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Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells — up regulation of Nrf2 expression and down regulation of NF-κB and COX-2

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ABSTRACT

Background: Ochratoxin A (OTA), a mycotoxin, causes extensive cell damage, affecting liver and kidney cells. OTA toxicity is fairly well characterized where oxidative stress is believed to play a role, however, the sequence of molecular events after OTA-exposure, have not been characterized in literature. Further, antidotes for alleviating the toxicity are sparsely reported. The aim of this study was to understand the sequence of some molecular mechanisms for OTA-induced toxicity and the cytoprotective effect of quercetin on OTA-induced toxicity.
Methods: Time course studies to evaluate the time of intracellular calcium release and ROS induction were carried out. The time of activation and induction of two key redox- sensitive transcription factors, NF-κB and Nrf-2 were determined by nuclear localization and expression respectively. The time of expression of inflammatory marker COX-2 was determined. Oxidative DNA damage by comet assay and micronucleus formation was studied. The ameliorative effect of quercetin on OTA-induced toxicity was also determined on all the above-mentioned parameters.
Results: OTA-induced calcium release, ROS generation and activated NF-κB nuclear translocation and expression. Pre-treatment with quercetin ameliorated ROS and calcium release as well as NF-κB induction and expression. Quercetin induced Nrf-2 nuclear translocation and expression. Quercetin's anti-inflammatory property was exhibited as it down regulated COX-2. Anti-genotoxic effect of quercetin was evident in prevention of DNA damage and micronucleus formation.
Conclusion: Quercetin modulated OTA-induced oxidative stress and redox-signaling in HepG2 cells.
General significance: The results of the study demonstrate for the first time that quercetin prevents OTA-induced toxicity in HepG2 cells.

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1. Introduction

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by several food-borne fungi such as *Aspergillus* sp. and *Penicillium* sp. OTA's wide spread occurrence and persistence in the food chain may lead to a significant exposure to humans [1]. OTA has grabbed global attention over the last few years as it causes genotoxicity, carcinogenicity (group 2B human carcinogen), teratogenicity, hepatotoxicity and nephrotoxicity in both human and farm animals [2]. Toxicity studies have revealed that OTA mainly affects the kidney and liver, after absorption from the gut and circulation via the portal vein, inducing hepatotoxicity in rats and hepatocellular carcinomas in mice [3]. The mechanism of OTA toxicity includes reactive oxygen species (ROS) formation and lipid peroxidation (LPO) [4], inhibition of protein synthesis [5], disturbance of calcium homeostasis and impairment of mitochondrial oxidation reactions [6,7].

At the molecular level, OTA-induced oxidative stress results in cellular counter mechanisms involving enzymatic and non-enzymatic defense systems [8], modulation of transcription factors such as nuclear factor E2 p45-related factor 2 (Nrf-2) and nuclear factor-kappa B (NF-κB) [9,10] as well as activation of cyclooxygenase-2 (COX-2), a common inflammatory marker [11]. Although these molecular redox mechanisms are reported in literature, time course studies to evaluate time and sequence of induction have not been carried out.

Abbreviations: ANOVA, Analysis of variance; AO/EB, Acridine orange/ethidium bromide; ARE, Antioxidant responsive element; $[Ca^{2+}]_i$, Intracellular calcium level; CAT, Catalase; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; COX-2, Cyclooxygenase-2; CBMN, Cytokinesis block micronucleus assay; DAB, Diaminobenzidine; DAPI, 4,4-diamidino-2-phenylindole; DCF, 2,7-dichlorofluorescein; DCF-DA, 2,7-dichlorodihydrofluorescein diacetate; DiOC6, 3,30-di-hexyloxacarbocyanine iodide; DMEM, Dulbecco's modified Eagle's medium; DTT, Dithiothreitol; EDTA, Ethylenediamine tetraacetic acid; FBS, Fetal bovine serum; GPx, Glutathione peroxidase; GSH, Glutathione; GST, Glutathione S transferase; IC20, Inhibitory concentration 20; iNOS, Inducible nitric oxide synthase; LDH, Lactate dehydrogenase; LPS, Lipopolysaccharide; LPO, Lipid peroxidation; MN, Micronucleus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, Nitric oxide; Nrf-2, Nuclear factor E2 p45-related factor 2; NF-κB, Nuclear factor-kappa B; OTA, Ochratoxin; OPT, Ortho-phthalaldehyde; PCC, Protein carbonyl content; PMSF, Phenyl methyl sulfonyl fluoride; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

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In the present study, we have attempted to understand the sequence of some underlying molecular redox mechanisms of OTA toxicity. Parameters such as calcium induction, ROS generation, modulation of redox transcription factors such as NF-κB and Nrf-2 and induction of COX-2 were evaluated.

A growing body of evidence indicates that oxidative stress plays an important role in the pathogenesis of many clinical conditions involving cardiovascular diseases, liver diseases, lung diseases, gastrointestinal disorders and aging. Several environmental and food toxins also are believed to exert their detrimental effect in cells through oxidative stress. In recent years, the naturally occurring substances have been receiving increased attention by researchers and have been subject to many rigorous scientific and clinical studies. Antioxidants could be a new therapeutic tool to improve the clinical manifestation of these illnesses [12]. Quercetin, a ubiquitous flavanoid (3,3′,4′,5,7-pentahydroxyflavone) in particular has shown great promise in a number of areas relevant to human health [13]. It has been shown to be an excellent *in vitro* antioxidant through its potent scavenger of ROS, including $O2^{\cdot -}$ [14], RNS like NO [15] and ONOO— [16]. It also exerts various bioactive effects which includes, anti-inflammatory [17,18] and antimutageneic [19].

Several studies in literature have proved the efficacy of quercetin as an antioxidant and potent free radical scavenger; however, this is the first study where the cytoprotective effect of quercetin on OTA-induced toxicity has been studied in liver cells. Although the ameliorative actions of retinol, ascorbic acid, and alpha tocopherol have been studied against OTA-induced toxicity, Quercetin was chosen for this study as it is ubiquitous in nature, can be consumed a part of food and devoid of toxicity compared to fat soluble vitamins. It is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference antioxidant trolox [20].

We present here for the first time, the sequence of some underlying molecular mechanisms for OTA-induced toxicity as well as the cytoprotective effect of quercetin on OTA-induced toxicity in HepG2 cells, with specific reference to oxidative stress, intracellular calcium flux, levels of protective antioxidant enzymes as well as nuclear localization and expression of two key transcription factors Nrf-2 and NF-κB p65. In addition, expression of inflammatory marker COX-2 as well as DNA damage and micronucleus (MN) formation was studied.

2. Materials and methods

2.1. Materials

OTA was purchased from Sigma Aldrich, Bangalore. Quercetin, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), 3-4,5-dimethylthiazol-2-yl, 2,5-diphenyltetrazoliumbromide (MTT) and all other analytical grade chemicals were obtained from Hi-media Laboratories, Mumbai, India. Primary monoclonal antibodies for NF-κB p65 (sc-1008), COX-2 (sc-19999), Nrf2 (sc-365949), β-actin (sc-10731) and Lamin B2 (sc-58667) were obtained from Santa Cruz Biotech, CA. Western blot membranes were obtained from Whatman, USA. Dichlorofluorescein diacetate (DCF-DA), Diehxyloxacarbocyanine iodide (DiOC6), and Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (CCCP) were obtained from Calbiochem, USA. DAB/H_2O_2 color development kit for western blots was obtained from Bangalore Genei, India.

2.2. Cell culture

HepG2 (human hepatoma) cell line obtained from National centre for cell science (NCCS), Pune, was grown to confluence in 25 cm^2 flasks supplemented with DMEM and 10% FBS (v/v), containing, 100 units/ml penicillin and 30 μg/ml streptomycin in a CO_2 incubator with 5% CO_2.

2.3. Cytotoxicity assay by MTT test

Since OTA affects cells of liver, HepG2 cells were chosen for this study. Cytotoxicity was determined colorimetrically by MTT method. Cells were seeded in 96 well plate at a density of 1×10^4 cells per well, in 0.2 ml DMEM and exposed to increasing concentration of OTA (10–50 μM/ml) dissolved in 50% (*v*/*v*) ethanol (solvent concentration not exceeding 0.1%) for different time intervals (24, 48 and 72 h). The concentrations of OTA were chosen (10–50 μM) based on previous reports which reflect concentrations found in plasma and other tissues in rats after OTA was fed orally at 0.5 mg/kg [21]. Quercetin dissolved in dimethyl sulfoxide (DMSO) was added to the cells to reach a final concentration of 5, 10 and 15 μM (final solvent concentration did not exceed 0.1% v/v) to determine pre, co and post treatment efficacy. The concentrations of quercetin were based on sera concentrations of patients given an oral dose of the flavonoid [22]. Based on the dose response studies, effective OTA (10 μM for 48 h) and quercetin (10 μM pre-treatment for 24 h) concentrations were chosen as optimum which was followed for further studies. Control cells received appropriate carriers, final concentration not exceeding 0.1%. After the exposure times, MTT was added to a final concentration of 0.5 mg/ml medium and the plates were incubated for 4 h at 37 °C. The purple formazan crystals formed were dissolved in DMSO and read at 570 nm in a microquant plate reader (Bio-Tek Instruments). The results were expressed as % viability.

2.4. Measurement of intracellular calcium level ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using fluorescent dye Fura-2 AM [23]. Cells were treated with OTA for different time intervals (10, 15, 30, 45 and 60 min). In the quercetin pre-treatment groups, cells were pre-treated with quercetin for 24 h, followed by OTA exposure. As a positive control, cells were treated with 50 μM H_2O_2 for 30 min. Cells from the treatment groups were washed in calcium buffer (HEPES 10 mM, NaCl 132 mM, KCl 3 mM, glucose 10 mM, $CaCl_2$ 1 mM and K_2HPO_4 at pH 7.4), loaded with Fura-2 AM in calcium buffer (5 μM) and incubated for 1 h at 37 °C in a CO_2 incubator. Cells were then washed with PBS, trypsinized and suspended in 1.0 ml PBS. Changes in $[Ca^{2+}]_i$ were measured in a spectrofluorometer, excitation wavelength 340 nm and 380 nm and emission wavelength 500 nm. The ratio of intensities at 340 and 380 nm is proportional to $[Ca^{2+}]_i$. The values were expressed as % relative fluorescence compared to control.

2.5. Measurement of ROS in cells with fluorescent dye DCFH-DA

OTA-induced cellular oxidative stress was evaluated by seeding cells in a 24 well plate. After overnight attachment, the cells were incubated for 30 min at 37 °C in PBS containing 25 μl of 50 μM H_2DCF-DA, [24] followed by OTA exposure (10 μM) for different time intervals (15, 30, 60, 180 and 300 min). In the quercetin pre-treatment groups, cells were pre-treated with quercetin for 24 h and then exposed to H_2DCF-DA as described above and then exposed to OTA. For positive control, 50 μM H_2O_2 for 60 min was included. The cells were centrifuged, washed and resuspended in PBS, and 2,7-dichlorofluorescein (DCF) formed was measured in a Hitachi spectrofluorometer, (excitation 480 nm, emission 520 nm). The estimations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

2.6. Immunofluorescence by confocal microscopy

Nuclear localization of NF-κB and Nrf2 was detected by immunofluorescence as described previously [25]. Cells were seeded on poly lysine coated cover slips and allowed to attach overnight. To determine NF-κB nuclear translocation, cells were exposed to OTA for different time intervals (15, 30, 60 and 180 min) to determine time of optimum translocation. Pre-treatment with quercetin was carried out for 24 h, followed by exposure to OTA for 60 min, which was the time point of maximum translocation. To determine Nrf2 translocation, cells were treated with quercetin for different time points (1, 2, 4 and 6 h). Optimum translocation was observed at 4 h and this was used in the combination with OTA. Following treatment, cells were washed in PBS, fixed with 4% paraformaldehyde, permeabilized and incubated overnight with primary antibody anti-Nrf2, anti NF-κB (1:1000) dilution. Following this, cells were washed with PBS and incubated with FITC conjugated secondary antibody (1:2000) for 1 h at room temperature, nuclei stained with 4,4-diamidino-2-phenylindole (DAPI) for few min and viewed in a Leica SP2 confocal laser scanning microscope.

2.7. Western blot analysis

For the evaluation of COX-2 expression, cells were seeded at a density of 5×10^6 in a 6 well plate, treated with OTA (10 μM) at different time intervals (10, 15 and 24 h), trypsinized and extracted using mammalian cell lysis buffer. To determine the subcellular localization of NF-κB p65 and Nrf2 expression, nuclear extracts were prepared. Cells were treated with OTA for 1, 3 and 5 h for NF-κB p65 and quercetin at 4, 6, 8 and 10 h for Nrf2. At the determined times, cells were suspended in extraction buffer containing 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.6% Nonidet P40, 1 mM ethylene diamine tetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 1 μl/ml protease inhibitor cocktail. After incubation for 20 min on ice, nuclei were pelleted by centrifugation at 4 °C and 14,000 rpm for 15 min. The extract was homogenized in nuclear extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 1 μl /ml protease inhibitor cocktail). 50 μg of total protein was separated on a 10% SDS-PAGE gel followed by protein transfer to a nitrocellulose membrane. Membrane was probed with specific primary antibodies (1:200 dilution) and incubated over night at 4 °C. The blots were washed and incubated with the secondary antibody (antimouse HRP conjugate 1:5000) for 1 h at room temperature and developed using DAB/H_2O_2 color development system. β-actin and Lamin B2 were used as the internal control to ensure equal sample loading. Densitometric analysis was carried out using UN-SCAN-IT image digitalizing software.

2.8. Other oxidative stress parameters

2.8.1. Nitrite determination

Nitrite accumulation, an indicator of nitric oxide (NO) synthesis, was measured in the culture medium by Griess reagent. Cells were seeded at a density of 0.5 million cells per well in a 6 well plate, treated with 10 μM OTA for 48 h, with and without 10 μM quercetin pre-treatment for 24 h, after attachment. One group, treated with 10 μM quercetin alone for 24 h served as a quercetin control. 50 μM H_2O_2 for 60 min, was included as a positive control. After the treatment period, 50 μl of culture supernatant was reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine, 1% sulfanilamide in 5% H_3PO_4) for 10 min at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader Bio-Tek Instruments, Inc.). Nitrite concentrations were calculated using linear regression using a sodium nitrite standard curve.

2.8.2. Determination of lipid peroxidation

Lipid peroxidation was assayed by the measurement of the TBARs (thiobarbituric acid reactive substances) level as described previously [26]. The concentration of TBARS was calculated using standard curves of increasing 1,1,3,3-tetramethoxypropane concentrations, and expressed as nmol/mg of protein.

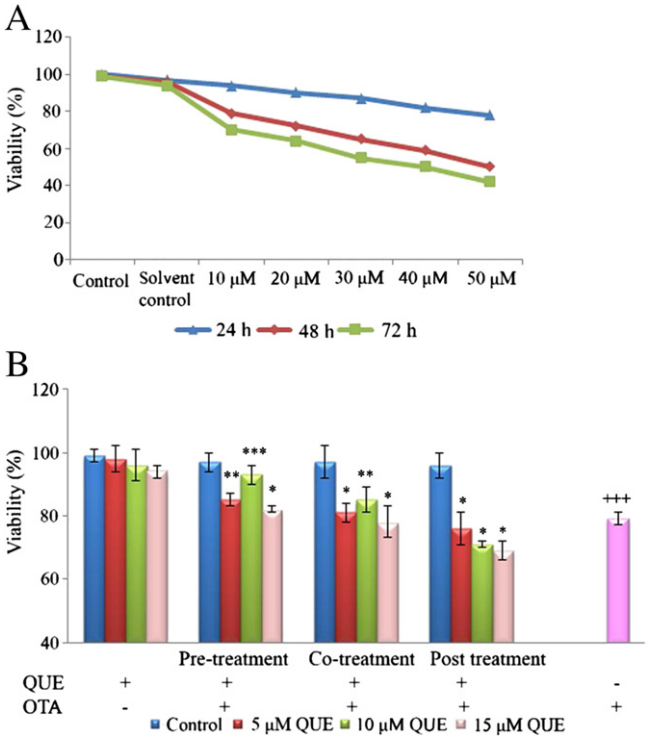


Fig. 1. Effect of quercetin on OTA-induced loss of cell viability in HepG2 cell line. (A) Dose and time dependent reduction in cell viability. Cells treated with OTA for different concentration (10–50 μM) for different time periods (24, 48 and 72 h). Results expressed as % viability. (B) Protective effect of quercetin (5–15 μM) on OTA treated HepG2 cells — pre-, co- and post-treatment. For pre-treatment, cells were treated with quercetin (5, 10 and 15 μM for 24 h) followed by OTA (10 μM for 48 h), for co-treatment, cells were treated with OTA (10 μM for 48 h) and quercetin (5, 10 and 15 μM for 24 h), for post-treatment cells were treated with OTA (10 μM for 48 h) followed by quercetin (5, 10 and 15 μM for 24 h). Results expressed as % Viability expressed as Mean ± SEM ($n = 9$). Significant differences versus OTA treated group were indicated by $^{***}p < 0.001$, $^{**}p < 0.05$, $^{*}p < 0.01$ respectively. Significant differences versus control were indicated by $+++$ $p < 0.001$. # — Not significantly different from control.

2.8.3. Determination of protein carbonyl content

Protein carbonyl content (PCC) content was determined in cells by measuring the reactivity of carbonyl groups with 2, 4-dinitrophenylhydrazine (2,4-DNPH) as described earlier [27]. Carbonyl content was calculated using extinction coefficient of $22 \times 10^3\ M^{-1}\ cm^{-1}$ and expressed as μM carbonyls formed per mg protein.

2.8.4. Estimation of glutathione

Intracellular reduced glutathione was quantified by a spectrofluorometric assay using ortho-pthalaldehyde (OPT) [28]. Cells were seeded in 6-well culture plates and treated as described in NO assay, harvested and suspended in 0.1 M phosphate EDTA buffer, pH 8.0. Following sonication, precipitation of the supernatant with 25% ortho-phosphoric acid, OPT was added to the supernatant to obtain a final concentration of 5 mg/ml. Samples were incubated for 10 min in dark and analyzed in a spectrofluorometer (excitation 350 nm and emission 420 nm). All experiments were carried out thrice in triplicate to ensure reproducibility and expressed as μmoles GSH/mg protein. Glutathione concentrations were calculated based on a standard curve of reduced glutathione using linear regression.

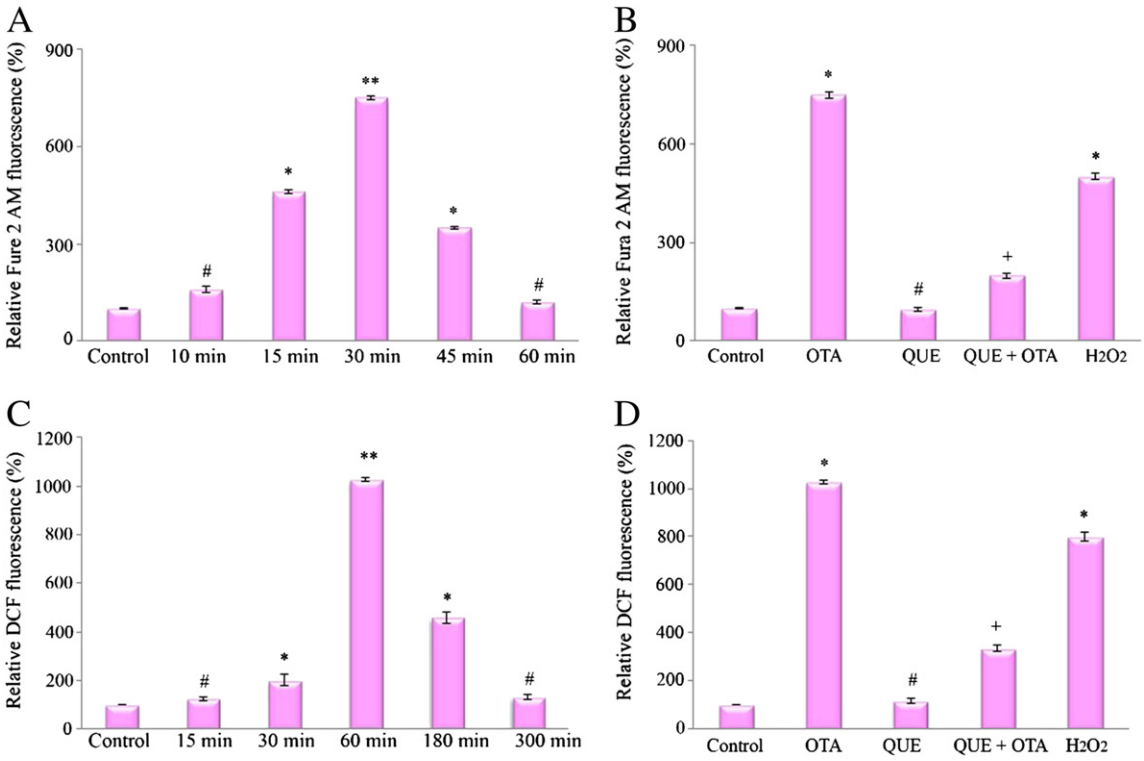


Fig. 2. Effect of quercetin on OTA-induced oxidative stress. (A) OTA-induced increase in intracellular calcium level $[Ca^{2+}]_i$, in time dependent manner. Cells were treated with OTA (10 μM) for different time intervals at 15, 30, 45 and 60 min. Results expressed as % relative fluorescence of Fura/2 AM with respect to control. Data presented were Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{**}p < 0.001$, $^{*}p < 0.05$. # — Not significantly different from control. (B) Quercetin inhibits OTA-induced intracellular calcium level $[Ca^{2+}]_i$. Intracellular calcium level was measured by using Fura/2 AM. Cells were treated OTA (10 μM) for 30 min in presence and absence of quercetin pre-treatment (10 μM for 24 h). Cells treated with 50 μM H_2O_2 for 30 min was used as a positive control. Results expressed as % relative fluorescence of Fura/2 AM with respect to control. (C) OTA-induced ROS generation in time dependent manner. Cells were treated with DCF-DA for 30 min followed by OTA (10 μM) for 15, 30, 60, 180 and 300 min. Results are expressed as % relative fluorescence of DCF-DA with respect to control. Data presented were Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{**}p < 0.001$, $^{*}p < 0.05$. # — Not significantly different from control. B) Quercetin inhibits OTA-induced ROS generation. Cells were treated with OTA (10 μM) for 60 min following DCF-DA treatment for 30 min. For quercetin pre-treatment, cells were exposed to quercetin (10 μM for 24 h) and then exposed to DCF-DA for 30 min followed by OTA (10 μM) for 60 min. Cells treated with 50 μM H_2O_2 for 60 min was used as a positive control. Results expressed as % relative fluorescence of DCF-DA with respect to control. Data presented were Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{*}p < 0.05$. Significant differences versus OTA treated group were indicated by $+ p < 0.05$. # — Not significantly different from control.

2.8.5. Estimation of enzymic antioxidants

Activities of superoxide dismutase (SOD) [29], catalase (CAT) [30], Glutathione peroxidase (GPx) [31] and Glutathione S transferase (GST) [32] were determined as described previously. Cells were seeded in 6-well culture plates and treated with 10 μM OTA for 48 h, with and without 10 μM quercetin pre-treatment for 24 h. A control group with 10 μM quercetin alone for 24 h and a treatment group with 50 μM H_2O_2 for 60 min were included to serve as a positive control. Cell extracts were prepared by sonication in 50 mM Tris, 5 mM EDTA, 10 μg/ml phenyl methyl sulfonyl fluoride (PMSF), pH 7.6, followed by centrifugation at 4000 rpm for 5 min at 4 °C. All assays were carried out in the triplicate and specific activities expressed as units per mg of protein, protein content determined by the Lowry method [33].

2.9. Alkaline gel electrophoresis (comet assay) in HepG2 cells

Comet assay was carried out by alkaline gel electrophoretic method as described earlier [34]. HepG2 cells were seeded in a 24 well plate, allowed to attach overnight and treated with quercetin and OTA as described in NO assay. In brief, frosted microscopic slides were covered with 1% normal melting agarose (NMA) in PBS at 60 °C for 1 h. A second layer of 0.5% low melting agarose (LMA) containing 50 μl of cell suspension was added, cover slip placed immediately and incubated at 4 °C for 10 min to allow the agarose to solidify. After solidification of the LMA, cover slips were carefully removed and another layer of 150 μl of 0.5% low melting agarose (LMA) was added, incubated at 4 °C for 10 min and placed in the chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris HCl, 1% DMSO, 1% Triton X 100 and 1% sodium sarcosinate, pH 10) for 1 h at 4 °C. Electrophoresis was carried out at 25 V, 180 mA for 30 min in freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA pH ≥ 13) and washed with neutralisation buffer (0.4 M Tris HCl). The slides were stained with ethidium bromide (EtBr) (20 μg/ml) and visualized using a Olympus fluorescent microscope, screening 100 cells/slide per culture and captured by progress camera. The quantification of the DNA strand breaks of the stored images was done using the CASP software by which % tail DNA, % head DNA and olive tail moment could be obtained directly.

2.10. Cytokinesis block micronucleus assay in HepG2 cells (CBMN)

The CBMN technique was carried out by the method of Eastmond and Tucker with some modifications [35]. Cells were seeded in a 6-well plate and treated with quercetin and OTA as described in NO assay. Cells were rinsed with PBS and Cytochalasin B (4 μg/ml) was added and incubated for 28 h. Post-harvest, a mild cold hypotonic treatment with 0.075 M KCl, followed by immediate centrifugation at 800 rpm for 8 min was carried out. The supernatant was discarded and fixative along with few drops of formaldehyde was added and incubated at room temperature for 10 min to preserve cytoplasm. Following this, samples were centrifuged and cells were fixed in fresh fixative and

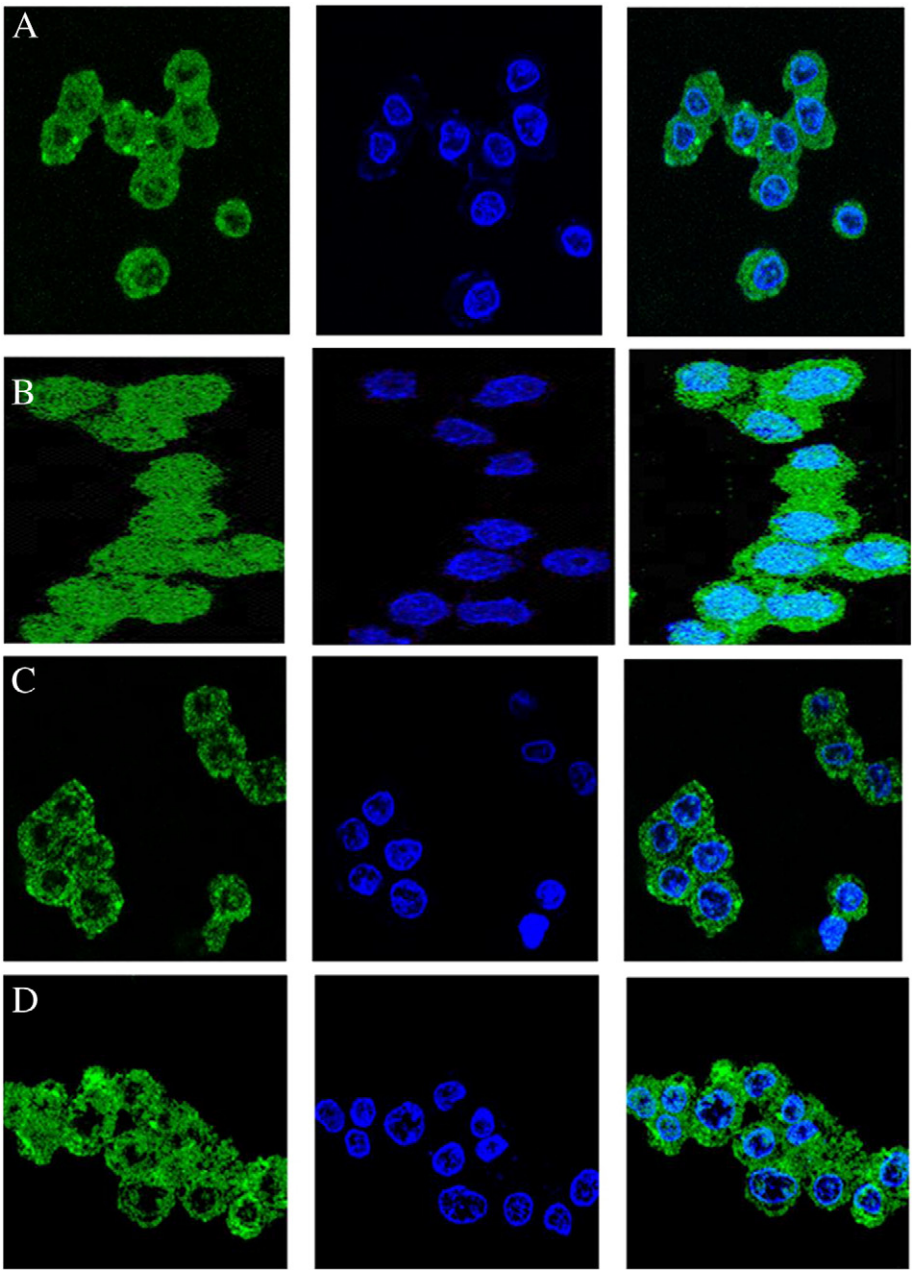


Fig. 3. Effect of quercetin on OTA-induced NF-κB P65 translocation. HepG2 cells were treated with OTA (10 μM) for 60 in presence and absence of quercetin pre-treatment (10 μM for 24 h). Cells were studied for localization of NF-κB P65 through indirect immunofluorescence using FITC conjugated secondary antibody. The nucleus was stained with DAPI. Cells viewed at 400× magnification. A) Control cells showing cytoplasmic localization of NF-κB. B) Cells exposed to OTA for 60 min showing that NF-κB has migrated into the nucleus, colocalization of FITC and DAPI gives characteristic turquoise blue color C) Cells pre-treated with quercetin for 24 h followed by OTA exposure for 60 min, showing NF-κB retention in the cytoplasm. D) Cells treated with quercetin alone, showing retention of NF-κB in the cytoplasm.

dropped on glass slides. The slides were stained with acridine orange solution (0.03 mg/ml) and examined using Olympus fluorescence microscope at 400× magnification. Selection of binucleated cells and scoring of micronuclei was performed. Triplicate cultures were used for each group. A minimum of thousand BNC were scored from each culture and the frequency of micronucleated binucleate cells (MNBNC) was determined and the values were expressed as Mean ± SEM from three independent experiments.

2.11. Statistical analysis

The data obtained were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison using SPSS 16 software. Values were considered significantly different at $p < 0.001$, $p < 0.01$ and $p < 0.05$.

3. Results

3.1. Quercetin protects OTA-induced loss of cell viability in HepG2 cells

In order to select the appropriate concentration of OTA, dose and time response study was conducted by exposing the HepG2 cells to different concentrations of OTA (10–50 μM) for different time periods (24, 48 and 72 h). The results of the dose response study revealed a dose and time dependant decrease in cell viability, with an IC_{20} value of 10 μM at 48 h which was used for further studies (Fig. 1A).

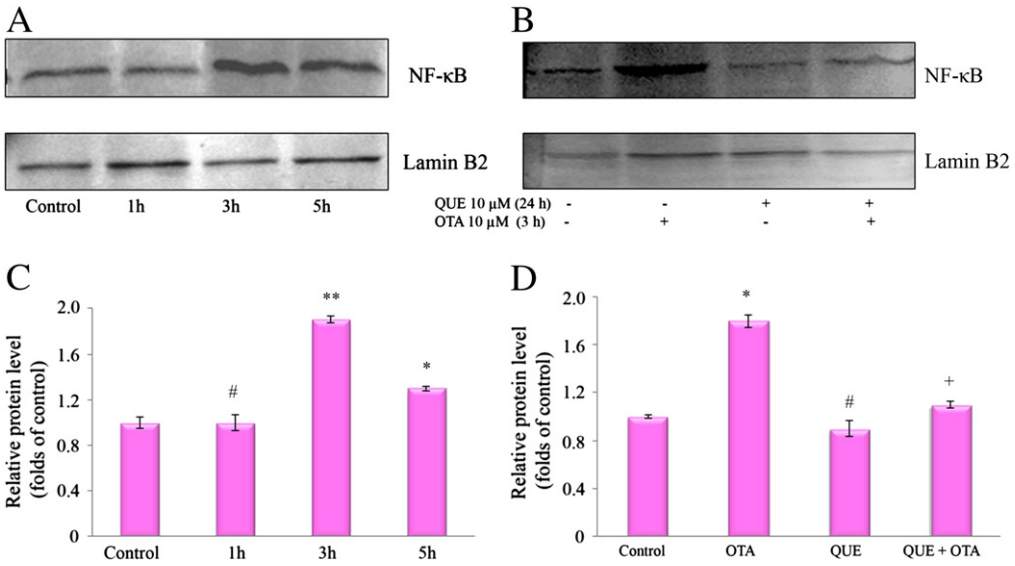


Fig. 4. Effect of quercetin on OTA-induced NF-κB P65 expression. (A) Time course of NF-κB P65 expression in response to OTA treatment. Cells were treated with OTA (10 μM) for 1, 3 and 5 h. (B) Quercetin downregulates OTA-induced NF-κB P65 expression. Cells were treated with quercetin (10 μM for 24 h) followed by OTA treatment for 3 h. (C & D) Bars represent data from densitometric analysis and represent mean ± SEM ($n = 3$) bars are expressed relative to the protein level of the control group. Significant differences versus control were indicated by $^{**}p < 0.05$, $^{*}p < 0.01$. Significant differences versus OTA treated group were indicated by $+ p < 0.05$. # — Not significantly different from control.

Effect of pre-, co-, and post-treatment of quercetin, revealed that pre-treatment of cells with 10 μM of quercetin significantly ($p < 0.001$) restored cell viability to 93% whereas cells pre-treated with 5 and 15 μM of quercetin showed 85% and 82% cell viability respectively (Fig. 1B). Cells treated with 10 μM quercetin alone showed cell viability on par with that of control. As pre-treatment with quercetin showed better protective effect than co- and post-treatment, it was decided to continue further experiments with the 10 μM quercetin pre-treatment for 24 h followed by exposure to 10 μM of OTA for 48 h.

3.2. Effect of quercetin on OTA-induced $[Ca^{2+}]_i$ and ROS

3.2.1. OTA-induced increase in $[Ca^{2+}]_i$, occurred before ROS generation and was ameliorated by quercetin

Cells exposed to OTA for different time intervals (10, 15, 30, 45 and 60 min) resulted in significantly ($p < 0.05$) elevated intracellular calcium level which peaked at 30 min (Fig. 2A). Pre-treatment of cells with quercetin significantly attenuated OTA-induced increase in $[Ca^{2+}]_i$, when compared to OTA treated group (Fig. 2B). Quercetin treatment alone showed $[Ca^{2+}]_i$ on par with the control. H_2O_2 positive control cells showed significant ($p < 0.05$) increase of intracellular calcium level compared to control at 30 min exposure.

3.2.2. Quercetin attenuated ROS generation, which peaked at 60 min OTA exposure

In order to determine the time at which ROS generation was maximum, a time course of ROS generation by OTA was carried out at 15, 30, 60, 180 and 300 min. Increase in ROS generation started as early as 30 min, peaked at 60 min and subsequently decreased at 180 and 300 min of OTA exposure (Fig. 2C). Since OTA-induced ROS generation peaked at 60 min exposure time, this time period was chosen to evaluate the protective effect of quercetin on OTA-induced ROS generation. Pre-treatment of cells with quercetin for 24 h, followed by OTA for 60 min significantly ($p < 0.05$) prevented OTA-induced ROS generation when compared to OTA treated group alone (Fig. 2D). H_2O_2 positive control cells showed significant ($p < 0.05$) increase of ROS generation compared to control at 60 min exposure. Treatment with quercetin alone did not affect intracellular ROS production when compared to control, and was statically non-significant ($p < 0.05$).

3.3. Effect of quercetin and OTA on redox signalling

3.3.1. Quercetin prevented OTA-induced nuclear localisation and NF-κB expression — nuclear localization as early as 60 min and maximum expression at 3 h

Under normal physiological conditions, NF-κB remains in an inactive form in the cytoplasm by binding with its inhibitory protein IκB. Exposure of cells to various extracellular stimuli including oxidative stress, pro-inflammatory cytokines, UV radiation and physical stress results in the activation of IκB kinase (IKK) that phosphorylates IκB. This leads to ubiquitinylation and degradation of IkB by proteasomes, resulting in activation and translocation of NF-κB into the nucleus, where it up-regulates the expression of genes involved in inflammatory responses. To determine NF-κB translocation, cells were exposed to OTA treatment for different time intervals (15, 30, 60 and 180 min) (Supp. Fig. 1). Our results indicate that NF-κB migrates into the nucleus in OTA treated cells at 60 min and remains in the nucleus up to 180 min. Pre-treatment of cells with quercetin, resulted in retention of NF-κB in the cytoplasm (Fig. 3).

In order to determine the effects of OTA on NF-κB expression, Western blot analysis were carried out at different time periods of 1, 3 and 5 h (Fig. 4A). Densitometric analysis of the blots indicate that NF-κB P65 expression was increased in OTA treated cells at 3 h but decreased at 5 h. Pre-treatment of cells with quercetin (10 μM for 24 h) followed by OTA treatment for 3 h resulted in significant suppression of NF-κB expression, indicating the anti-inflammatory activity of quercetin (Fig. 4B).

3.3.2. Quercetin modulated OTA-induced nuclear localization and expression of transcription factor Nrf2 — nuclear localization at 4 h quercetin pre-treatment and maximum expression at 8 h pre-treatment

In the present study, effect of OTA and quercetin on nuclear translocation of Nrf2 was studied through indirect immunofluorescence, with an FITC conjugated secondary antibody, primary antibody directed against Nrf2 and nucleus stained with DAPI. Co-

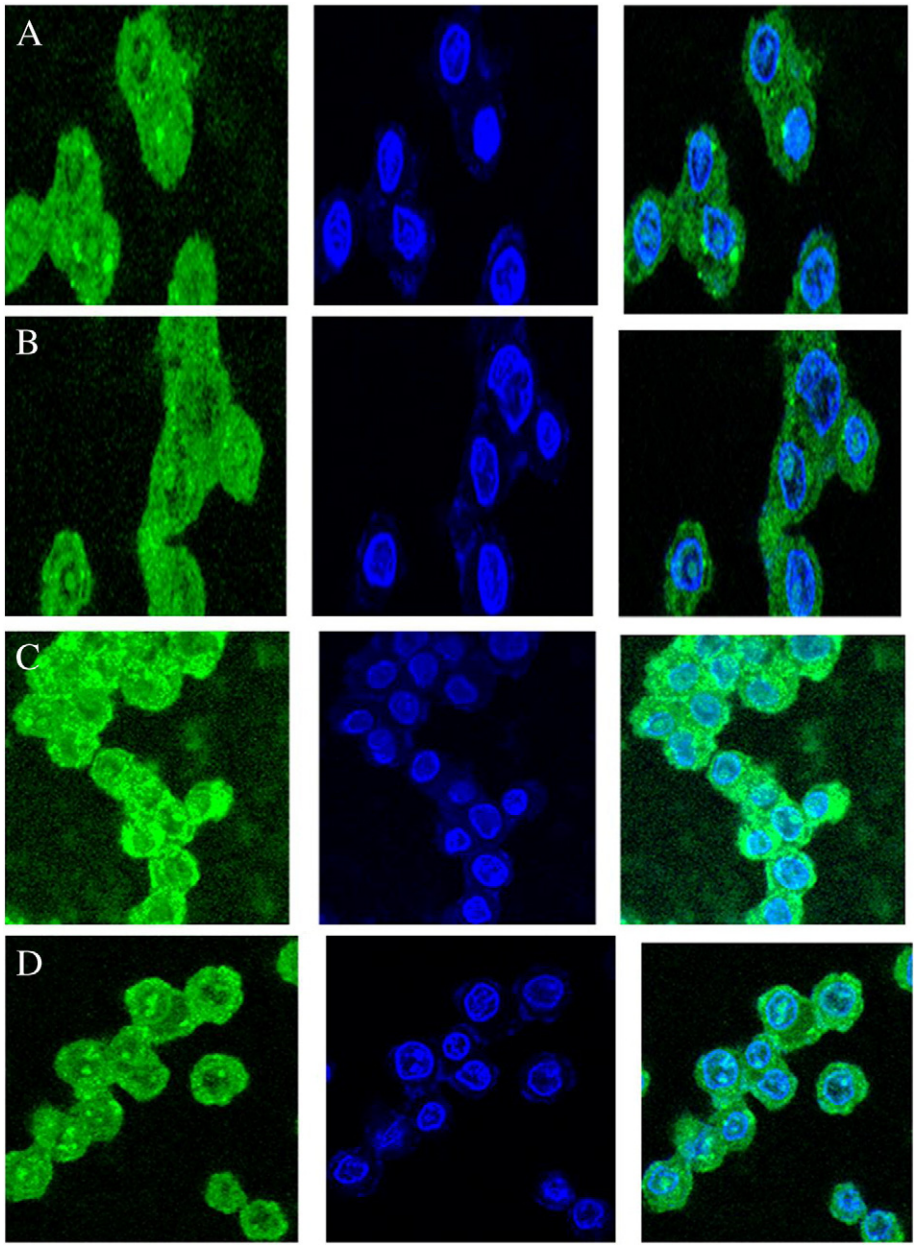


Fig. 5. Effect of quercetin and OTA on Nrf2 translocation. HepG2 cells were treated with OTA (10 μM) for 48 h in presence and absence of quercetin pre-treatment (10 μM for 4 h). Cells were studied for localization of Nrf2 through indirect immunofluorescence using FITC conjugated secondary antibody. The nucleus was stained with DAPI. Cells viewed at 400× magnification. (A) Control cells showing cytoplasmic localization of Nrf2. (B) Cells treated with OTA alone for 48 h showing retention of Nrf2 in the cytoplasm. (C) Cells exposed to quercetin alone for 4 h showing that Nrf2 has migrated into the nucleus, colocalization of FITC and DAPI gives characteristic turquoise color. (D) Cells pre-treated with quercetin for 4 h followed by OTA exposure showing migration of Nrf2 into the nucleus colocalization of FITC and DAPI gives characteristic turquoise color.

localization of FITC with DAPI results in a characteristic turquoise blue color. Under unstimulated conditions, Nrf2 is associated with Keap1 (Kelch-like ECH-associated protein 1), which is sequestered in the cytoplasm. Once stimulated, the Keap1–Nrf2 complex dissociates and Nrf2 translocates into the nucleus, where it binds to ARE sites in the promoter regions of many detoxification and antioxidant genes. Cells treated with OTA (10 μM) for 6, 12, 24 and 48 h did not show any activation of Nrf2, which was retained in the cytoplasm, revealing that OTA inhibits Nrf2 translocation. A time course assay of quercetin treatment for 1 h, indicated Nrf-2 migration from 2 h, which was maximum at 4 h and decreased at 6 h of exposure. Since 4 h exposure to quercetin resulted in nuclear localization of Nrf-2, this time point was chosen for the pre-treatment group with OTA (Supp. Fig. 2). Pre-treatment of cells with quercetin for 4 h followed by OTA exposure for 48 h resulted in Nrf2 translocation in the nucleus (Fig. 5).

In order to determine the effects of OTA and quercetin on Nrf2 expression, Western blot analysis was carried out at different time periods of quercetin exposure (4, 6, 8 and 10 h). Densitometric analysis of the blots indicates that Nrf2 expression was increased 1.5 fold at 6 h and 1.7 fold at 8 h and decreased at 10 h in quercetin treated cells (Fig. 6A). Since Nrf2 expression was maximum at 8 h quercetin treatment, this time period was chosen to evaluate the effect of quercetin on OTA treated cells. Quercetin pre-treatment followed by OTA treatment resulted in 1.7 fold increased expression of Nrf2 when compared to OTA treated cells alone, indicating that quercetin has a

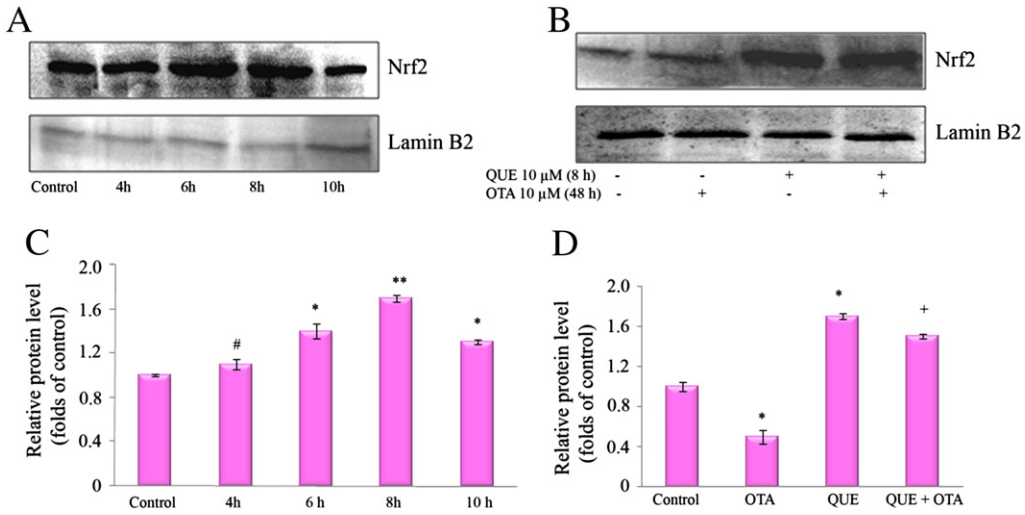


Fig. 6. Effect of quercetin on OTA-induced Nrf2 expression. (A) Time course of Nrf2 expression in response to quercetin treatment. Cells were treated with quercetin (10 μM) for 4, 6, 8 and 10 h. (B) Quercetin prevents OTA-induced Nrf2 downregulation. Cells were pre-treated with quercetin (10 μM) for 8 h followed by OTA (10 μM) for 48 h. (C& D) Bars represent data from densitometric analysis and represent mean ± SEM ($n = 3$) bars are expressed relative to the protein level of the control group. Significant differences versus control were indicated by $^{**}p < 0.05$, $^{*}p < 0.01$. Significant differences versus OTA treated group were indicated by $+\ p < 0.05$. # — Not significantly different from control.

protective effect on the cells (Fig. 6B). In OTA treated cells alone, the Nrf2 expression was downregulated when compared to control cells.

3.4. Effect of quercetin and OTA on inflammatory marker COX-2 Quercetin suppress OTA-induced COX-2 expression — maximum induction at 15 h OTA treatment

Cyclooxygenase-2 is an inducible enzyme, which is one of the early response genes in the inflammatory process and is commonly used as an inflammatory marker. In the present study, we studied the effect of quercetin and OTA on COX-2 expression. Cells were exposed to OTA for different time intervals (10, 15 and 24 h) for the determination of COX-2 induction. Our results indicate that COX-2 expression was induced (2 fold increase) at 15 h exposure to OTA (Fig. 7). Pre-treatment of cells with quercetin followed by OTA for 15 h substantially decreased expression of COX-2 in HepG2 cells.

3.5. Effect of quercetin on other oxidative stress parameters, induced by OTA

3.5.1. Quercetin inhibited OTA-induced NO accumulation

Nitrite is the stable product of nitric oxide released in response to oxidative stress, contributed by iNOS. Treatment of HepG2 cells with OTA resulted in a statistically significant increase in nitrite production, from 12 micromoles nitrite in the control to 39 micromoles in OTA treated samples ($p < 0.05$). Pre-treatment of cells with quercetin lowered OTA-induced nitrite production in a statistically significant manner to 15 micromoles ($p < 0.01$). Treatment with quercetin alone did not exhibit a significant increase or decrease in nitrite production

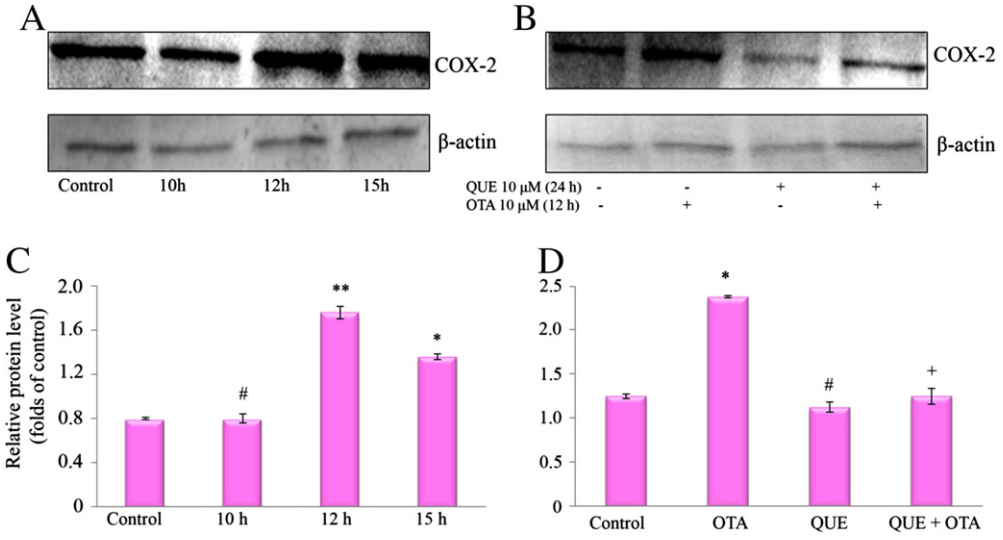


Fig. 7. Effect of quercetin on OTA-induced COX-2 expression. (A) Time course of COX-2 expression in response to OTA treatment. Cells were treated with OTA (10 μM) for 10, 12 and 15 h. (B) Quercetin downregulates OTA-induced COX-2 expression. Cells were treated with quercetin (10 μM for 24 h) followed by OTA treatment for 15 h. (C & D) Bars represent data from densitometric analysis and represent mean ± SEM ($n = 3$) bars are expressed relative to the protein level of the control group. Significant differences versus control were indicated by $^{**}p < 0.05$, $^{*}p < 0.01$. Significant differences versus OTA treated group were indicated by $+\ p < 0.05$. # — Not significantly different from control.

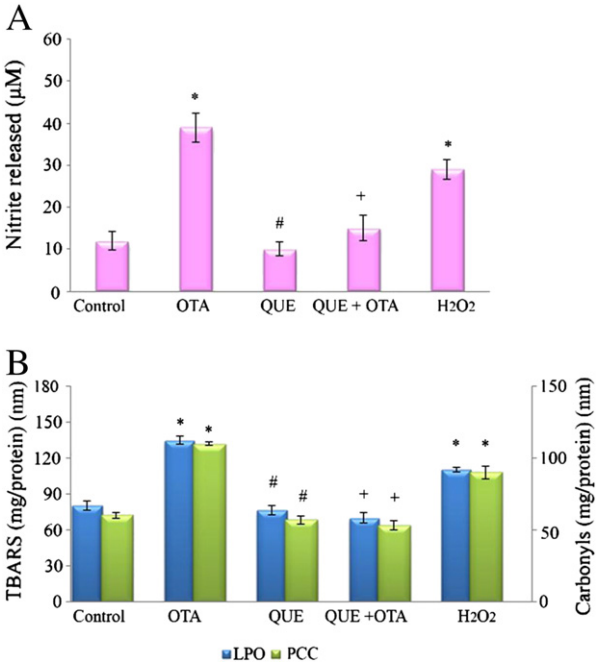


Fig. 8. Effect of quercetin on OTA-induced delayed oxidative stress. **(A)** Quercetin inhibited OTA-induced NO production. Cells were treated with OTA (10 μM for 48 h) in the presence and absence of quercetin pre-treatment (10 μM for 24 h). Results expressed as μM of nitrite released. (B) Effect of quercetin on OTA-induced LPO and PCC production. Cells were pre-treated with quercetin (10 μM) for 24 h, followed by OTA (10 μM) for 48 h. Cells treated with 50 μM H_2O_2 was used as a positive control. Results expressed as nmoles of TBARS/mg protein and nmoles of carbonyl/mg of protein. Data presented were Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{*}p < 0.05$. Significant differences versus OTA treated group were indicated by $+ p < 0.05$. # — Not significantly different from control.

when compared to the control (Fig. 8A). Treatment of cells with H_2O_2, used as a positive control for oxidative stress showed significant ($p < 0.05$) increase in nitrite when compared to control.

3.5.2. Quercetin decreased OTA-induced lipid peroxidation and protein carbonylation

OTA treatment resulted in significantly elevated levels of LPO and PCC ($p < 0.05$) compared to the control, which may be attributed to oxidative stress. Pre-treatment of cells with quercetin resulted in LPO and PCC levels which were near control values. Cells treated with quercetin alone did not show significant changes (Fig. 8B), while cells treated with 50 μM H_2O_2 indicated significant increase of carbonyl content when compared to control.

3.5.3. Quercetin prevented GSH depletion in OTA treated cells

In the present study, GSH levels were significantly ($p < 0.05$) lowered from 130 μM in the control to 60 μM in OTA treated cells. Pre-treatment with quercetin followed by OTA resulted in restoration of glutathione levels to 110 μM which was statistically significant when compared to the OTA treated cells ($p < 0.05$). Quercetin by itself did not have significant ($p < 0.05$) effect on GSH level (Table 1). Treatment of cells with H_2O_2, which was used as a positive control, also reduced GSH levels significantly ($p < 0.05$) indicating that GSH is consumed during oxidative stress.

Table 2
Effect of quercetin on OTA-induced DNA damage.

Treatment groups	% Head DNA	% Tail DNA	Olive tail moment
Control	96.63 ± 0.56	3.31 ± 0.39	0.52 ± 0.05
OTA	49.41 ± 2.18 *	50.58 ± 4.89 *	23.16 ± 4.25 *
QUE	93.98 ± 0.75 #	6.01 ± 0.59 #	1.36 ± 0.04 #
QUE + OTA	85.75 ± 2.28 +	14.42 ± 2.54 +	2.48 ± 0.85 +

HepG2 cells were treated with quercetin (10 μM) for 24 h in presence and absence of OTA (10 μM) for 48 h to determine OTA induced DNA damage. Results expressed as Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{*}p < 0.05$. Significant differences versus OTA treated group were indicated by $^{+}p < 0.05$. # — Not significantly different from control.

3.5.4. Quercetin modulated antioxidant enzyme activity in HepG2 cells treated with OTA

Antioxidant enzyme activity is an indicator of oxidative stress. In the present study SOD, CAT, GST, and GPx were significantly ($p < 0.05$) decreased in OTA treated cells. Pre-treatment with quercetin significantly ($p < 0.05$) increased antioxidant enzyme activities when compared to the OTA treated cells (Table 1), thereby indicating that the antioxidant and free radical scavenging of quercetin has a role to play in cytoprotection.

3.6. Effect of quercetin on OTA-induced genotoxicity

3.6.1. Quercetin protected HepG2 cells from OTA-induced DNA damage

The Comet assay was used to determine the association between genotoxicity in the MN assay and the induction of oxidative damage caused by OTA exposure. In cells treated with OTA percentage Tail DNA and olive tail moment significantly increased to 50.58 ± 4.89 and 23.16 ± 4.25 from 3.31 ± 0.39 and 0.52 ± 0.05 observed in control cells (Table 2). Cells pre-treated with quercetin significantly reduced percentage Tail DNA and olive tail moment to 14.42 ± 2.54 and 2.48 ± 0.85 which was a significant decrease compared to OTA treated group. Quercetin treatment alone showed 6.01 ± 0.59 and 1.36 ± 0.04 of percentage tail DNA and olive tail DNA respectively, which was not significantly different from control.

3.6.2. Quercetin reduced OTA-induced micronucleus formation

The analysis of MN may be considered as a useful biomarker of genotoxic effects in cells exposed to genotoxicants [36]. Cells treated with OTA-induced 95 micronuclei per 1000 binucleate cells which was a statistically significant difference ($p < 0.05$) compared to the control (Fig. 9). Pre-treatment with quercetin reduced the genotoxic effect of OTA as evidenced by reduction in the frequency of MNBNC induction to 20 micronuclei per 1000 binucleate cells, which was a statistically significant ($p < 0.05$) decrease compared to the OTA treated cells.

Table 1
Effect of quercetin on OTA-induced decrease of antioxidant enzyme activities.

Treatment groups	SOD U/mg protein	CAT U/mg protein	GST U/mg protein	GpxU/mg protein	GSH U/mg protein
Control	150.75 ± 3.56	35.57 ± 2.85	120.25 ± 4.27	10350 ± 85.21	102.58 ± 1.58
OTA	65.85 ± 3.54*	15.25 ± 0.85*	60.35 ± 3.40*	6345.66 ± 75.91*	50.54 ± 3.97*
QUE	148.76 ± 2.76	33.52 ± 5.42 #	118.65 ± 2.89 #	10340 ± 92.79 #	99.45 ± 2.45 #
QUE + OTA	142.5 ± 2.38 +	30.25 ± 2.53+	110.92 ± 3.78+	10200.92 ± 65.79 +	95.85 ± 1.95#
H_2O_2	78.56 ± 3.81*	20.28 ± 3.19*	78.85 ± 4.79*	7546.65 ± 91.02*	65.17 ± 2.56*

HepG2 cells were treated with quercetin (10 μM) for 24 h in presence and absence of OTA (10 μM) for 48 h to determine OTA induced oxidative stress. SOD 1 U = The amount of enzyme required to give 50% inhibition of pyragallol auto-oxidation. CAT 1 U = The amount of enzyme that consumes 1 nmol H_2O_2 /min, GST 1 U = The amount of enzyme that conjugates 1 μmol CDNB /min, GPx 1 U = The amount of enzyme that converts 1 μmol GSH to GSSG in the presence of H_2O_2/min. Results expressed as Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{*}p < 0.05$. Significant differences versus OTA treated group were indicated by $^{+}p < 0.05$ # — Not significantly different from control.

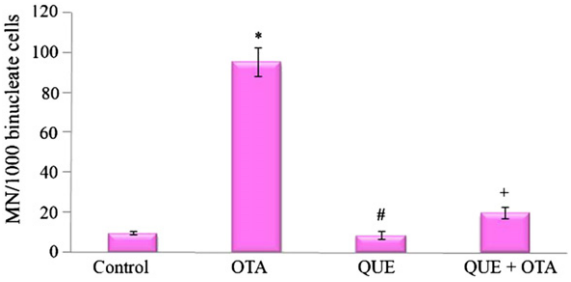
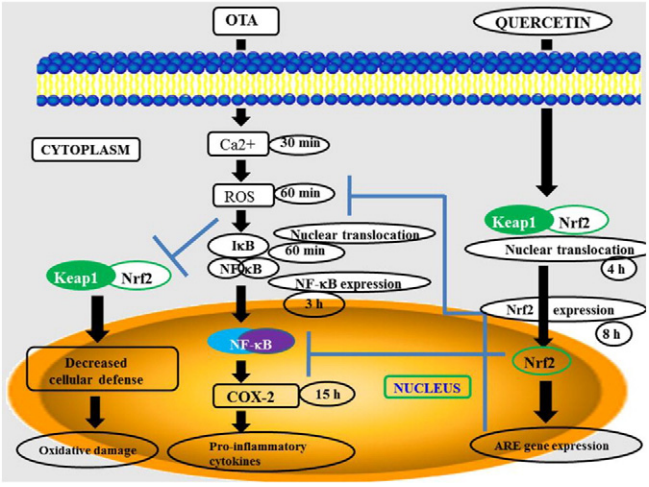


Fig. 9. Effect of quercetin on OTA-induced micronucleus induction. Cells were pre-treated with quercetin (10 μM) for 24 h, followed by OTA (10 μM) for 48 h. Results expressed as micronucleus per 1000 binucleate cells. Data presented were Mean ± SEM ($n = 9$). Significant differences versus control were indicated by $^{*}p < 0.05$. Significant differences versus OTA treated group were indicated by $+\ p < 0.05$. # — Not significantly different from control.

4. Discussion

It is now well accepted that reactive oxygen species are mediators of intracellular signalling cascades and induce several changes at the cellular level. Excessive production of ROS leads to oxidative stress, loss of cell function and lipid peroxidation [37]. OTA is a mycotoxin that affects cells by turning on one or more redox signalling molecules that have a significant effect on cellular processes. Although some of these molecular mechanisms are reported in literature, time course studies to elucidate the sequence of these events are not reported. The present study evaluates some molecular redox signalling molecules induced by OTA and the time points of induction as illustrated.



Dietary intake of antioxidants is a plausible and effective way to augment and fortify endogenous defence systems, since many antioxidants act as free radical scavengers and immunomodulators, resulting in cytoprotection. Although OTA-induced toxicity is widespread, antidotes to its toxicity are sparsely reported in literature. In the present study, we evaluated the effect of quercetin, a flavonoid on OTA-induced oxidative stress as well as some molecular redox signalling molecules. Although some other antioxidants such as catechins [38] and rosmarinic acid [39] have been evaluated against OTA-induced toxicity, this is the first study where quercetin has been evaluated to ameliorate the harmful effects of OTA.

There are several reports in literature which cite that increase in intracellular calcium leads to ROS generation [40,41]. It has been proposed that intracellular Ca^{2+} homeostasis is important for cellular homeostasis and that disturbances or accumulation of Ca^{2+} may be a common step in the development of cytotoxicity and cell injury [42]. Our study demonstrates that increase in ROS is a downstream event to that of induction of $[Ca^{2+}]i$,occurring at 60 min and 30 min respectively. It has been reported in literature that OTA exposure increases intracellular ROS; however, these studies were carried out at later time points of 24 h [38]. A study by Bouaziz et al. [43] reported that ROS generation did not exceed 1.6 folds of control after 24 h exposure of OTA. In our study, we report for the first time that OTA-induced ROS induction starts after 30 min exposure to OTA and peaks at 60 min which is 10 fold increases relative to control. The times at which these two studies were carried out, might account for the observed difference between the two studies. Calcium release seems to be an early intracellular event in OTA-induced toxicity, leading to oxidative stress. Gekle et al. and Klaric et al. [44,42] provide evidence for OTA-induced effect on intracellular Ca^{2+} homeostasis. Normally, the Ca^{2+} concentration in the cytosol of unstimulated cells is maintained between 0.05 and 0.2 mM. Extracellular Ca^{2+} levels are approximately 1.3 mM. This produces a large electrochemical gradient that is mainly balanced by active Ca^{2+} extrusion through the plasma membrane and by co-ordinated activity of Ca^{2+} sequestering systems located in the mitochondrial, endoplasmatic reticular and nuclear membranes. Disturbances of these processes can result in enhanced Ca^{2+} influx, release from intracellular stores and inhibition of Ca^{2+} extrusion at the plasma membrane. When the cytoplasmic $[Ca^{2+}]$ increases, mitochondria take up Ca^{2+}, functioning as a Ca^{2+}-buffering organelle. However, excessive accumulation of mitochondrial Ca^{2+} is known to trigger ROS generation from the mitochondrial Electron transport chain. It is well documented in literature that elevation of intracellular calcium concentration $[Ca^{2+}]_i$ leads to an augmentation of ROS in cells [40]. Functionally, cellular effects of interactions between Ca^{2+} and ROS signaling systems depends on the type of target proteins, the ROS species, the dose, duration of exposure, and the cell contexts.

In the present study H_2O_2 has been used only as positive control. It has been documented in literature that increased intracellular calcium by H_2O_2 can lead to elevated levels of ROS [23], which corroborates with our findings.

In the present study, pre-treatment with quercetin resulted in reduced ROS as well as intracellular calcium. It is possible that quercetin's free radical scavenging activity may have an important role to play in modulating ROS and calcium levels, thereby acting as a cytoprotectant. Quercetin being a powerful antioxidant has been shown previously to reduce oxidative stress mediated cell death by inhibiting the increase in intracellular calcium and ROS in H_2O_2 and tertiary-butyl hydroperoxide exposed rat thymocytes and C6 glial cells [45,46].

Following ROS release, several downstream events are known to occur. In our study we chose to evaluate 2 critical redox signalling molecules namely NF-κB and Nrf-2. NF-κB was chosen as it is a central mediator of immune responses and is known to be induced by ROS [47]. Nrf-2 was chosen as it is a critical redox signalling transcription factor known to be modulated by antioxidants in response to oxidative stress [48].

Our study demonstrates that NF-κB nuclear localization after OTA treatment occurs as early as 60 min and remained in the nucleus up to 180 min. Maximum NF-κB expression was induced at 3 h, indicating that nuclear localization of NF-κB and its expression can occur simultaneously. Our results corroborate with previous studies where significant increase of NF-κB expression was observed after OTA exposure in rat embryonic midbrain cells [10], however, this is the first study to report the exact time point of nuclear localization of this key redox transcription factor.

Another important redox transcription factor is Nrf-2, which migrated into the nucleus at 4 h after quercetin treatment. Activation of Nrf-2 is known to increase expression of ARE genes, the product of which are antioxidant enzymes [49]. Treatment of cells with OTA

resulted in retention of Nrf-2 in the cytoplasm and concomitant decrease in Nrf-2 expression and antioxidant enzyme activity, clearly indicating that OTA causes imbalance in the cellular oxidative defense machinery, attributable to oxidative stress. Quercetin has been shown previously to modulate increase in ARE gene expression in human hepatocytes during ethanol treatment [50]. Our study demonstrates that quercetin plays a cytoprotective function, through induction of nuclear localization of Nrf-2 as well as upregulation of Nrf-2 expression leading modulation of ARE encoded genes.

Increase in ARE gene products by quercetin through Nrf-2 mediated activation and expression is reflected in the cells ability to enhance enzymatic and non-enzymatic defences. Quercetin through its anti-oxidant and redox modulatory effects clearly enhances the cell's cytoprotective machinery. Surh reported that modulation of NF-κB and Nrf-2 expression by antioxidants and anti-inflammatory phytonutrients plays a major role in cytoprotection [51]. Some phytochemicals, such as curcumin and sulforaphane, can inhibit overexpression of NF-κB and activate Nrf2, and these actions may involve the modification of the cysteine moiety in p50 of NF-κB and Keap1 [52]. It is possible that quercetin plays a role in such redox modulation and may play a role in the potential cross talk between NF-κB and Nrf-2. Further studies are required to elucidate such roles.

COX-2 is an immediate early gene induced in response to NF-κB activation. In our study we observed that COX-2 induction followed NF-κB expression at 15 h and quercetin down regulated COX-2 expression. Earlier studies have reported OTA-induced COX-2 expression in peritoneal macrophages and murine macrophages [53], however, this is the first study demonstrating an increased expression of COX-2, in HepG2 cells. Similarly, it is documented that quercetin downregulates COX-2 mRNA and protein level expression in different cell types, in other studies exposed to stimulants such as cadmium and cooking oil fumes [54,55]. However this is the first study where quercetin has been shown to down regulate OTA-induced COX-2 expression.

Oxidative stress results when the oxidative insult supercedes the cell's ability to counter it. Several oxidative stress indicators such as NO, LPO, PCC are enhanced by OTA, clearly indicating a prolonged and sustained oxidative stress [56], possibly leading to DNA damage.

It is well documented in literature that free radicals lead to DNA damage as well as strand breakage (double-strand breaks, DNA single-strand breaks and alkali-labile sites). It has been previously reported that induction of micronucleus in HepG2 cell lines by OTA is partially due to its chromosome breaking effect and spindle disruption [57]. Our findings corroborate with previous reports where OTA-induced oxidative DNA damage and MN have been demonstrated both *in vivo* and *in vitro* [21,58]. Quercetin treatment offers protection against OTA-induced DNA damage and MN possibly due to its free-radical scavenging activity, preventing sustained oxidative stress, protecting the DNA from damage.

5. Conclusion

In this study we have demonstrated for the first time the sequence of some of the molecular mechanisms implicated in OTA-induced toxicity and the protective role of quercetin in preventing oxidative damage through its anti-oxidant, redox-modulatory and immuno-modulatory properties. These studies make quercetin an attractive phytochemical for protection against environmental and mycotoxins. Further studies are required to identify the time of activation of some of the kinases such as p38, ERK1/2 and MAPK, which have been previously documented to play a role in OTA-induced toxicity. Also, redox cross talk between NF-κB and Nrf-2 and the role of quercetin in its modulation will need to be explored in the future, which will deepen our understanding of the molecular mechanisms of action of quercetin in such applications.

Conflict of interest statement

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.10.024.

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