated with isoproterenol, quercetin, and their combination were checked for the amount of generated MDA. Based on the results, the cells treated with the combination of isoproterenol and quercetin showed a considerable decrease in MDA compared to those treated with isoproterenol alone (Figure 2). This shows the protective effects of quercetin.

4.1.2. Reduced Glutathione

GSH serves as the cellular reducing equivalent and readily neutralizes ROS, thereby offering protection against oxidative injury. Thus, intracellular GSH level provides a relevant and accurate measure of the cells oxidative status. In our study, the cells treated with isoproterenol showed decreased GSH levels. On the other hand, the cells treated with the combination of isoproterenol and quercetin showed a significant increase in GSH level, which signifies the protective effect of quercetin (Figure 3).

Table 1. The Primer Sequences Used for PCR Analysis

Primers	Sequence
STAT3 (Fw)	GGAGGAGTTGCAGCAAAAAG
STAT3 (Rev)	TGTGTTTGTGCCCAGAATGT
FOXO3 (Fw)	ATGTTTCAGCTCTTCCACCTACAGA
FOXO3 (Rev)	CCAGAGAGAGCTCAGATACGTTGAC

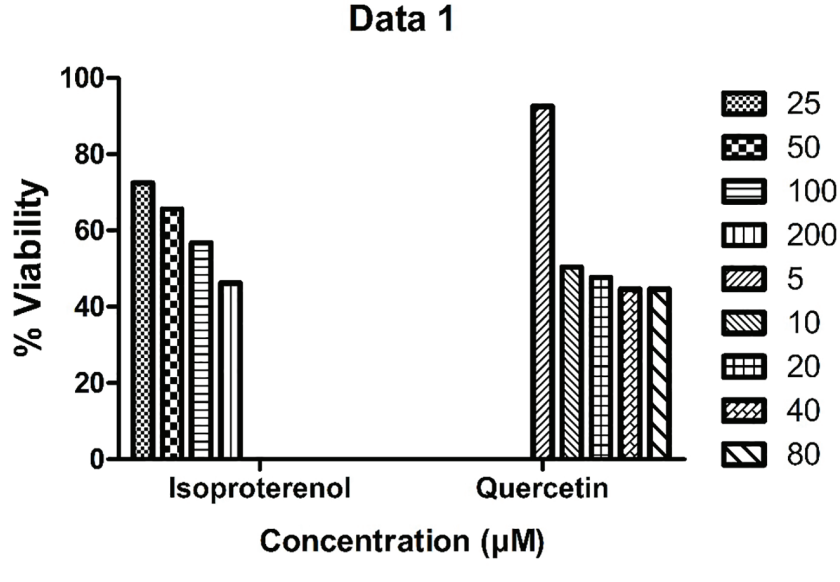

Figure 1. MTT Assay for Determination of Cytotoxic Concentrations of Isoproterenol and Quercetin

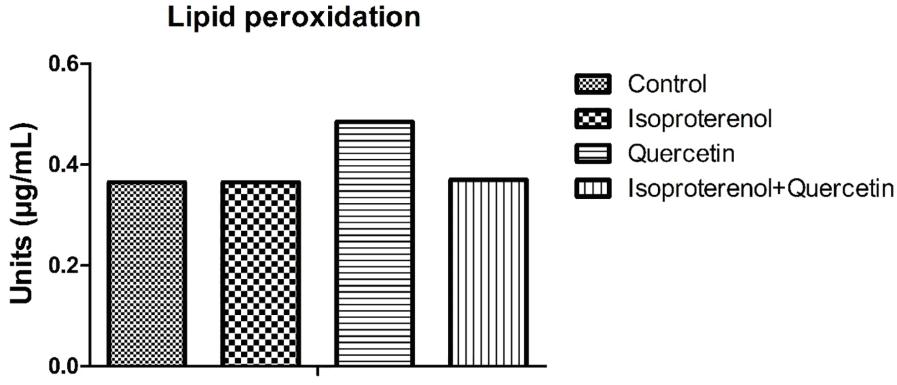

Figure 2. Determination of the Protective Effect of Quercetin on Lipid Peroxidation against the Isoproterenol-Induced Toxicity

important roles in physiology as well as pathophysiology.

Figure. 3. Estimation of GSH Content

4.1.3. Catalase

Catalase enzyme protects cells from oxidative damage by ROS, especially hydrogen peroxide. Therefore, recovery of catalase content to the normal range from a depleted state after treatment with quercetin is an indication of its protective effect against hydrogen peroxide (Figure 4).

4.2 Analysis of the Effect of Quercetin-Induced Regulation of FOXO3 Gene on Isoproterenol-Treated H9c2 Cells

The gene expression analysis of FOXO3 and STAT3 were carried out by reverse transcriptase PCR (Figure 5). The results revealed that FOXO3 expression was down regulated by isoproterenol and was upregulated upon treatment with quercetin. On the other hand, STAT3 expression was down regulated in the presence of quercetin. This suggests that the action of quercetin was mediated through regulation of FOXO3 gene.

5. Discussion

Based on the "free radical theory" of disease, researchers have been trying to elucidate the role of oxidative stress in cardiovascular diseases. Considerable data have indicated that ROS and oxidative stress are important features of cardiovascular diseases, including atherosclerosis, hypertension, and congestive heart failure. Blanket strategies with antioxidants to ameliorate cardiovascular diseases have not generally yielded favorable results. However, our understanding of ROS realizes that these species have Indeed, there are several sources of ROS that are known to be active in the cardiovascular system (17).

Reviews on protective effects of antioxidants on cardiotoxicity were validated by checking the effect of quercetin, a flavonoid, on isoprotenol-induced cardiotoxicity. Isoproternol causes a myocardial damage similar to the one observed in Acute Myocardial Infraction (AMI) in humans. Isoproterenol has also been reported to induce oxidative stress and subsequently imbalance in cellular oxygen supply, which is similar to events of cardiac hypertrophy. Furthermore, it has been claimed that there is an elevation of Ca++ overcharge inside cardiac cells. In addition, Ca++ ions are related to activation of the adenylatecyclase enzyme and depletion of ATP levels. Eventually, there occurs an oxidative stress augmentation because of several metabolic products originated from isoproterenol, leading to generation of ROS and the subsequent cell death (18-20).

The results of the present study proved the cytoprotective effect of quercetin by MTT assay. The decrease in mitochondrial metabolism could be attributed to toxicity of isoproternol, which was effectively ameliorated by addition of quercetin. Additionally, intracellular antioxidant enzymes and markers were determined to assess the oxidative damage produced by isoproternol, which was in accordance with the previous findings (21, 22). Similar trends were also observed for catalase and GSH levels.

Several phytochemicals were reported to elicit antioxidant

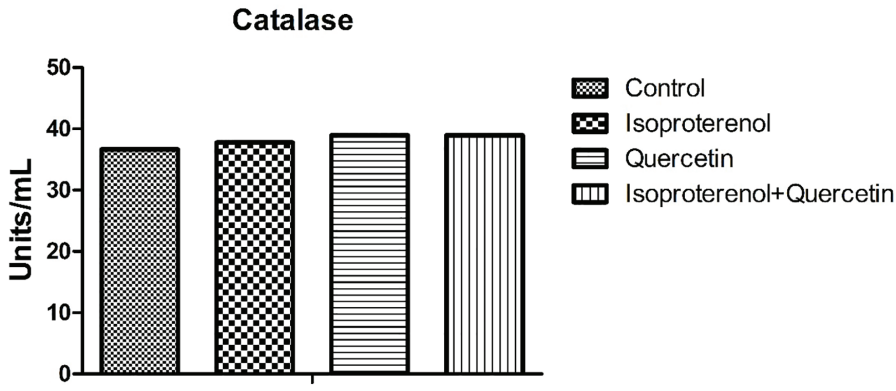

Figure 4. Estimation of Catalase Content

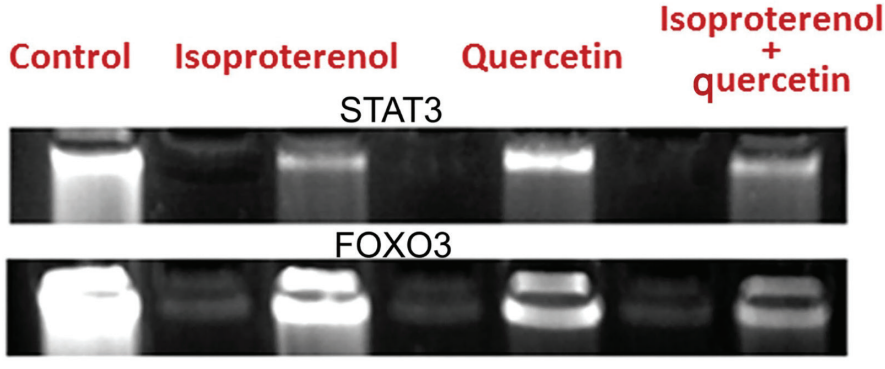

Figure 5. Expression Analysis of FOXO3 and STAT3 Gene

response by targeting SIRT1, a member of the sirtuin family of nicotinamide adenine dinucleotide dependent deacetylases. SIRT1 functions by deacetylating several transcription factors in chromatins, such as p53, FOXO, and NF-kB. Therefore, SIRT1 plays a vital role in cell differentiation, survival, and well-being. Being deacetylated by SIRT1, FOXO3a genes are triggered, which opens the antioxidant pathways (23). The major targets of FOXO3 include antioxidant enzymes, such as MnSOD, catalase, and thioredoxins (24). In the current study, the increased expression of FOXO3 gene and raised levels of catalase and GSH in the quercetin-treated H9c2 cells compared to the isoproterenol controls revealed involvement of FOXO3 genes in the cytoprotective effects of quercetin. Nonetheless, the mechanism by which quercetin influences FOXO3 remains to be explored. Yet, the capability of quercetin to trigger antioxidant enzymes in response to oxidative stress potentiates its therapeutic applications, especially against ROS-born cardiomyopathies.

5.1. Conclusions

ROS and oxidative stress are the important aggravators of cardiovascular diseases, including atherosclerosis, hypertension, and congestive heart failure. The present study explored the protective effects of quercetin on the cardiotoxicity induced by isoproterenol in H9c2 cardiomyoblasts. The protective effects were elicited through the increased levels of antioxidant enzymes and GSH reserve, which were in turn mediated by upregulation of FOXO3 genes. Thus, the results potentiated the application of quercetin for management of oxidative stress and the associated cardiotoxicity.

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Authors' Contribution

All the three authors carried out the experiments and the first author interpreted and wrote the manuscript.

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