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ORIGINAL ARTICLE

An *in vitro* study ascertaining the role of H_2O_2 and glucose oxidase in modulation of antioxidant potential and cancer cell survival mechanisms in glioblastoma U-87 MG cells

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Abstract Glial cells protect themselves from the elevated reactive oxygen species (ROS) via developing unusual mechanisms to maintain the genomic stability, and reprogramming of the cellular antioxidant system to cope with the adverse effects. In the present study non-cytotoxic dose of oxidants, H_2O_2 (100 μM) and GO (10 μU/ml) was used to induce moderate oxidative stress via generating ROS in human glioblastoma cell line U-87 MG cells, which showed a marked increase in the antioxidant capacity as studied by measuring the modulation in expression levels and activities of superoxide dismutase (SOD1 and SOD2) and catalase (CAT) enzymes, and the GSH content. However, pretreatment (3 h) of Curcumin and Quercetin (10 μM) followed by the treatment of oxidants enhanced the cell survival, and the levels/activities of the antioxidants studied. Oxidative stress also resulted in an increase in the nitrite levels in the culture supernatants, and further analysis by immunocytochemistry showed an increase in iNOS expression. In addition, phytochemical pretreatment decreased the nitrite level in the culture supernatants of oxidatively stressed U-87 MG cells. Elevated ROS also increased the expression of COX-2 and APE1 enzymes and pretreatment of Curcumin and Quercetin decreased COX-2 expression and increased APE1 expression in the oxidatively stressed U-87 MG cells. The immunocytochemistry also indicates for APE1 enhanced stress-dependent subcellular localization to the nuclear compartment, which advocates for enhanced DNA repair and redox functions of APE1 towards survival of U-87 MG cells. It can be concluded that intracellular oxidants activate the key enzymes involved in antioxidant mechanisms, NO-dependent survival mechanisms, and also in the DNA repair pathways for glial cell survival in oxidative-stress micro-environment.

Keywords Oxidative stress · Antioxidants · APE1 · Curcumin · Quercetin · iNOS

Introduction

Cellular oxidative stress is generated by the imbalance of the redox status in a cell. One of the important key players of the oxidative stress is reactive oxygen species (ROS) produced by various metabolic activities in the cell. Oxidative stress plays an important role in the development and progression of cancer (Birben et al. 2012; Sosa et al. 2013). Cells possess a number of antioxidant defense mechanisms to minimize the ROS generation and evolve various stress response mechanisms which are activated or induced upon oxidative stress that may result in survival or death of a cell (Martindale and Holbrook 2002). Glioblastoma Multiforme (GBM) is the most deadly, aggressive and malignant grade IV form of brain tumors having a mean survival rate of one year. Despite available therapies, the prognosis is extremely poor (Holland 2001). Etiology of glioblastoma remains largely unknown. Exposure to ionization radiation and genetic predispositions are the risk factors associated with the occurrence of GBM

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(Preusser et al. 2011). The ROS scavenging machinery includes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) enzymes. GSH-Px deficiency leads to accumulation of ROS and death of GBM cells suggesting that GSH-Px is a crucial enzyme over the other antioxidant enzymes (Dokic et al. 2012). The hydrogen peroxide (H_2O_2) produced by the SOD or the action of oxidases such as xanthine oxidase is reduced to water (H_2O) by GSH-Px and CAT as reviewed earlier (Birben et al. 2012).

Oxidative stress causes genomic instability. ROS induces oxidized bases in the DNA that are repaired via the base excision repair (BER) pathway. APE1 is the major multifunctional enzyme in the BER - pathway involved in the repairing of abasic (AP) sites in oxidized DNA and activation of transcription factors via its redox function as reviewed (Bhakat et al. 2009; Tell et al. 2010). Oxidative stress has been reported to elevate the expression of APE1 in glioma cells and contributes to the resistance to alkylating agent-based chemotherapy (Silber et al. 2002). APE1 activity is also related to the radioresistance in the glioma (Naidu et al. 2010). APE1 knockdown in glioma cells has been documented for enhanced cytotoxic effect of temozolomide (TMZ) drug, indicating APE1 role in contributing resistance to chemotherapeutic drugs in GBM (Montaldi et al. 2015). Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are important enzymes that mediate inflammatory process, and their altered expression has been associated with progression of various tumors. Several chemopreventive phytochemicals have been shown to inhibit the COX-2 and iNOS expression via inhibiting NF-KB activation (Surh et al. 2001). High expression of COX 2 is associated with the glioma aggressiveness (Shono et al. 2001). Overexpressed NOS and NO production in the cancer cell induces invasion, angiogenesis, immunosuppression, differentiation, and therapeutic resistance in gliomas as reviewed recently (Tran et al. 2016).

Life expectancy of GBM patients is still poor even after the development of the surgical techniques, chemotherapeutic agents, and radiotherapy strategies involving GBM management. There are many factors involved in the drug resistance in glioblastomas reviewed in detail such as including drug efflux, hypoxic areas of tumor cells, cancer stem cells, DNA damage repair, and miRNAs (Haar et al. 2012). Phytochemicals are proven to be very valuable in the regulation of cancer. Curcumin modulates the growth of tumor cells through regulating cell proliferative and cell survival pathways (Ravindran et al. 2009). Curcumin has been found to suppress initiation, progression, and metastasis of a variety of tumors and found to induce apoptosis (Shanmugam et al. 2015). Curcumin sensitizes the glioma cells to several clinical therapeutic agents, radiation, and helps in overcoming glioma cell radioresistance and chemoresistance (Dhandapani et al. 2007). Quercetin shows anti-oxidative property and modulates the signalling pathways which are leading to the inflammation and carcinogenesis (Murakami et al. 2008). Quercetin exhibits an inhibitory effect on ROS-induced cell death in C6 glioma cells (Chen et al. 2006).

In the present study, the human glioblastoma (U-87 MG) cells were oxidatively stressed using moderate concentration of H_2O_2 and glucose oxidase (GO) to ascertain the levels/activities of the antioxidant enzymes (SOD and CAT) and a cellular antioxidant GSH; how the phytochemicals (Curcumin and Quercetin) can modulate the expression of these antioxidant enzymes. We also investigated the oxidative stress-induced altered expression and modulatory effect of dietary phytochemicals on the other enzymes- NOS2, COX-2, and APE1 associated with cell survival mechanisms by which the GBM might be enrouting towards metastasis and severity of the disease.

Materials and methods

Cell culture and treatments Human glioblastoma U-87 MG cells (gifted by Prof. Gursharan Kaur, Guru Nanak Dev University, Amritsar) were maintained and cultured in the DMEM media (Gibco) supplemented with 10% FBS (Gibco) and 1× penicillin-streptomycin solution (HiMedia) in humidified CO_2 incubator (New Brunswick Scientific) at 37 °C. For different treatments, U-87 MG cells were seeded in 96 well plates at a density of 1×10^4 cells per well or in 10 cm dishes at 1×10^6 cells per plate. H_2O_2 (MP-Biomedicals) and GO (Sigma-Aldrich) at various concentrations as indicated were used to induce the oxidative stress in the U-87 MG cells. The treatments, 3 h pretreatment of 10 μM phytochemicals Curcumin (Sigma-Aldrich) and Quercetin (HiMedia) and followed by oxidative stress for 24 h period as suggested in various previous studies (Alía et al. 2006; Duthie et al. 1997).

Cell survival assay U-87 MG cell survival was analyzed with MTT as described previously with slight modifications in the protocol (Dhiman et al. 2012). Cells were seeded (8000 cells/well) in 96 well plates and were incubated at 37 °C, being allowed to grow for 24 h. Cells were serum starved for overnight and treated with various concentrations of oxidants H_2O_2 (0 to 200 μM) and GO (0 to 40 μU/ml) for 24 h. Afterward, 100 μl of MTT (0.5 mg/ml) reagent (Thermo Scientific) was added to each well, and then the plates were incubated for 3 h in the dark at 37 °C. The formazan product formed was dissolved in acidified DMSO and measured by using a microplate reader (Biotek) at 570 nm. The percent viability was calculated by using absorbance of treatment vs. the untreated controls.

Intracellular ROS measurement Intracellular ROS was measured using H_2DCFDA reagent as described previously with minor modifications (Knerr et al. 2006). Approximately

0.8×10^4 U-87 MG cells/well were seeded in 96 well plate. Cells were serum starved overnight, and oxidant (H_2O_2 and GO) treatment was given to the cells for 24 h. Afterward, cells were incubated with 100 μl of 50 μM H_2DCFDA (Thermo Scientific) solution for 30 min in the dark and washed with 1× PBS. A blank reading was recorded at excitation/emission 478/518 nm, and cells were further incubated for 30 min in the dark, and relative fluorescence intensity was measured.

Nitric oxide (NO) level NOS activity was measured by determining the increase in the concentration of nitrite (NO_2^-), a breakdown product of NO in the cell culture medium using Griess reaction as described previously with slight modifications (Bryan and Grisham 2007; Dhiman et al. 2009). U-87 MG cells were seeded in 96 well plates at an initial density of 0.8×10^4 cells per well for 24 h. The medium was replaced with H_2O_2 and GO containing serum free media for 24 h. 100 μl of cell supernatants were added in 1:1 ratio with Griess reagent. After 5 min, the OD of the red-pink colour mixture was measured spectroscopically at 540 nm on the multiplate reader (Biotek). The nitrite content of each sample was evaluated with a standard curve obtained by linear regression made with sodium nitrite and was expressed in μM of nitrite.

Enzyme linked immunosorbent assay (ELISA) ELISA was used to quantify the level of MnSOD, CuZn-SOD, and COX-2 proteins as previously described with slight modifications in the protocol (Dhiman et al. 2012). Briefly, 96 well plates were precoated with protein samples (20 μg) of total cell lysates, cytosol, and mitochondrial extracts) and incubated overnight at 4 °C. After blocking with 1% BSA at room temperature for 1 h, plates were incubated with primary antibodies against MnSOD (1:500; Santa Cruz) and CuZn-SOD (1:500; Santa Cruz) and COX-2 (1:500; Santa Cruz) for an hour. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). The plates were then probed with HRP conjugated secondary antibodies (1:1000; Santa Cruz). Finally, the color was developed using the tetramethylbenzidine (TMB) substrate for the HRP reaction. The reaction was stopped with 2M H_2SO_4, and OD was measured at 450 nm using a microplate reader (Biotek). The OD is directly proportional to the reaction product formed due to the interaction between antigen and antibody.

Western blot analysis APE1 expression was measured by Western blot analysis in U-87 MG cells. Cells were lysed using RIPA buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitor cocktail (Amresco) as described previously with few modifications (Mantha et al. 2012). Protein concentration was determined by BSA protein assay reagent (Amresco), according to the manufacturer's instructions. Protein samples (30 μg) were separated by electrophoresis on 10% SDS-PAGE gel and transfered onto PVDF membrane (Pall Corporation). The membranes were then blocked for 1 h in 5% non-fat dry milk [NFDM] (Santa Cruz) in 50 mM Tris-HCl (pH 7.4), containing 0.05% Tween 20 (TBST). Blocked membranes were incubated with primary antibody against APE1 (1:1000; Santa Cruz) and further incubated with the secondary antibody (1:5000; Invitrogen). β-Actin (1:1000; Santa Cruz) was used as endogenous control for normalization. The immune detection was accomplished using ECL (BioRad), and protein expression was visualized using fluor chem hd2 (Protein Simple).

In-gel assay for SOD activity Protein samples (50 μg) were separated using 10% native PAGE in electrophoresis buffer (200 mM glycine, 25 mM Tris-HCl) at 4 °C as described previously with slight modifications (Beauchamp and Fridovich 1971). Gels were incubated with 2 mM NBT and 28 μM riboflavin for 20 min in the dark. The gels were incubated with 1% TEMED in the dark followed by 20 min exposure to white light under a fluorescent lamp. To confirm the CuZn-SOD activity, one gel was incubated in the presence of KCN, an inhibitor of SOD. The dark purple stained gel shows clear bands of SOD enzyme as described previously.

In-gel assay for CAT activity Protein samples (50 μg) were separated using 10% native PAGE in electrophoresis buffer (200 mM glycine, 25 mM Tris-HCl) at 4 °C as described previously with slight modifications (Weydert and Cullen 2010). Then the gels were washed with deionized H_2O for 10 min. Afterward, these gels were incubated in 0.003% of H_2O_2 for 10 min. Then these gels were incubated in a solution of 1% ferric chloride and 1% KCN until the dark bands appeared. Then these gels were washed extensively with H_2O to stop the reaction. The green color stained gels with dark bands at ~220 kDa showing the CAT activity.

Spectrophotometric determination of GSH levels Glutathione level was analyzed using the previously defined protocol (Rahman et al. 2006). Protein samples (50 μg) were diluted with 0.1 M sodium phosphate buffer (pH 8.0) and incubated with 25% trichloroacetic acid (TCA) for 1 h at −20 °C which allows all the proteins to be precipitated except GSH and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatants were transferred to fresh centrifuge tubes. A calibration curve was set using different concentrations of reduced GSH. The samples were then incubated with 20 μL of Ellman's regent (DTNB) at a final concentration of 0.6 mM. After 10 min of incubation, the OD was measured at 412 nm using microplate reader (Biotek) as described previously.

Immunocytochemistry Cellular localization of APE1 was determined using immunocytochemistry staining as described in detail previously (Singh and Englander 2012). A constant cell suspension was poured on sterile coverslips placed in each well and U-87 MG cells were treated with oxidants for 24 h.

After incubation, cells were fixed with 4% formaldehyde solution for 20 min and permeabilized using 0.1% Triton X-100 for 10 min. Cells were blocked using 10% FBS for 1 h at room temperature. U-87 MG cells were washed and incubated at 4 °C overnight with primary APE1 (1:100; Santa Cruz) and NOS2 (1:100; Santa Cruz) antibodies. U-87 MG cells were washed three times with sterile 1× PBS and incubated with Alexa Fluor 488 (Invitrogen) for 1 h. Then the cells were washed three times with sterile 1× PBS and incubated with DAPI for 10 min, and images were captured under 60× oil objective confocal microscope (Olympus) at Central Instrumentation Laboratory (CIL), CUPB.

Statistical analysis

The mean and standard deviation of each treatment group was calculated for all the experiments. Statistical analysis was done using the student's t-test to compare two means with $p < 0.05$ representing statistical significance.

Results

GO and H_2O_2 induces cytotoxicity in the U-87 MG cells A broad range micromolar concentrations of oxidants GO (0–40 μU/ml), and H_2O_2 (0–200 μM) were used to study the cytotoxicity of these oxidants in U-87 MG cells. U-87 MG cells were exposed to GO treatment for 24 h. Our results show that (Fig. 1a) with an increase in the concentration of GO, U-87 MG cells showed an increase in cell proliferation (up to 15 μU/ml) and on further increase in the concentration resulted in the cell proliferation rate as compared to the untreated control cells. In further studies, GO at the non-cytotoxic concentration i.e. 10 μU/ml was used throughout the study. U-87 MG cells were also treated with H_2O_2 to induce oxidative stress in a concentration range up to 200 μM. H_2O_2 treatment at 100 μM showed no cytotoxic effect in the U-87 MG cells (Fig. 1b) which was used throughout the study. U-87 MG cell viability was also analyzed in the presence of phytochemicals Curcumin (10 μM) and Quercetin (10 μM) along with the 10 μU/ml GO and 100 μM H_2O_2. After the phytochemicals treatment, no significant change in the U-87 MG cell viability was observed, and the pretreatment of them certainly enhanced cell proliferation rate after the treatment of oxidants (Fig. 1c).

GO and H_2O_2 induces oxidative stress via enhancing ROS levels in the U-87 MG cells Oxidative stress was analyzed using ROS measurement in U-87 MG cells. U-87 MG cells were treated with a non-cytotoxic concentration of GO (10 μU/ml) and H_2O_2 (100 μM) to induce moderate oxidative stress. There was an increase in production of intracellular ROS by 36% in GO treated and 48% in H_2O_2 treated U-87 MG cells as compared to untreated control cells. Curcumin pretreatment decreased the ROS level in GO treated cells by 22%, and 27% in H_2O_2 treated U-87 MG cells when compared to the untreated control cells. However, Curcumin pretreatment in GO treated cells decreased the ROS level when compared with only GO

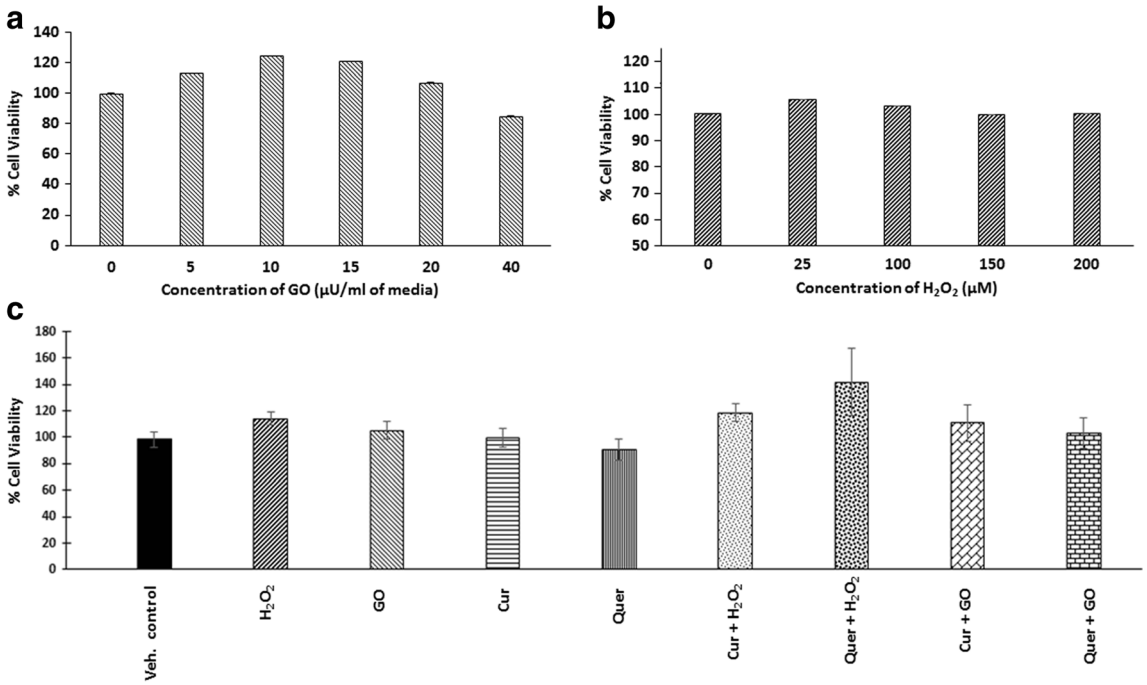

Fig. 1 The percent cell viability of U-87 MG cells when treated with increasing concentrations of: (**a**) GO for 24 h; (**b**) H_2O_2 for 24 h; and (**c**) U-87 MG cells when treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. The results are expressed as a mean ± standard deviation ($n = 3$)

stressed U-87 MG cells by 35% and 51% drop in ROS level in Curcumin pretreated H_2O_2 stimulated cells when compared to H_2O_2 stressed U-87 MG cells. However, there was 20% increase in ROS level when U-87 MG cells were treated with Curcumin only as compared to the untreated U-87 MG cells. Quercetin pretreatment of U-87 MG cells decreased the ROS level (22%) when compared to untreated control U-87 MG cells. Quercetin pretreatment in GO and H_2O_2 stressed cells caused a decline in 33% and 27% ROS levels, respectively (Fig. 2).

Curcumin and Quercetin modulates the activity and levels of antioxidant enzymes in the oxidatively stressed U-87 MG cells In order to test the role of ROS in modulating the antioxidant enzymes and their levels, the moderate oxidative stress was induced by the oxidants in U-87 MG cells. Hypothetically the cell must respond to ROS by mounting the antioxidant enzymes to prevent the cellular oxidative stress. Naturally occurring phytochemicals (Curcumin and Quercetin) supplementation may boost the functionality of the antioxidant enzymes under oxidative stress influenced tumor microenvironment.

SOD1 activity and level Cytosolic SOD1 (Cu-Zn-SOD) activity was found to be increased when U-87 MG cells were exposed to GO, H_2O_2, Curcumin, and Quercetin treatment independently. In Curcumin and Quercetin pretreated, H_2O_2 stressed U-87 MG cells, there was an increase in the cytosolic SOD1 activity as compared with only H_2O_2 stressed U-87 MG cells. Whereas, there was a decrease in the activity of SOD1 in Curcumin and Quercetin pretreated GO stressed U-87 MG cells when compared only with that of GO stressed U-87 MG cells (Table 1). Using ELISA, we found that GO, H_2O_2, Curcumin, and Quercetin independent treatments in U-87 MG cells enhanced the SOD1 level in the total cell lysates. U-87 MG cells when pretreated with Curcumin/Quercetin followed by H_2O_2 and GO treatment elicited a significant increase in SOD1 level as compare to the untreated control U-87 MG cell lysates. An increase in SOD1 level was also observed upon Quercetin + H_2O_2 treatment and Quercetin + GO treatment in U-87 MG cells. Curcumin pretreatment in the H_2O_2 stressed U-87 MG cells caused 25% elevation in SOD1 level and Curcumin pretreatment in GO stressed U-87 MG cells had no effect on the SOD1 level when compared only with that of respective oxidant-stressed U-87 MG cells. Quercetin pretreatment caused an increase in SOD1 level in H_2O_2 stressed U-87 MG cells, as compared with that of only H_2O_2 stressed U-87 MG cells, and vice-versa results were observed in GO stressed U-87 MG cells (Fig. 3a). Taken together these results show that phytochemicals can enhance the level and activity of the antioxidant enzyme, SOD1 during oxidative stress.

SOD2 activity and level SOD2 (Mn-SOD) activity was found to be increased in the mitochondrial lysates when U-87 MG cells were exposed to GO, H_2O_2, Curcumin, and Quercetin. Curcumin and Quercetin pretreatment in H_2O_2 and GO stressed U-87 MG cells, induced an increase in SOD2 activity in the mitochondrial lysates when compared with only H_2O_2 and GO stressed U-87 MG cells (Table 1). However, H_2O_2, GO, Curcumin and Quercetin treatment in U-87 MG cells showed a decrease in SOD2 level as compared to the untreated control total

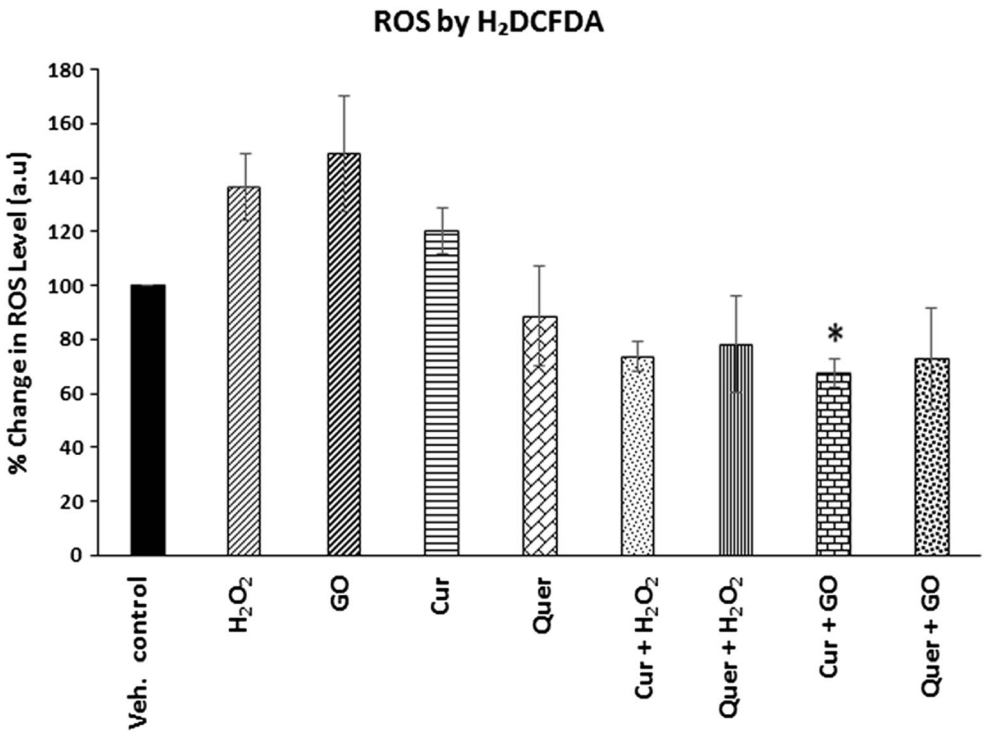

Fig. 2 The fluorescence intensity (%) change in U-87 MG cells when treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. The results are expressed as a mean ± standard deviation ($n = 3$)

Table 1 The CuZn-SOD activity in the cytosol and MnSOD activity in the mitochondrial samples of untreated control U-87 MG cells and in the oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations treated U-87 MG cells after 24 h time point

Treatments	CuZn-SOD Activity (Cytosol) (OD ± SD)	% Change in Activity (Cytosol)	MnSOD Activity (Mitochondria) (OD ± SD)	% Change in Activity (Mitochondria)
Veh. Control	1685 ± 7	100	1495 ± 49	100
H_2O_2	1740 ± 85	103	2700 ± 325*	180
H_2O_2 + Cur	1935 ± 70	115	2835 ± 35	189
H_2O_2 + Quer	1910 ± 42	113	2945 ± 35	197
GO	2040 ± 14**	121	2640 ± 240*	176
GO + Cur	1890 ± 42$^{\#}$	112	2770 ± 240	185
GO + Quer	1915 ± 21$^{\#}$	114	2685 ± 49	179
Cur	1980 ± 28**	117	2495 ± 176*	167
Quer	1930 ± 28	114	2965 ± 346*	198

The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $*p \leq 0.05$ and $**p \leq 0.005$ when untreated control cytosolic samples were compared with Curcumin and GO treated; and $\#p \leq 0.05$ when GO + C, GO + Q, H_2O_2 + C, H_2O_2 + Q treated cytosolic fractions were compared with only GO and H_2O_2 treated samples, respectively, for CuZn-SOD activity. The data was considered as statistically significant at $*p \leq 0.05$ when mitochondria of untreated cells were compared with H_2O_2 and GO treatments along with the phytochemicals Curcumin and Quercetin for MnSOD activity determination. The results are presented as showed in mean ± standard deviation ($n = 3$)

cell lysates from U-87 MG cells. Further, the SOD2 level in the pretreatment of phytochemical and followed by oxidants caused decrease by 14% in Curcumin + H_2O_2, 23% in Quercetin + H_2O_2, 19% in Curcumin + GO and 12% in Quercetin + GO in U-87 MG cells when compared to the untreated control U-87 MG total cell lysates. There was no significant effect of Curcumin pretreatment in the H_2O_2 stressed cells when compared only with H_2O_2 stressed U-87 MG cells. Quercetin pretreatment caused a decrease in SOD2 level in H_2O_2 and GO stressed U-87 MG cell lysates when compared with respective stressed U-87 MG cell lysates (Fig. 3b).

In-gel Mn-SOD activity In addition, the in-gel assay for Mn-SOD was also performed. H_2O_2, GO, Curcumin and Quercetin treatment in U-87 MG cells showed a significant increase in the Mn-SOD activity. In combination treatment groups Curcumin + H_2O_2, Quercetin + H_2O_2, and Curcumin + GO, there was a significant increase in Mn-SOD activity. However, it was recorded that no significant change in Mn-SOD activity in Quercetin + GO treated U-87 MG cells as compared with untreated control U-87 MG cells. Curcumin and Quercetin pretreatment in the H_2O_2 stressed U-87 MG cells resulted in an increase in the Mn-SOD activity

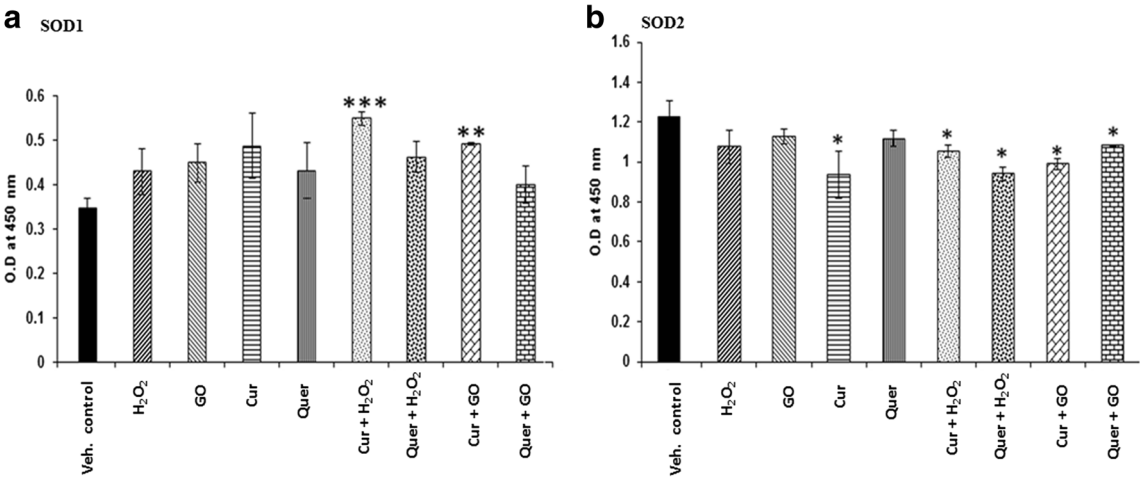

Fig. 3 (**a**) SOD1 levels were measured by ELISA in total cell lysates of the U-87 MG cells treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. The results are expressed as a mean ± standard deviation ($n = 3$). The data was statistically significant at $^{**}p = 0.005$–0.001 in Cur + GO treated samples and at $^{***}p < 0.001$ in Cur + H_2O_2 treated samples when compared with the untreated control U-87 MG total cell lysates. (**b**) SOD2 levels were measured by ELISA in total cell lysates of the U-87 MG cells treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Qurecetin; and their combinations. The results are expressed as a mean ± standard deviation ($n = 3$). The data was considered as statistically significant at $*p = 0.05$–0.005 when only phytochemical Quercetin and the oxidants + phytochemicals treated samples were compared with the untreated control U-87 MG total cell lysates

whereas in GO stressed U-87 MG cells showed a decrease in the Mn-SOD activity when compared to respective stressed U-87 MG cells (Fig. 4a, b). BSA was used as a standard marker which gives a band at ~66 kDa, and the Mn-SOD bands appeared slightly higher to it at ~88 kDa. Our results indicate that oxidative stress enhances the Mn-SOD activity and Curcumin acts a potent antioxidant as compared to Quercetin in enhancing the Mn-SOD activity during the oxidative stress.

CAT activity CAT activity was analyzed using in-gel assay using U-87 MG total cell lysates. H_2O_2, GO, Curcumin, and Quercetin treatment elevated the CAT activity. Also, in combination treatments viz.: Curcumin + H_2O_2, Quercetin + H_2O_2, Curcumin + GO, and Quercetin + GO showed an increase in the CAT activity as compared to the untreated control U-87 MG cells. Curcumin + H_2O_2 showed 75%, and Curcumin + GO showed 57% increase in the CAT activity when compared to the respective stressed U-87 MG cell lysates. CAT activity found to be increased by 36% in Quercetin + H_2O_2 and 52% in the Quercetin + GO when compared to the respective stressed U-87 MG cell lysates only (Fig. 5a, b). Our results indicate that CAT activity increases under the oxidative stress conditions and phytochemicals (Curcumin and Quercetin) can enhance the CAT activity.

Glutathione (GSH) level H_2O_2 and GO treatment in U-87 MG cells showed marginal decrease in GSH level. The Curcumin and Quercetin treatment showed increase in GSH levels in U-87 MG cells. Further, in combination treatment, GSH level was decreased by 24% in Curcumin + H_2O_2, 22% in Curcumin + GO, and 22% in Quercetin + GO when compared to control U-87 MG cells. A decrease in the level of GSH was found in Curcumin and Quercetin pretreatments in H_2O_2 and GO stressed U-87 MG cells when compared to respective oxidants stressed U-87 MG cells (Fig. 6).

Oxidative stress enhances NO production and induces NOS2 expression

NO level NO level was determined from the extracellular media of oxidatively stressed U-87 MG cells. There was a slight increase in the mean concentration of nitrite (measurement of extracellular NO produced) in H_2O_2, GO, Curcumin and Quercetin treated media of U-87 MG cells. In combination of Curcumin + H_2O_2 and Quercetin + H_2O_2 treatment, there was a decrease in the concentration of nitrite was observed when compared only with that of H_2O_2 stressed U-87 MG cells. Curcumin + GO showed significant decrease and Quercetin + GO showed a slight increase in NO levels when compared only with GO stressed U-87 MG cell media (Fig. 7a).

NOS2 expression The expression of inducible nitric oxide synthase (iNOS/NOS2) was analyzed using the immunocytochemistry. NOS2 level was found to be increased in the cytosolic subcellular localization upon oxidative stress induced by the GO and H_2O_2 as compared with untreated control U-87 MG cells. Whereas, GO induced a significant increase in expression of NOS2 in U-87 MG cells as compared to the H_2O_2

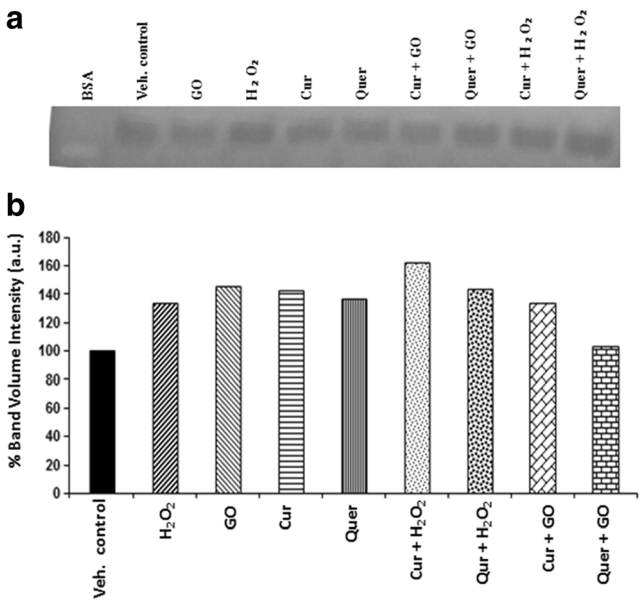

Fig. 4 (**a**) In-gel activity of SOD enzyme in U-87 MG total cell lysates made after the treatment with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. **b** The corresponding SOD activity intensities are represented as band intensities (a.u.)

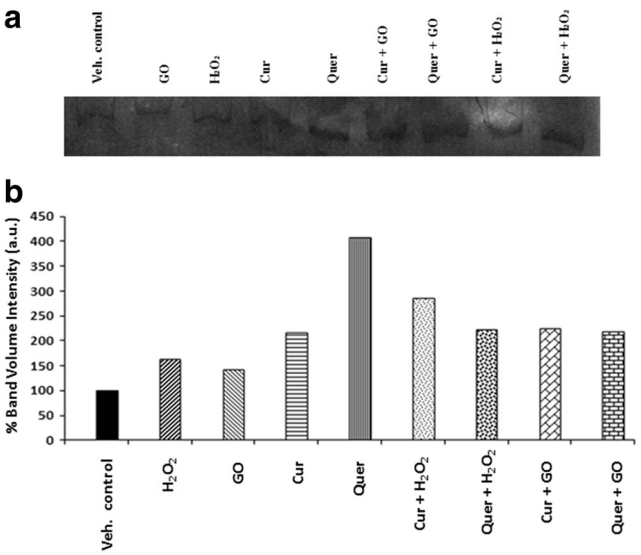

Fig. 5 (**a**) In-gel activity of CAT in U-87 MG total cell lysates made after the treatment with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. (**b**) The corresponding CAT band in native PAGE (250 kDa) are represented as band intensities (a.u.)

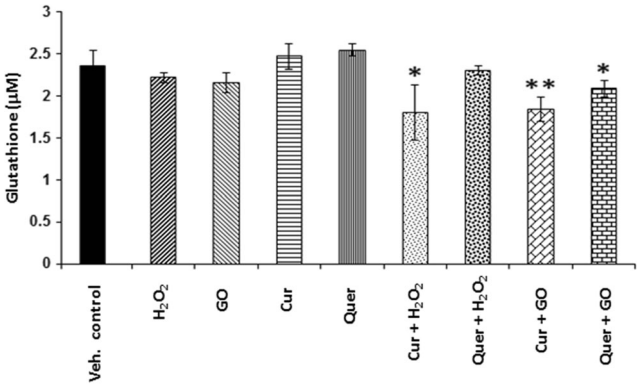

Fig. 6 Spectrophotometric measurement of GSH content in the total cell lysates of the untreated control U-87 MG cells, and the cells treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. The data was considered as statistically significant at $*p = 0.05–0.005$ in Cur + H_2O_2 and Quer + GO treated samples; and at $**p = 0.005–0.001$ in Cur + GO treated samples when compared with that of untreated control U-87 MG cells ($n = 3$)

induced expression (Fig. 7b). Our results indicate that NOS2 expression can ascertain the production of extracellular NO and intracellular NO [not determined, but literature supports this notion (Caneba et al. 2014; Glynn et al. 2010)] during the oxidative stress. However, the NO level was decreased in stressed U-87 MG cells treated with phytoprotectants.

Oxidative stress modulates cyclooxygenase (COX-2) expression H_2O_2 and GO treatment of U-87 MG cells showed 30% and 11% increase in COX-2 levels, respectively as measured using the total cell lysates by ELISA. The Curcumin and Quercetin treatment showed a decrease in COX-2 level in U-87 MG cells. Curcumin in combination with H_2O_2 and GO showed a decrease in COX-2 level. COX-2 level was increased in Quercetin + H_2O_2 and decrease in Quercetin + GO treated U-87 MG cells when compared to the untreated control U-87 MG cells. Curcumin pretreatment in H_2O_2 and GO stressed U-87 MG cells caused 40% and 14% decrease in COX-2 level, respectively, when compared with respective stressed U-87 MG cells. Quercetin pretreatment in H_2O_2 and GO stressed U-87 MG cells caused 12% and 36% decrease in COX-2 level as compared to the respective stressed U-87 MG cells (Fig. 8). Our results indicate that oxidative stress induces COX-2 level and phytochemical (Curcumin and Quercetin) supplementation, decreases its level during the oxidative stress conditions.

Oxidative stress stimulates DNA repair APE1 enzyme The increase in the APE1 protein expression by oxidative stress was confirmed by Western blot analysis. The result showed that expression level of APE1 protein enhanced by 2 folds in H_2O_2 and GO treated U-87 MG cells, compared with untreated U-87 MG cells (Fig. 9a). Curcumin and Quercetin treatment also enhanced the expression level of APE1 by 29% and 27%, respectively. Pretreatment of Curcumin followed by H_2O_2 treatment in U-87 MG cells resulted in 30% increase in APE1 expression whereas Curcumin pretreatment in GO stressed U-87 MG cells showed 35% decrease in the APE1 expression as compared to the respective stressed U-87 MG cells. Quercetin pretreated H_2O_2 stressed cells showed 43% decrease in the APE1 level and GO stressed cells pretreated with Quercetin showed 11% decrease in the APE1 level as compared to the respective stressed U-87 MG cells (Fig. 9a). APE1 expression induced by oxidative stress was further confirmed by

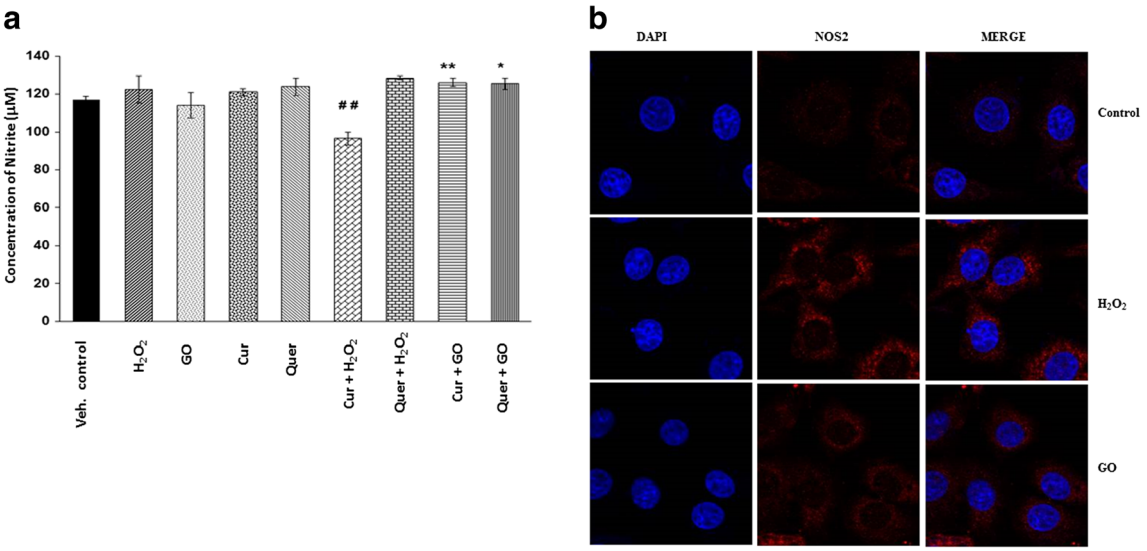

Fig. 7 (**a**) Spectrophotometric measurement of extracellular NO levels in the cell culture media of the untreated control U-87 MG cells, and the cells treated with oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations. The data was considered as statistically significant at $*p \leq 0.05$ and $**p \leq 0.005$ when untreated control cells were compared with H_2O_2 and GO treated cells. $^{\#\#}p \leq 0.005$ when GO + Curcumin treated cells were compared with only GO treated U-87 MG cells. (**b**) Localization of inducible nitric oxide synthase (iNOS/NOS2) after the treatment of oxidants H_2O_2 and GO by immunocytochemistry in the U-87 MG cells

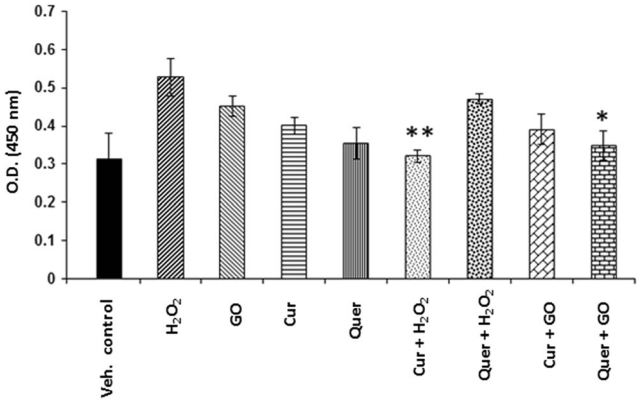

Fig. 8 The COX-2 levels in the total cell lysates of the untreated control U-87 MG cells, also the cells treated with the oxidants: 100 μM H_2O_2 and 10 μU/ml GO, phytochemicals (10 μM) such as Curcumin and Quercetin and their combinations. The data was considered as statistically significant at *p= 0.05–0.005 in Quer + GO samples and at **p= 0.005–0.001 in Cur + GO samples, when compared with only H_2O_2 and only GO-treated U-87 MG cells (n = 3)

immunocytochemistry which revealed that APE1 expression was increased upon GO and H_2O_2 treatment in the nucleus (predominantly) and localized to the subcellular sites in the cytosol as compared to the untreated control U-87 MG cells. GO was found to be more potent oxidant in inducing the expression of APE1 in the nuclear compartment for taking care of oxidized base damage repair via BER-pathway (Fig. 9b).

Discussion

Oxidative stress is the key player in the development and progression of cancer. Generation of ROS is a normal physiological process which helps in cellular signalling. However, altered production of ROS results in oxidative stress. The purpose of this study was to investigate the role of intracellular oxidants and modulatory role of phytochemicals toward the progression of GBM using glioblastoma U-87 MG cells as an experimental model in the moderate oxidative stress environment induced by the oxidants (GO and H_2O_2). The role of ROS is already well explored in various cancer studies. However, the role of dietary phytochemicals in oxidative stress environment and cellular consequences in GBM remains elusive. Due to the accelerated metabolism in cancer cells, there is more ROS production which is associated with the high proliferative rate of cancer cells (Sosa et al. 2013). In the present study, moderate oxidative stress was induced in the U-87 MG cells by using non-cytotoxic doses of H_2O_2 (100 μM) and GO (10 μU/ml). In the current study Curcumin and Quercetin in combination with the non-cytotoxic dose of H_2O_2 and GO were also used which enhanced the U-87 MG proliferation rate. Curcumin in combination with H_2O_2 significantly enhanced the PC-12 cellular proliferation (Siddiqui et al. 2010). Quercetin showed a protective effect after 10 μM concentration and did not affect the cellular viability during H_2O_2 induced oxidative stress condition in lymphocyte study (Duthie et al. 1997). Curcumin plays a potential therapeutic role in glioma and also reported to reduce U-87 MG viability (Dhandapani et al. 2007). Our data clearly indicates the role of low concentration of Curcumin (10 μM) towards the enhanced survival of U-87 MG cells through accelerating the antioxidant profile of U-87 MG cells. ROS is significantly reduced by pretreatment of Quercetin (10 μM) in *tert*-butyl hydroperoxide induced oxidative stress in HepG2 cells (Alía et al. 2006). Pretreatment of Quercetin enhanced the cellular viability of PC-12 cells in H_2O_2 induced oxidative stress (Heo and Lee 2004). Similarly in our study,

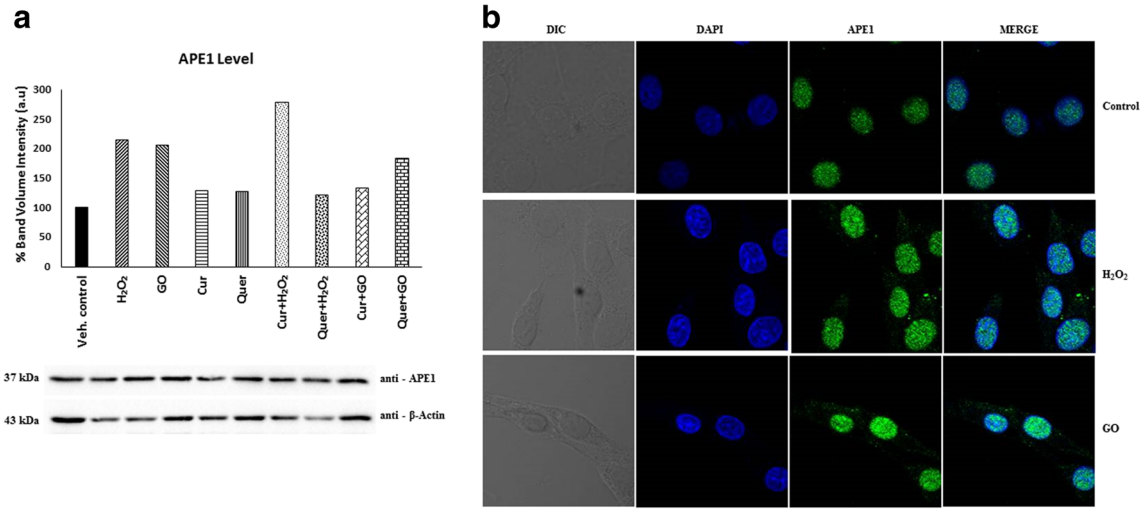

Fig. 9 (**a**) Western blot analysis of APE1's expression in the total cell lysates of the untreated control U-87 MG cells and in the oxidants: 100 μM H_2O_2 and 10 μU/ml GO; phytochemicals (10 μM) such as Curcumin and Quercetin; and their combinations treated U-87 MG cells after 24 h time point. (**b**) Expression analysis of multifunctional base excision repair enzyme, APE1 after the treatment of oxidants H_2O_2 and GO by immunocytochemistry in the U-87 MG cells

pretreatment of low concentration of Quercetin (10 μM) enhanced the survival of oxidatively stressed U-87 MG cells. SOD is the major antioxidant enzyme responsible for the elimination of superoxide radicles. SOD level was found to be increased in the oxidative environment as well as along with the pretreatment of phytochemicals (Curcumin and Quercetin). Past studies advocate the role of phytochemicals in cancer development and progression and indicating SOD as a potential useful anticancer drug target (Che et al. 2016). Mn-SOD is the major superoxide scavenger in the mitochondria. There is heterogeneity in the expression and activity of SOD2 in various cancer types which suggests for a differential regulation of Mn-SOD in different cancer cells (Dhar and St Clair 2012). Remarkably our findings are in accordance with the earlier done studies as to protect against H_2O_2 induced oxidative stress in epithelial cells as there is an upregulation of antioxidant enzyme transcript of GSH and CAT in H_2O_2 resistant epithelial cells (Carper et al. 2001). According to the literature, CAT activity has been shown to be higher in brain tumor tissue as compared to the normal tissue. CAT activity has been shown to be increased in Curcumin treated rats showing its effectiveness against glutamate neurotoxicity and oxidative stress (Kuo et al. 2011). Inhibiting the CAT leads to increase in the level of H_2O_2 (Sen et al. 2012). In our study the H_2O_2 and GO treated samples showed a significant increase in CAT activity. Further, the Curcumin and Quercetin treated samples also showed increase in the activity of CAT in comparison with the control U-87 MG cells. Antioxidant GSH has a pivotal role in the proliferation of tumor as combined inhibition of GSH and thioredoxin (TRX) antioxidant pathways resulted in synergistic cancer cell death [both *in vitro* and *in vivo*] (Harris et al. 2015). In the present study, GSH level was found to be decreased in the oxidatively stressed cells. Curcumin and Quercetin individually enhanced the GSH level, but the level was decreased in combination treatments. According to the previously done studies, Quercetin at lower concentration act as antioxidant and at higher concentration act as prooxidant. It decreases H_2O_2 induced oxidative stress in HepG2 cells (Kim and Jang 2009). Quercetin treatment resulted in decrease of intracellular H_2O_2 and a reduction in GSH content was also reported in human histiocytic lymphoma U937 cells (Ferraresi et al. 2005).

The NOS2 expression is involved in the inflammation process. In the current study, NO level was found to be increased in H_2O_2 and GO treated U-87 MG cells, which may be due to the stimulation of NOS2 in the stressful conditions. Also, there was an up-regulation in the cytoplasmic distribution of NOS2 during the oxidative stress in U-87 MG cells. These results are in accordance with the previously done studies that showed NOS2 expression was increased in the tumor cells (Ambs et al. 1998). Previously it was also suggested that μM concentration of H_2O_2 has greatly augmented the iNOS expression (Milligan et al. 1996). The COX-2 expression is associated with pro-inflammatory activities and cause chronic diseases (Minghetti 2004). Oxidative stress generated via reactive oxygen intermediates and H_2O_2 induces the expression of COX-2 (Feng et al. 1995; Park et al. 2009). In the present findings, COX-2 level was increased in oxidatively stressed U-87 MG cells which was decreased upon Curcumin and Quercetin pretreatment in the oxidatively stressed U-87 MG cells. According to the literature, Curcumin had been shown to markedly inhibit the COX-2 expression at both mRNA and protein level in HT-29 cells (Goel et al. 2001).

Oxidative stress elevates the level of APE1 and also its AP endonuclease activity (Silber et al. 2002). APE1 is the major multifunctional enzyme in the BER-pathway which not only involved in the repair of AP-sites but also regulates the expression and activation of various redox-regulated transcription factors via its redox effector factor (Ref-1) activity as reviewed (Bhakat et al. 2009; Tell et al. 2009; Thakur et al. 2014). In the current study GO, and H_2O_2-generated oxidative stress elevated the APE1 level, and treatment of phytochemicals (Curcumin and Quercetin) further increased the APE1 level. It can be said that increased ROS level is the induction factor for the enhanced expression of APE1 in U-87 MG cells. Nuclear and cytoplasmic APE1 expression was upregulated in tumor specimens as studied in the non-small lung cancer carcinoma (Yoo et al. 2008). It has been suggested that this nuclear-cytoplasmic translocation was dependent on NO induced S-nitrosation modification of APE1 at cysteine 93 and 310 residues (Qu et al. 2007). Similarly, present data also advocates for the increased NO level and enhanced nuclear as well as cytoplasmic distribution of APE1 in GO and H_2O_2 stressed U-87 MG cells. The results of this study suggest that increased ROS production in the U-87 MG cells altere the expression of antioxidant system and other associated enzymes necessary for the cancer cell survival.

Summary and conclusion

Aberrant metabolic activities in the cancer cells lead to excessive ROS production in the cancer microenvironment that can cause cellular damage or cell death. Cancer cells have to adapt to this environment to maintain the capacity for tumor progression. Cellular antioxidant system ameliorates the cellular microenvironment by scavenging the excessive ROS. The current study explores the role of oxidants such as H_2O_2 and GO induced oxidative stress which forces U-87 MG cells to rely on antioxidant enzymes to tackle the damaging effect of oxidative stress via modulating the level/activity of antioxidant machinery and explores the role of phytochemicals such as Curcumin and Quercetin at non-cytotoxic concentrations toward the survival of U-87 MG cells during the oxidative stress condition. In addition to this, also there is an up-regulation of other oxidative stress induced proteins such as NOS2, COX-2, and APE1. Their levels were also modulated

by the pretreatment of Curcumin and Quercetin during the stress environment. So, phytochemicals Curcumin and Quercetin have potent antioxidant properties and may have therapeutically advantage for the GBM patients if used along with the current therapies. Chemotherapeutic approaches should be designed using dietary phytochemicals that aim towards altering the tumor cell redox state via modulation of the antioxidant response system. Future research is required to gain additional insights into signalling networks which are altered during the oxidative stress in tumor microenvironment.

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Compliance with ethical standards

Conflict of interest Authors declare that no conflict of interest exists.

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