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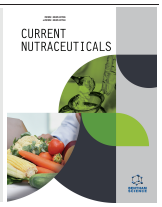
RESEARCH ARTICLE



Hydrogen Peroxide and Quercetin Induced Changes on Cell Viability, Apoptosis and Oxidative Stress in HepG2 Cells



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Abstract: ***Background:*** Different cellular responses influence the progress of cancer. In this study, the effects of hydrogen peroxide and quercetin induced changes on cell viability, apoptosis, and oxidative stress in human hepatocellular carcinoma (HepG2) cells were investigated.

Methods: The effects of hydrogen peroxide and quercetin on cell viability, cell cycle phases, and oxidative stress related cellular changes were investigated. Cell viability was assessed by WST-1 assay. Apoptosis rate, cell cycle phase changes, and oxidative stress were measured by flow cytometry. Protein expressions of p21, p27, p53, NF-Kβ-p50, and proteasome activity were determined by Western blot and fluorometry, respectively.

Results: Hydrogen peroxide and quercetin treatment resulted in decreased cell viability and increased apoptosis in HepG2 cells. Proteasome activity was increased by hydrogen peroxide but decreased by quercetin treatment.

Conclusion: Both agents resulted in decreased p53 protein expression and increased cell death by different mechanisms regarding proteostasis and cell cycle phases.

Keywords: HepG2 cells, oxidative stress, hydrogen peroxide, quercetin, apoptosis, cell cycle.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of cancer, causing over 700,000 deaths per year and covers around 90% of all histopathology types of primary liver cancers [1, 2]. Signaling mechanisms related to growth factors, cell differentiation, and angiogenesis play a significant role in the development of HCC. Reactive oxygen species (ROS) signaling is particularly important in its progression [1, 3]. Among different types of ROS, hydrogen peroxide acts as a signaling molecule at low concentrations. Hydrogen peroxide has a longer half-life than others and can easily cross cell membranes [4]. Besides, it has been useful as an oxidative stress-inducing agent in different disease models in cells and tissues [5-7].

Cellular oxidant/antioxidant balance is influenced by dietary agents, such as flavonoids. Flavonoids are present in a large variety of foods and beverages such as fruits, vegetables, aromatic plants, medicinal herbs, tea, and red wine [8]. Cell culture studies and experiments on animal models have shown that different flavonoids exert protection against carcinogenesis [9]. They may also act as pro-oxidants and induce apoptosis in cancer cells [10]. Quercetin is one of these molecules as it depletes intracellular glutathione and increases ROS to levels that may cause cell death [10, 11].

Quercetin has anti-cancer activities and was shown to enhance the efficiency of radiotherapy and chemotherapy in animal models [12]. It is also a chemo-preventive agent against a number of diseases, including tumors [13]. Anti-cancer activity of quercetin involves its effects on cell cycle, cancer cell growth, and apoptosis [14-16]. Quercetin has different effects on cell cycle-related proteins p53, p21, p27, cyclin B, cyclin D, and cyclin depending kinases depending on the cell type [14]. PI3K, EGFR, and Her2/neu are also involved in the inhibition of cancer cell proliferation by

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quercetin. Quercetin related apoptosis is regulated by Akt, NFkB, p53, Bcl-2 family, and FasL [17, 18].

HepG2 is an immortalized cell line consisting of human liver carcinoma cells, derived from the liver tissues of a 15-year-old Caucasian male who had a well-differentiated HCC. In this study, hydrogen peroxide was used as a pro-oxidant, while quercetin was used as both pro-oxidant and antioxidant to investigate their effects on HepG2 cells. Concentrations that support cell viability and increase intracellular ROS production were selected to test changes in cell cycle phases, apoptotic mechanisms, and proteasome activity.

2. MATERIALS AND METHODS

Experiments were performed with HepG2 cells, which were obtained from American Type Culture Collection (ATCC-HB-8065). Stress agents were hydrogen peroxide and quercetin. The stock solution of quercetin (40 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. For hydrogen peroxide, the stock solution (40 mM) was prepared fresh before each application.

2.1. Cell Viability Assay

HepG2 cells were cultured in DMEM (Biochrom-Germany) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. In viability experiments, $5x10^3$ cells/well were seeded in 96-well plates in 100 ml of culture media and were exposed to different concentrations of quercetin (50-200 mM) and H_2O_2 (50-200 mM). After 30, 90, 180 minutes, and 72 hours, 10 mL of WST-1 was added to each well and incubated for 2 hours at 37°C. The plates were then shaken thoroughly on the shaker for 1 minute and the absorbance of reporter substrate was measured at 450 nm using a microplate reader (Perkin Elmer, USA).

2.2. Apoptosis and Cell Cycle Analysis

Annexin V/PI stain was used for the determination of apoptosis. Cells (3×10^5) were seeded in 6-well plates. Cells were harvested and centrifuged 30 minutes after the application of each stress agent. For each assay, 1×10^4 cells were washed once with PBS and stained. After 30 minutes of incubation, they were analyzed on a FACSCalibur flow cytometry system (Becton Dickinson, USA). In cell cycle analysis, after treatment with H_2O_2 (50-200 μM) and quercetin (50-200 μM) for 24 hours, cells were washed twice with PBS and fixed with 70% ethanol for 1 hour at 4°C. They were then suspended with PBS containing 20 µg/ml PI (Sigma) and 50 µg/ml RNase A. Cells were incubated at room temperature in the dark for 30 minutes and the cell cycle was analyzed by flow cytometry (FACS Calibur TM). Analysis of data was done using Cell Quest Pro 6.0 (BD Bioscience).

2.3. Western Blot Analysis

Cells (1.3×10^6) were seeded in 10 cm culture dishes. Protein was extracted from each group with a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin). Samples were kept on ice for 15 minutes and the supernatants were collected after centrifugation at 18,000 x g for 10 minutes. The amount of protein in samples was determined with BCA assay, and approximately 40 µg of total protein was loaded to each well. After performing SDS-PAGE, proteins were transferred to a nitrocellulose membrane by wet-transfer. Membranes were blocked with 5% BSA in TBST and immunoblotted overnight at 4°C with corresponding primary antibodies (p21: Cell Signaling-2947S, p27: Cell Signaling-3698S, p53: Cell Signaling-9282S, NF-Kβ-p50: Santa Cruz-sc-114). This was followed by the appropriate horseradish peroxidase-linked secondary antibody binding. Detection was performed using the West Pico chemiluminescent substrate (Thermo-Scientific, USA) and the ChemiDoc MP System (Bio-Rad Laboratories, USA) was used to analyze the results.

2.4. Measurement of Proteasome Activity

Cells were incubated in proteasome lysis buffer (225 mM Tris buffer (pH 7.8) containing 7.5 mM MgOAc, 7.5 mM $MgCl_2$, 45 mM KCl and 1 mM dithiothreitol), lysed in repeated freeze-thaw cycles by keeping 15 seconds in liquid nitrogen and then for 1 minute in 40°C water bath (three times). The lysates were centrifuged at 15,000 x g for 30 minutes at 4°C. The fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-methyl coumarin (suc-LLVY-MCA) was used as a substrate at a concentration of 200 µM to measure chymotrypsin-like activity (CT-L) of the proteasome. Different conditions were created by the addition and depletion of ATP to discriminate ATP dependent and independent proteasome activities. After incubation at 37°C for 60 minutes, methyl coumarin (MCA) liberation was measured with a fluorescence reader at 360 nm excitation and 485 nm emission. Results were calculated using free MCA as standard.

2.5. Oxidative Stress Assay

General oxidative stress assay was performed using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) staining. Cells (1.7×10^5cells/well) were seeded in 6-well plates and the stressors were applied for 30 minutes and 24 hours. Afterwards, cells were washed two times with PBS, incubated with 10 µM CM-H2DCFDA for 30 minutes and then analyzed by flow cytometry (FACS Calibur ™).

2.6. Statistical Analysis

Statistical analysis of all data was performed with Prism 7.04 (GraphPad Software Inc., USA).

3. RESULTS

3.1. Cell Viability

Two different concentrations were used to study the effects of the two stressors, hydrogen peroxide and

quercetin, at different time points (30, 90, 180 minutes and 72 hours). Cell viability was suppressed by 50 mM hydrogen peroxide after 30 minutes (~ 80%). But afterwards, it started to increase and was ~ 110% after 72 hours. The effect of quercetin was also followed at different time points. Cell viability was above 100% at all times, except at 72 hours (Fig. **1**).

3.2. Apoptosis Rates and Cell Cycle Phases

The percentage of apoptotic cells was significantly increased in both hydrogen peroxide and quercetin treated HepG2 cells compared to the control group. The highest apoptosis rate (~18%) was observed in 200 µM quercetin group (Fig. **2**). Cancer cells respond positively to stimulatory signals and grow continuously. The results showed that hydrogen peroxide had no significant effect on cell cycle phases. However, quercetin treatment resulted in cell cycle arrest at G0/G1 phase (~38%) and S phase (~49%) after 24 hours (Fig. **3**).

3.3. Western Blot Analysis

Cell cycle-related protein expression levels determined by Western blot are shown in Fig. (**4**). After hydrogen peroxide treatment expression of p53, NF-KB and p27 protein levels were decreased while p21 levels were increased. Decreased p53 protein expression was observed after quercetin treatment, but levels of other proteins were either unchanged or increased.

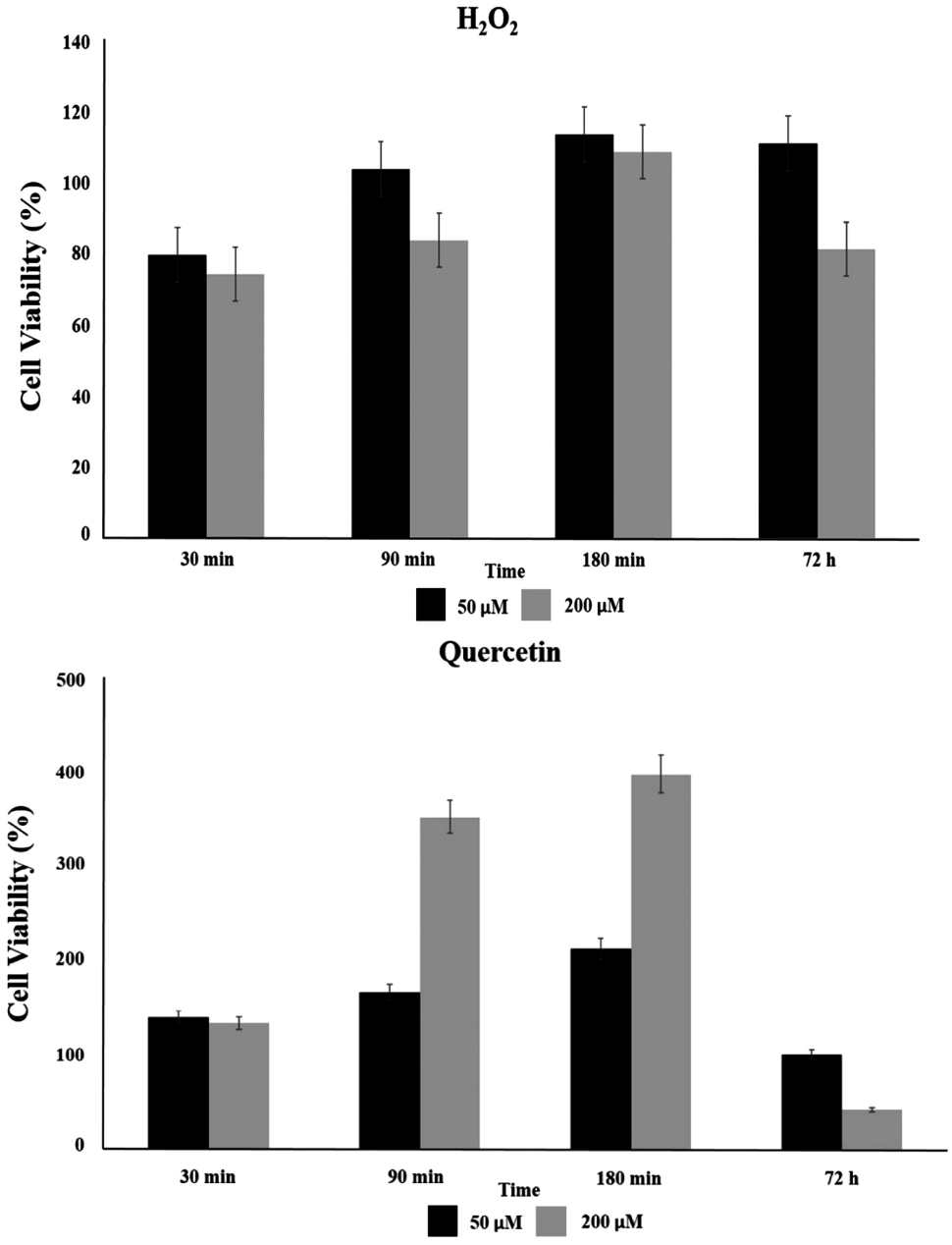


Fig. (1). Cell viability after 30, 90, 180 minutes, and 72 hours in H_2O_2 and quercetin treated HepG2 cells. Data shown are mean ± SD of three independent experiments.

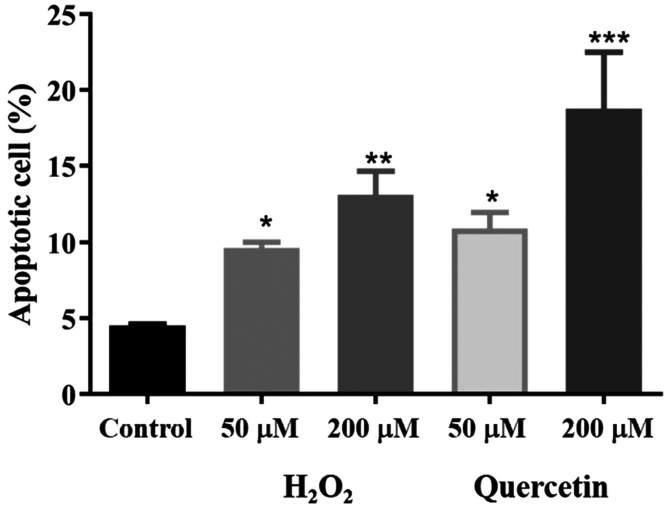
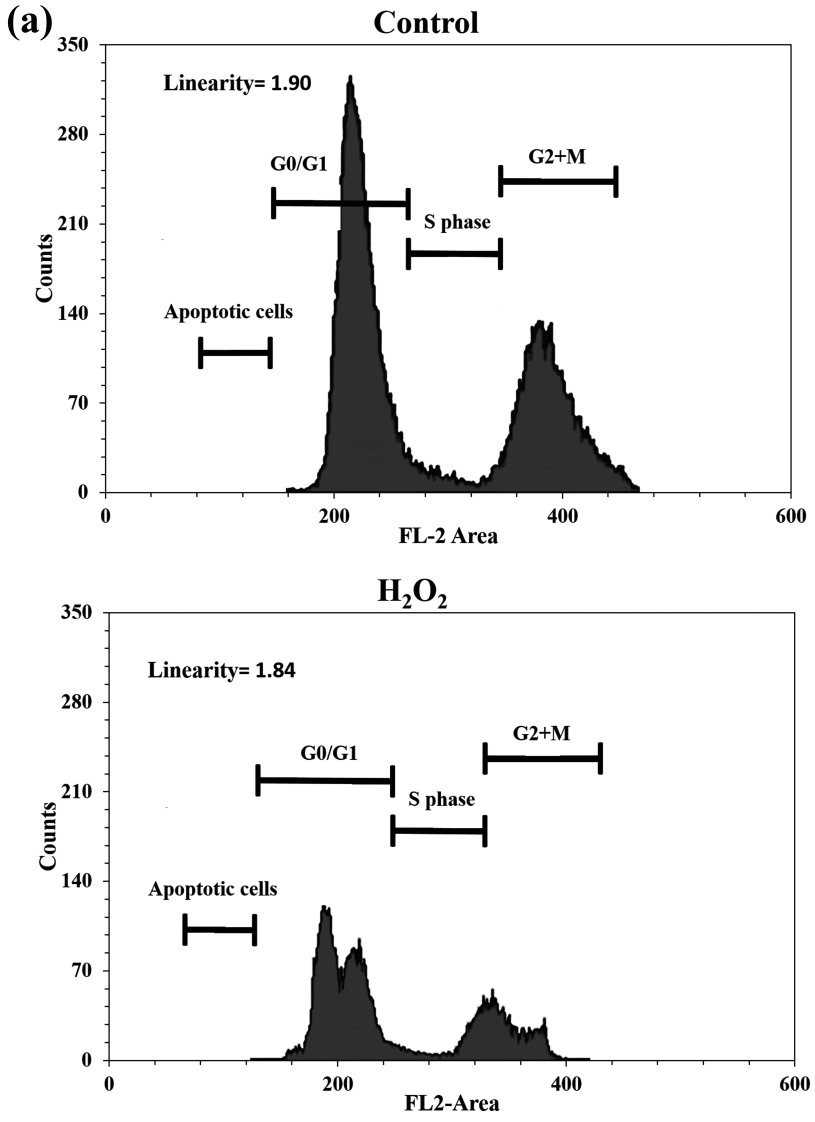


Fig. (2). Percentage apoptosis values of HepG2 cells treated with different concentrations of H_2O_2 and quercetin for 30 minutes. Values represent mean ± SD of three independent experiments. Data were analyzed by One-way ANOVA and Tukey multiple comparison post-test. *p<0.05, **p<0.01, ***p<0.001 *vs*. control group.



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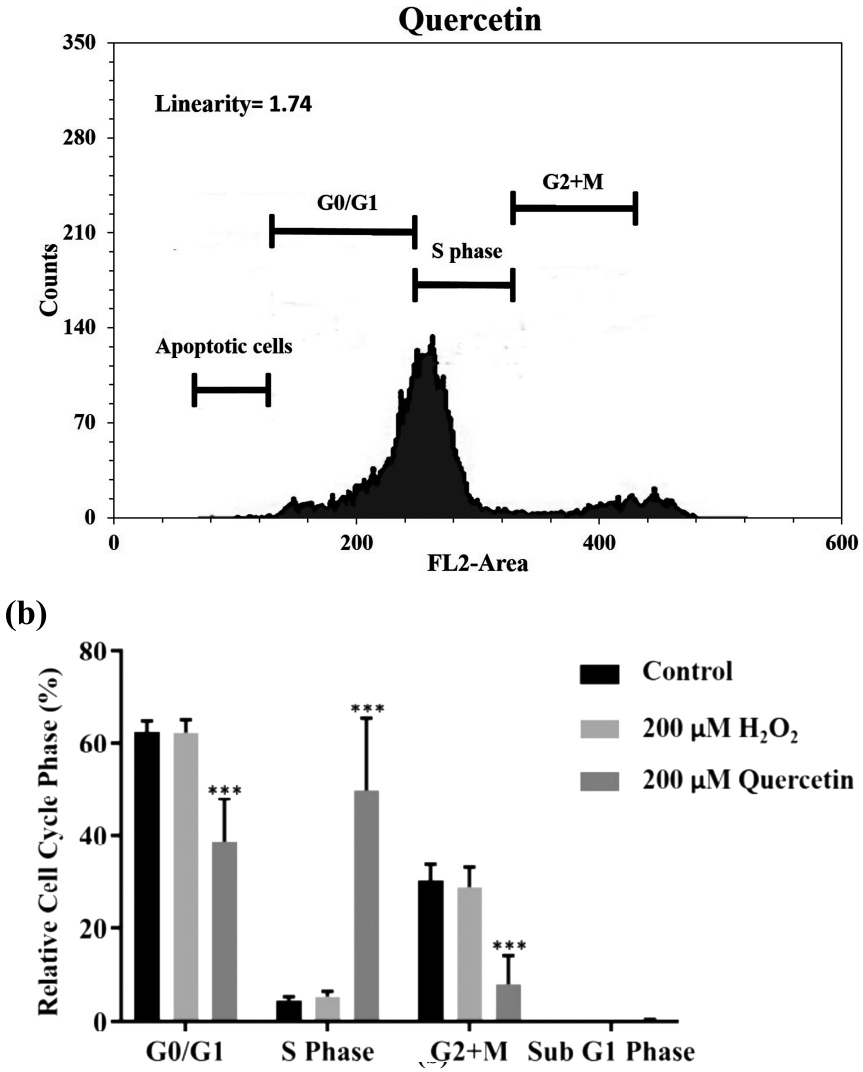

Fig. (3). Cell cycle analysis after 24 hours in H_2O_2 and quercetin treated HepG2 cells. The histogram plots illustrate the cell cycle phase. Vertical bars represent the mean ± SD of three independent experiments. Data were analyzed by one-way ANOVA and Tukey multiple comparison post-test. ***p<0.001 *vs*. control group.

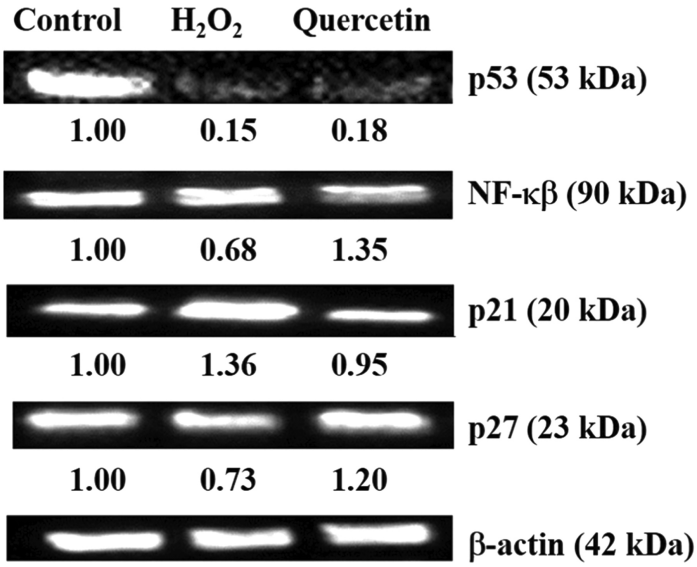

Fig. (4). Western blot analysis of protein expression levels after H_2O_2 and quercetin treatment in HepG2 cells. Fold-changes against the control group are given for each protein.

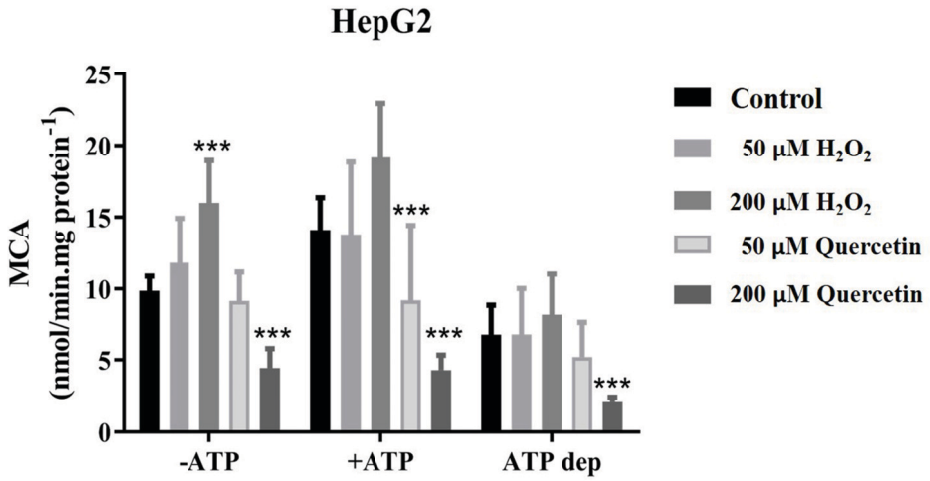

Fig. (5). Proteasome activity after H_2O_2 and quercetin treatment in HepG2 cells. Values represent mean ± SD of three independent experiments. Data were analyzed by One-way ANOVA and Kruskal-Wallis post-test. ***p<0.001 *vs*. control group.

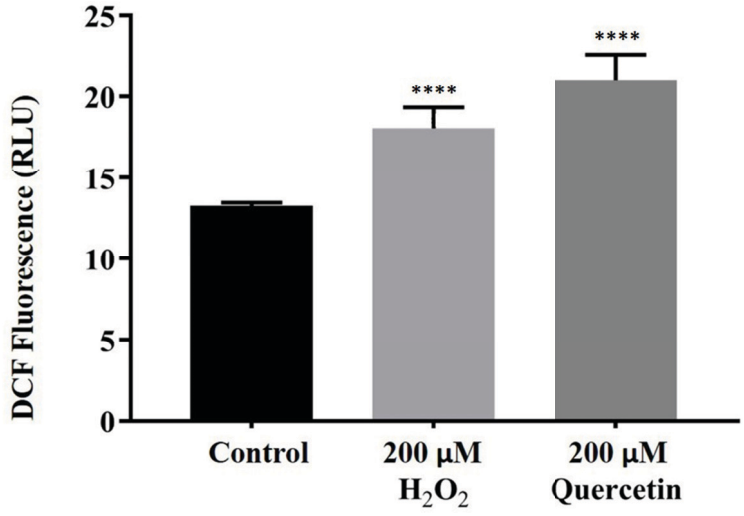

Fig. (6). Oxidative stress assay with CM-H2DCFDA. The bar graph shows ROS generation of HepG2 cells treated with H_2O_2 and quercetin. Values represent mean ± SD of three independent experiments. Data were analyzed by One-way ANOVA and Tukey multiple comparison post-test. ****p<0.0001 *vs*. control group.

3.4. Proteasome Activity

Fig. (**5**) shows the results of the fluorometric analysis of proteasome activity. The proteasome activity was investigated at three different conditions. Proteasome activity was increased by hydrogen peroxide in a concentration-dependent way. However, quercetin treatment inhibited proteasome activity at all conditions.

3.5. Oxidative Stress Assay

ROS levels were assayed by a general oxidative stress indicator CM-H2DCFDA after treatment of HepG2 cells with hydrogen peroxide and quercetin. Both agents significantly increased ROS production compared to controls (Fig. **6**).

4. DISCUSSION

The primary challenge in cancer therapy is to protect normal cells while killing cancer cells selectively by the agents used. However, standard chemotherapy and radiotherapy drugs do not discriminate cancer cells from normal cells effectively. Most drugs employed in standard chemotherapy are mutagenic and may lead to potential therapy-induced malignancies and side effects. The elevated levels of ROS observed in cancer cells compared to their normal counterparts represent a promising therapeutic strategy in selectively targeting malignant cells [19]. Thus, there is a need to develop novel strategies to understand the mechanism and homeostasis of ROS [20].

ROS act as secondary messengers in signal transduction pathways and are produced by various mechanisms in cells. ROS rheostat theory states that they regulate cellular mechanisms in a dose-dependent manner [21]. Low/medium levels of ROS promote mitogenic signaling through the formation of sulfenic acid [22] and reversible oxidation of disulfides [23], while high levels of ROS are cytotoxic and trigger inflammation and fibrosis. ROS may induce base modifications in nucleic acids, oxidation of proteins, and lipid peroxidation, eventually resulting in cell death. In

cancer cells, activation of oncogenic pathways boosts ROS production *via* mitochondrial electron transport chain and non-mitochondrial oxidases [24].

In this study, the induction of oxidative stress by hydrogen peroxide and quercetin in HepG2 cells was demonstrated. Wu *et al*. [25] have determined cell viability in both dose and time-dependent manner in HepG2 cells and observed that 100-200 μM H_2O_2 supported cell viability in a time-dependent manner similar to the results of this study. They also investigated the apoptotic rate of HepG2 cells and determined 30% apoptotic cells after 600 μM H_2O_2 treatment. One of the unique abilities of quercetin is its dual function and action as pro-oxidant and antioxidant [26]. Quercetin has significant effects on mitochondrial integrity, and, depending on the oxidation state of the cells, it can act as a pro-oxidant for balancing ROS. In this study, it was observed that both agents induced oxidative stress and increased intracellular ROS significantly.

Quercetin has been studied widely in hematological cancers and proved to possess beneficial effects. He *et al*. [27] showed that it inhibits the proliferation of multiple myeloma cell lines by inducing apoptosis as well as arresting cell cycle in G2/M phase. Their highest quercetin concentration was 80 μM, while in this study, 200 μM quercetin was used. At higher concentrations, quercetin decreased proteasome activity but, at the same time, increased oxidative stress, cell damage, and the number of cells at S phase of the cell cycle. Studies testing the effects of quercetin on cell cycle changes in liver cells demonstrate different results. In one study investigating the antioxidant and anti-cancer properties of quercetin, HepG2 cells were arrested in G1 phase with increased levels of tumor suppressor p53 and CDK inhibitors (p21 and p27) after 12 hours of administration [28]. In the present study, quercetin application at pro-oxidant concentration caused an increase in the S phase. The tumor suppressor protein, p53, was decreased, while p21 and p27 expressions varied depending on the stressor. It should be considered that the intensity of cellular redox conditions affects p53 activation. If the cell is under low redox stress, p53 upregulates the enzymatic antioxidant defense genes. Whereas in high redox stress, pro-oxidant and pro-apoptotic gene expressions increase [29].

In the present study protein expression levels of p21 and p27 were examined and no change in p21 expression was observed, while the expression of p27 was increased. Shortened survival seems to correlate with a high expression of p27. On the other hand, p53 is essential for decreasing intracellular ROS in oxidative stress conditions. Additionally, decreased p53 protects cells against hydrogen peroxide-induced damage, which leads to decreased viability, increased apoptosis, and elevated DNA oxidation. However, in cancer cells, p53 is down-regulated and its functions are significantly blocked. Thus, quercetin may improve ROS sensitivity resulting from p53 deficiency by stabilizing its levels [30].

Protein chaperones play a significant role in protein folding and maintenance. Disruption of ubiquitin proteasome system may lead to cell death. In many tumor cells, dysregulation of heat shock protein chaperones is induced by ionizing radiation. Recent findings revealed that quercetin inactivates the protein chaperone system [31]. Proteasome activity is also thought to increase in response to protein carbonyls, levels of which are increased under oxidative stress conditions. In the present study, increased proteasome activity was observed in hydrogen peroxide-treated groups while proteasome activity was inhibited in quercetin-treated groups. Previously it was also demonstrated that quercetin exerted pro-apoptotic effects in thyroid cancer cells by inhibiting proteasome activity and Hsp 90 protein expression [32].

CONCLUSION

Hydrogen peroxide and quercetin triggered oxidative stress in HepG2 cells, but at the same time, both supported cell viability. Proteasome activity was inhibited by quercetin, suggesting that 20S proteasome can be a potential target of quercetin in regulating the cell cycle. At high concentrations, quercetin may be involved in the resistance of cells to oxidative conditions due to S phase propagation. The effect of quercetin on cell cycle may also be related to reduced expression of p53 protein levels, which might have promoted cell proliferation by increasing the expression of genes that enable cells to adapt to oxidative stress conditions. Finally, different mechanisms seem to be involved in hydrogen peroxide and quercetin induced cell death in HepG2 cells.

LIST OF ABBREVIATIONS

HCC = Hepatocellular Carcinoma

ROS = Reactive Oxygen Species

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No human/animals were used in the study that is the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

FUNDING

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CONFLICT OF INTEREST

Authors declare no conflict of interest, financial or otherwise.

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Declared none.

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