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Curcumin mediates time and concentration dependent regulation of redox homeostasis leading to cytotoxicity in macrophage cells

Amit Kunwar [a,*], Santosh K. Sandur [b], Malini Krishna [b], Kavirayani I. Priyadarsini [a]

[a] Radiation & Photochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India
[b] Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India

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ABSTRACT

The present study was designed to test a hypothesis that curcumin may be modulating oxidative stress parameters including reactive oxygen species, non-protein thiols and expression of antioxidant genes in a concentration and time dependent manner in exhibiting cytotoxic effects in macrophage cell line RAW 264.7. The results have shown that curcumin elevated the reactive oxygen species levels accompanied by a decrease in levels of intracellular non-protein thiols at 2 h after its addition to cells. However, the levels of reactive oxygen species decreased and non-protein thiols content increased at 18 h after its addition. Whereas the expression of *glutathione peroxidase* (*GPx*), *catalase*, *Cu,Zn-superoxide dismutase* (*Cu,Zn-SOD*) and *heme oxygenase-1* (*HO-1*) increased with curcumin concentration and also with increase in time of incubation, the expression of *Mn- superoxide dismutase* (*Mn-SOD*) showed concentration dependant repression upon treatment with curcumin. The cell viability was significantly reduced at high concentration (25 μM) of curcumin treatment but not at low concentration (5 μM). Curcumin at 5 μM scavenged γ-radiation induced reactive oxygen species and inhibited cell death. On the contrary, at 25 μM, curcumin increased radiation induced reactive oxygen species production and augmented cell death. Interestingly pretreatment with reducing agents glutathione (GSH) or N-acetyl-cysteine (NAC), modified the curcumin mediated redox changes and cell death differentially, due to the inhibition of cellular uptake of curcumin by GSH but not by NAC. The important finding of the study is that the concentration and time dependent dual effect of curcumin may be attributed to changes in oxidative stress and antioxidant gene expression levels leading to inhibition or promotion of cell death.

1. Introduction

Curcumin (Diferuloylmethane), a principal phenolic pigment present in turmeric, is used commercially as a spice/food colouring agent and has been widely employed in ancient Indian and Chinese medicine (Chattopadhyay et al., 2004; Aggarwal et al., 2007). Recent scientific research has confirmed that curcumin possesses anti-inflammatory, antibacterial, anti-amyloid properties, suppresses proliferation of a wide variety of tumor cells and also acts as an antioxidant in normal tissues and cells (Sharma, 1976; Aggarwal et al., 2003; Sharma et al., 2005; Shishodia et al., 2005; Singh and Khar, 2006; Rahman et al., 2006; Singh, 2007; Fiala et al., 2007). The extensive research on curcumin's chemical biology in the past one decade suggested that it effectively scavenged reactive oxygen species like peroxyl and hydroxyl radicals. It inhibited reactive oxygen species induced lipid peroxidation and up regulated expression of several phase II detoxifying enzymes via the activation of the antioxidant response element (Priyadarsini, 1997; Motterlini et al., 2000; Balogun et al., 2003; Iqbal et al., 2003; Rahman et al., 2006; Rushworth et al., 2006; Wei et al., 2006; Surh et al., 2008).

In contrast to these reports, curcumin has also been shown to promote oxidative stress in transformed cells in culture. These effects are associated with loss of mitochondrial functions and oxidative DNA damage resulting in apoptosis, which has been attributed as one of the mechanisms associated with the antitumor activity of curcumin (Bhaumik et al., 1999; Morin et al., 2001; Syng-Ai et al., 2004; Jung et al., 2005; Fang et al., 2005; Sandur et al., 2007a; Li et al., 2008). Recently it has been reported that curcumin scavenged reactive oxygen species at low concentrations and induced their production at high concentrations in HL-60 cells (Chen et al., 2005). These observations indicated that curcumin may exhibit both antioxidant and prooxidant effects depending on the cell type. However, the mechanism responsible for such differential effects of curcumin is not clearly understood. It is suggested that curcumin may exert beneficial or cytotoxic actions through modulation of cellular reactive oxygen species levels, non-protein thiols content and the antioxidants enzymes including superoxide dismutase (SOD) family, catalase, glutathione peroxidase (GPx) and heme oxygenase (HO-1) (Balogun et al., 2003; Matés, 2000; Han et al., 2005; Maclachlan et al., 2005; Surh et al., 2008). The present study, therefore, was designed to test a hypothesis that curcumin may be

* Corresponding author. Fax: +91 22 25505151.
E-mail address: kamit@barc.gov.in (A. Kunwar).

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modulating oxidative stress parameters including reactive oxygen species, non-protein thiols and expression of antioxidant genes in a concentration and time dependent manner and subsequently influencing cell viability in murine macrophage cell line RAW 264.7. Since glutathione (GSH) has been shown to chemically interact with curcumin through Michael addition reaction (Awasthi et al., 2000), the curcumin induced cellular modifications in presence of GSH or N-acetyl cysteine (NAC) was also studied. Further, the ability of curcumin to modulate γ-radiation-induced oxidative stress and cell death was investigated in these cells. The information gained from these studies would provide a line of evidence to support the existence of a concentration and time dependent differential changes in oxidative stress and antioxidant gene expression levels as part of the molecular mechanism leading to either beneficial or cytotoxic actions as caused by curcumin.

2. Materials and methods

2.1. Chemicals

Curcumin (CI 7500), GSH, NAC, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), diethyl pyrocarbonate (DEPC), tris base and Dulbecco's modified essential medium (DMEM) were purchased from Sigma Chemical Company, USA. Penicillin, streptomycin, fetal calf serum and reverse transcription–polymerase chain reaction (RT-PCR) kit were procured from Invitrogen, USA. Total RNA isolation kit was obtained from Roche Biochem, Germany. Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular probes, USA. High performance liquid chromatography (HPLC) grade dimethyl sulphoxide (DMSO) was procured from Spectro Chem (India). All the other chemicals with maximum available purity were purchased from reputed local manufacturers/suppliers. The purity of curcumin was confirmed by thin layer chromatography and HPLC**.** The gene specific primers for RT-PCR were custom synthesized from the local agents. The reagent solutions were prepared in nanopure water from a Millipore Milli-Q system just before the use. Absorption spectra were recorded on a JASCO V-530 spectrophotometer and fluorescence intensity was recorded on a Hitachi F-4010 fluorimeter. PCR was performed on Techne thermocycler (Model no. — TC512, UK).

2.2. Cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from National Centre for Cell Science, India. The cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin and maintained at 37 °C under 5% CO_2 and humidified air. The cells in DMEM medium were seeded (1×10^6 cells/ml) in six-well culture plates and kept overnight for attachment. The medium was changed before treating the cells with different reagents for desired time points. 20 mM stock solution of curcumin was prepared in DMSO and diluted with the culture medium to get the desired concentration. The maximum concentration of DMSO was within permissible limits of toxicity ($\leq$0.1%). Appropriate DMSO controls were taken in all the experiments.

2.3. Measurement of reactive oxygen species

Intracellular levels of reactive oxygen species were estimated using a cell permeable oxidation sensitive probe DCF-DA whose fluorescence intensity increases after oxidation to dichlorofluorescein (DCF) by reactive oxygen species. The cells treated with different agents were harvested at different time points by centrifugation at 900 ×*g* and were washed three times with cold phosphate buffer saline (PBS). The cells were incubated with oxidation sensitive DCF-DA (a final concentration of 10 μM) in PBS for 30 min at 37 °C (Sandur et al., 2007a; Hail, 2008). The cells were again washed three times with cold PBS to remove the extracellular probe and were lysed in PBS containing 1% Tween 20. The supernatant was assayed for DCF fluorescence (excitation at 480 nm and emission at 530 nm). Curcumin has absorption maximum at 420 nm; therefore it does not show considerable absorption at 480 nm. However, to avoid any interference due to emission from curcumin excitation to the fluorescence emission, all the reactive oxygen species measurements in presence of curcumin using DCF-DA probe were subtracted with appropriate control samples treated with curcumin without adding the probe. The representative values are expressed as mean fluorescence intensity at 530 nm.

2.4. Measurement of intracellular non-protein thiols content

The concentration of non-protein thiols content in cells was determined using DTNB according to the reported method (Sedlak and Lindsay, 1968; Kunwar et al., 2007). This assay is based on the principle of formation of yellow color by sulfhydryl groups (-SH) with DTNB. The cells were suspended in to 0.2 ml of 10 mM Tris/HCl, pH 7.4, and disrupted two times using a bioruptor (Cosmos Bio, Tokyo, Japan) at 200 W for 30 s each. Protein estimation in the cell lysate was carried out by DC protein assay kit (Bio-Rad, U.S.A). Further the cell lysate was precipitated using 10% trichloro acid, and the supernatant was mixed with 6 μM DTNB and incubated for 10 min and absorbance measured at 412 nm against appropriate control samples that were processed in parallel without adding the DTNB. The non-protein thiols content was expressed as nmol of reduced thiol per μg of protein. Although GSH forms the majority of DTNB reactive thiols, the small-molecular-weight thiols other than GSH too react with DTNB. Therefore, the total DTNB reactive thiols have been addressed as non-protein thiols throughout the text.

2.5. RNA isolation and RT-PCR

All pipette tips, Eppendorf tubes and glassware were treated with DEPC to destroy ribonucleases. Cells were harvested at different time points and washed three times with cold PBS. Total RNA was isolated using RNA isolation kit (Roche Biochem, Germany) and was eluted in 50 μl deionised DEPC-treated water (Kurrey et al., 2005). 2 μg of total RNA was used for the synthesis of cDNA by reverse transcription (cDNA synthesis kit, Invitrogen, USA). cDNA was amplified using 1 μl of the reaction products in 25 μl with 10 pmole of the primers for 35 cycles. Each cycle consisted of 30 s of denaturation at 94 °C, 30 s of annealing and 60 s of extension at 72°C. The primers used for cDNA amplification (forward and reverse respectively) were as follows:

Mn-SOD	5′-GCACATTAACGCGCAGATCA-3′
	5′-AGCCTCCAGCAACTCTCCTT-3′
Cu,Zn-SOD	5′-AAGGCCGTGTGCGTGCTGAA-3′
	5′-CAGGTCTCCAACATGCCTCT-3′
Catalase	5′-GCAG ATACCTGTGAACTGTC-3′
	5′-GTAGAATGTCCGCACCTGAG-3′
GPx	5′-CCTCAAGTACGTCCGACCTG-3′
	5′-GTAGAATGTCCGCACCTGAG-3′
HO-1	5′-AACAAGCAGAACCCAGTC-3′
	5′-TGTCATCTCCAGAGTGTTC-3′
β-actin	5′-TGGAATCCTGTGGCATCCATGAAAC-3′
	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′
Bcl-2	5′-TGCACCTGACGCCCTTCAC-3′
	5′-TAGCTGATTCGACCATTTGCCTGA-3′
Bcl-XL	5′-TGGTCGACTTTCTCTCCTAC-3′
	5′-GAGATCCACAAAAGTGTCCC-3′

β-actin was used as internal control in all the reactions. The band intensity was quantified by gelquant software (version 2.7 DNR imaging systems Ltd. Israel). Fold changes were calculated after normalization to β-actin.

2.6. Irradiation protocol

The curcumin treated and untreated cells were exposed to γ-radiation to an absorbed dose of 2 Gy using a ^{60}Co γ-source at a dose rate of 4 Gy min^{-1} as measured by standard Fricke dosimeter (Spinks and Woods, 1990). After irradiation, fetal calf serum was added at a concentration of 5% v/v and cells were incubated at 37 °C in humidified incubator with 5% CO_2 in air for different time intervals.

2.7. Measurement of cell viability

The viability of cells was determined by trypan blue dye exclusion test using a hemocytometer (Tolnai, 1975). For this experiment 2×10^4 untreated (control) or curcumin treated cells were added to each well of 24-well plates in one ml of tissue culture medium in triplicate. After 24 h of addition floating cells in the medium of each well were transferred to centrifuge tubes. Adherent cells were detached and were added to the corresponding floating cells before centrifugation. The pellet was suspended and 0.14% trypan blue was mixed with the same volume of cell suspension and 5 min later the stained (dead) and unstained (viable) cells were counted in hemocytometer. The cell viability for each group was calculated as percentage (%). % viability = (the number of living cells ÷ total number of cells including living and dead) × 100.

2.8. Cell uptake studies

The cellular uptake of curcumin was calculated using the method reported earlier from our lab (Kunwar et al., 2006, 2008). In brief cells were plated at ~1×10^6 cells/ml in 24 well plate and incubated with 20 μM of curcumin for 4 h. The cell pellet after washing with ice cold PBS, was suspended in to 500 μl of methanol and sonicated for 5 min. The absorbance of the supernatant at 428 nm, after centrifugation was recorded and using the molar absorption coefficient, the amount of curcumin taken up by cells was estimated. The results were normalized to 1 nmol of curcumin added/million cells/ml and cell uptake was expressed as pmol/million cells.

2.9. Statistical analysis

The results have been presented as mean ± S.E.M. for two independent experiments run in triplicates. Data were analyzed by one-way and two-way ANOVA of SPSS software (version 8.0.0) to confirm the variability of data and for multiple comparisons of means. P values ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Biphasic effects of curcumin on reactive oxygen species levels and non-protein thiols content

Fig. 1A and B show the representative fluorescent intensities of DCF indicating levels of reactive oxygen species at 2 and 18 h after curcumin (1–25 μM) addition to cells. Curcumin increased reactive oxygen species levels steadily up to 25 μM concentration at 2 h after addition to cells. However the reactive oxygen species levels dropped below the control levels at 18 h after addition. The levels of non-protein thiols content were measured at the same time points after curcumin addition and it indicated that the basal levels of non-protein thiols content (0.251 ± 0.015 nmol/μg of protein) decreased at 2 h (Fig. 1C). Interestingly, at later time point (18 h) the levels of non-protein thiols increased significantly with increasing concentration (1–25 μM) of curcumin (Fig. 1D).

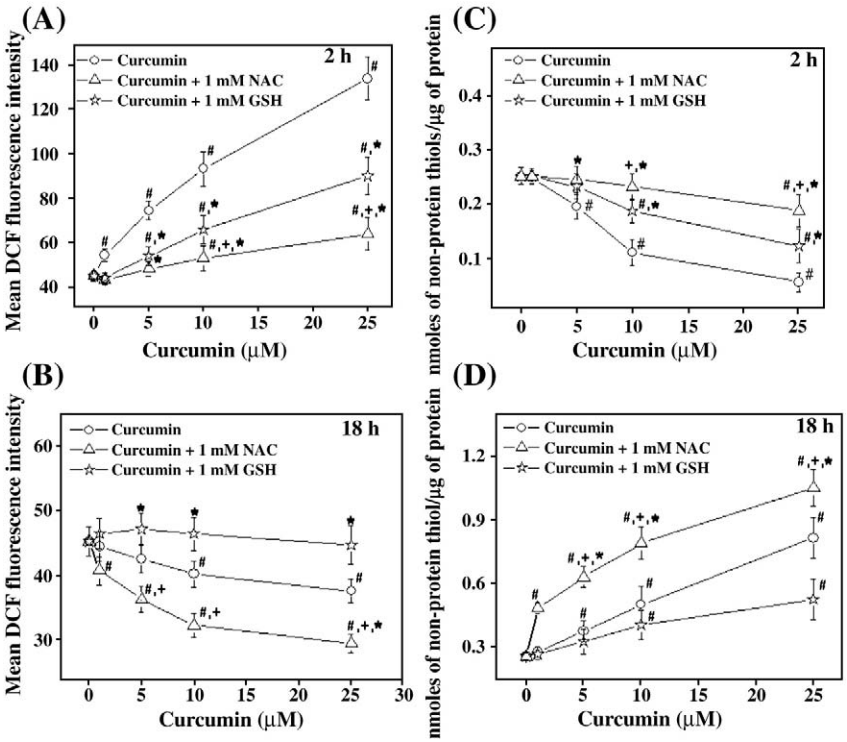

Fig. 1. Effect of curcumin (1–25 μM) on reactive oxygen species levels and non-protein thiols content at two different timepoints 2 h (A & C) and 18 h (B & D) after its addition and modulation by NAC (1 mM) or GSH (1 mM). The GSH or NAC was added to cell culture 2 h prior to addition of curcumin. Results presented as mean ± S.E.M. for $n=3$. $^{\#}P<0.05$ as compared to reactive oxygen species or non-protein thiols levels of control cells.$^{*}P<0.05$ as compared to reactive oxygen species or non-protein thiols levels of cells treated with curcumin at a particular concentration. $^{+}P<0.05$ as compared to reactive oxygen species or non-protein thiols levels of cells treated with curcumin at a particular concentration in presence of GSH.

Taken together these results suggest that curcumin modified the redox environment within cells in a concentration and time dependent manner. Further to know whether curcumin mediated changes in redox environment in cells could be restored by the addition of water soluble antioxidants like GSH or NAC, the cells were treated with 1 mM concentration of NAC or GSH for 2 h prior to addition of curcumin (1–25 μM). The reactive oxygen species levels and non-protein thiols content were measured at 2 and 18 h respectively. Exogenous addition of GSH or NAC prior to curcumin addition significantly decreased the reactive oxygen species generation at 2 h (Fig. 1A). The NAC was more effective than GSH in this respect. At later time point (18 h) the NAC and curcumin treated cells showed lower reactive oxygen species levels than the cells treated with curcumin alone (Fig. 1B). Similarly, pretreatment with NAC or GSH abrogated curcumin mediated decrease in the levels of non-protein thiols at early time point (2 h) (Fig. 1C). However, at a later time point (18 h), NAC potentiated the curcumin mediated increase in non-protein thiols levels while GSH decreased it (Fig. 1D). Thus NAC and GSH had an opposing effect on curcumin-mediated changes in levels of non-protein thiols at 18 h. The group treated with GSH alone did not bring down basal levels of reactive oxygen species. However, non-protein thiols content increased marginally from basal levels of 0.251 ± 0.015 nmol/μg of protein to 0.264 ± 0.018 nmol/μg of protein at 18 h. Interestingly, addition of NAC alone reduced basal levels of reactive oxygen species by 11% at 2 h and by 25% at 18 h, while non-protein thiols content increased to 0.371 ± 0.049 nmol/μg of protein at 2 h and 0.568 ± 0.085 nmol/μg of protein at 18 h compared to basal levels.

3.2. Effects of curcumin on the expression of antioxidant genes

Experiments were carried out to monitor the effects of low (5 μM) and high (25 μM) concentrations of curcumin on cellular antioxidant mechanisms. Antioxidant enzymes such as Mn-SOD, Cu,Zn-SOD, catalase, GPx and HO-1 play an important role in maintaining the redox homeostasis within the cells. Therefore expression of these enzymes at mRNA level was monitored in the cells at 6 and 18 h after curcumin addition. Curcumin upregulated the mRNA levels of *GPx*, *catalase* and *Cu,Zn-SOD* genes over the control in a concentration and time dependent manner with peak levels observed at 18 h after addition (Fig. 2A–D). The relative increase in the expression of *GPx* gene upon treatment with 5 and 25 μM curcumin was significantly higher as compared to increase in the expression of *catalase* and *Cu, Zn-SOD* genes. Although *HO-1* gene also showed an increase in the levels of mRNA with increase in concentration of curcumin from 5 to 25 μM, the peak level was observed at 6 h instead of 18 h (Fig. 2A–D). In contrast, the *Mn-SOD* gene showed time dependent decrease upon treatment with 5 and 25 μM curcumin (Fig. 2A–D).

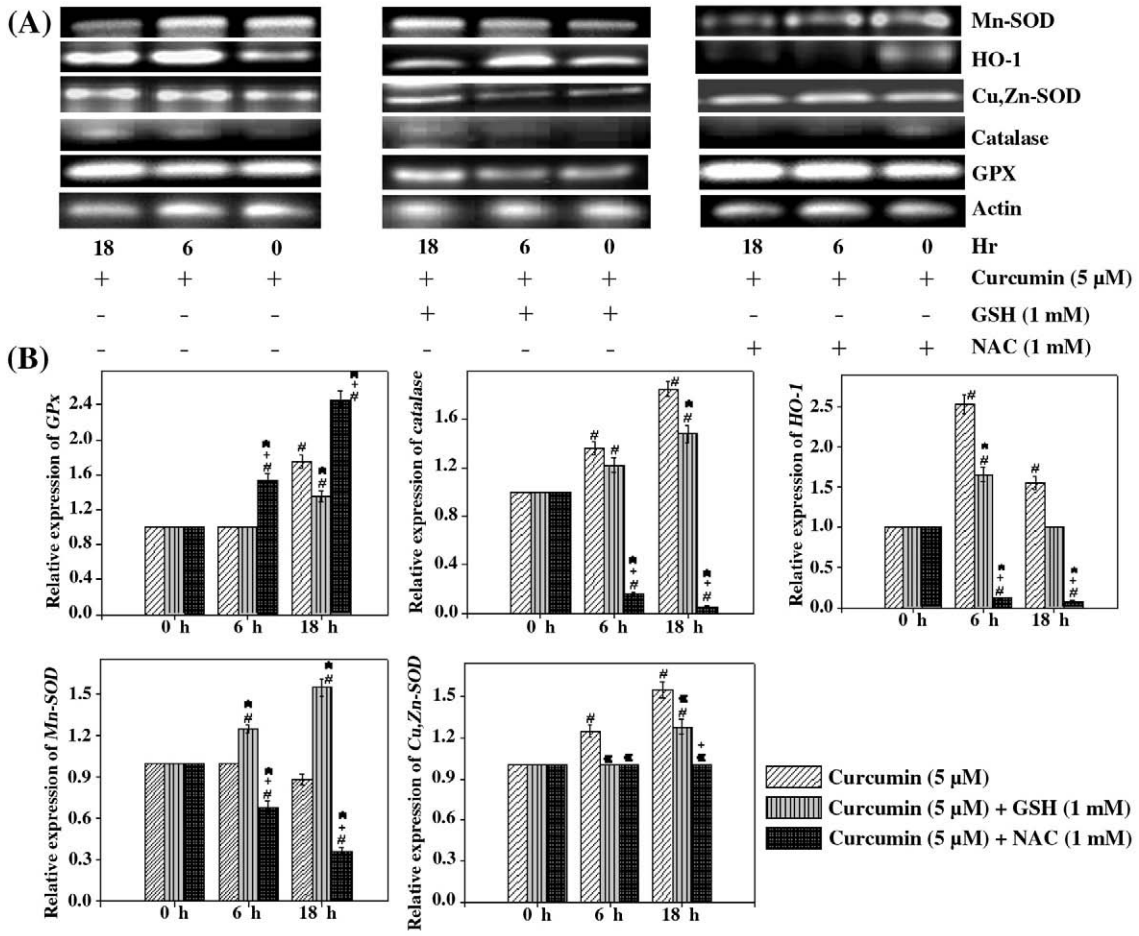

Fig. 2. Effect of curcumin concentration on the mRNA expression of antioxidant genes (*GPx*, *catalase*, *HO-1*, *Cu,Zn-SOD* and *Mn-SOD)* at 6 and 18 h after its addition and modulation by NAC (1 mM) or GSH (1 mM). (A) RT-PCR analysis at 5 μM curcumin in absence and presence of NAC or GSH. (B) Bar graph showing time dependant variation in the relative expression of the genes at 5 μM curcumin and in combination with NAC or GSH. (C) RT-PCR analysis of the genes at 25 μM curcumin both in absence and presence of NAC or GSH. (D) Bar graph showing time dependant variation in the relative expression of the genes at 25 μM curcumin and in combination with NAC or GSH. The GSH or NAC was added to cell culture 2 h prior to addition of curcumin. β-actin mRNA expression was used as an internal control. Results presented as mean ± S.E.M. for $n = 3$. $^{\#}P<0.05$ as compared to mRNA expression levels of control cells. $^{*}P<0.05$ as compared to mRNA expression levels of cells treated with curcumin at a particular timepoint. $^{+}P<0.05$ as compared to mRNA expression levels of cells treated with curcumin at any time point in presence of GSH.

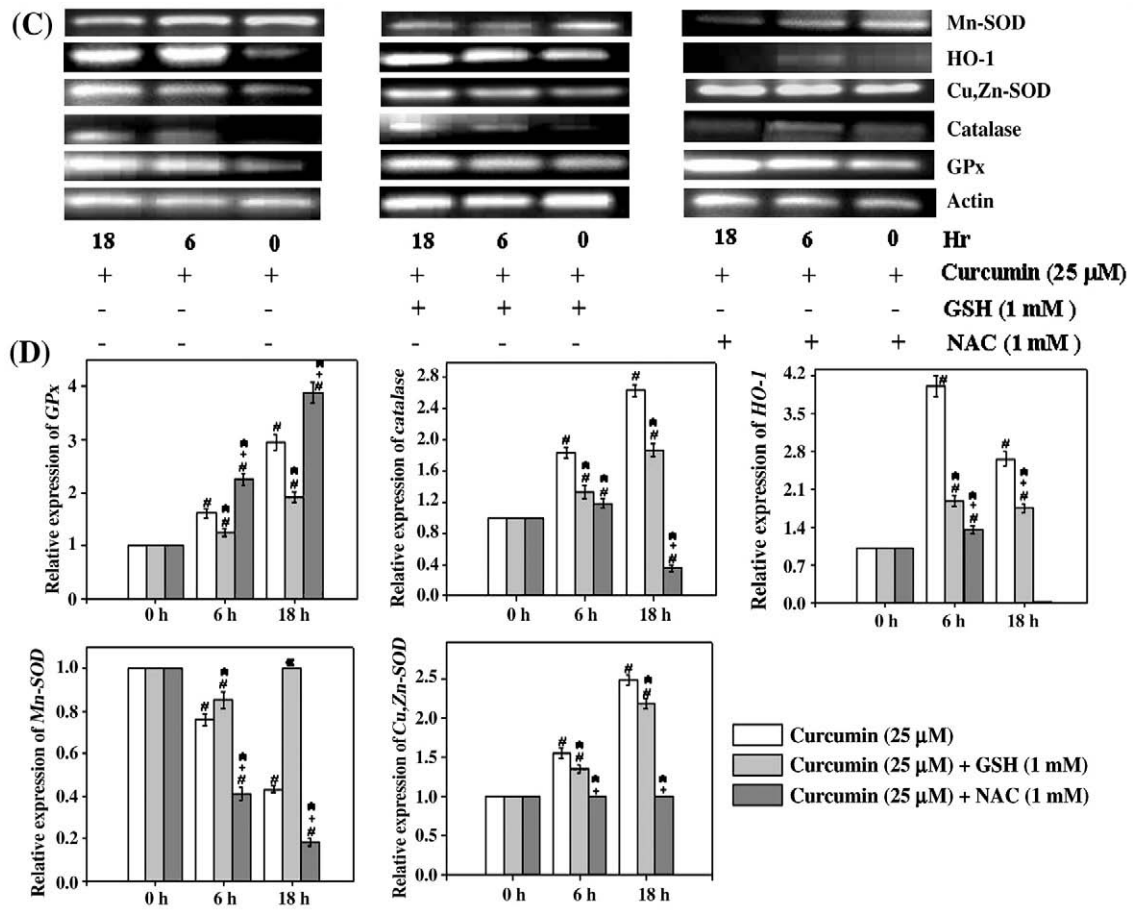

Fig. 2 (*continued*).

Pretreatment of cells with 1 mM GSH or NAC for 2 h modulated the curcumin's effect on gene expression. Pretreatment with GSH reduced the magnitude of induction in the expression of *GPx*, *catalase*, *Cu,Zn-SOD* and *HO-1* genes at both the time points (Fig. 2A–D). Interestingly, GSH also restored the suppression of *Mn-SOD* gene expression after treatment with curcumin (5 and 25 μM) (Fig. 2A–D). On the other hand cells pretreated with NAC showed an increase in the expression of *GPx* gene and decrease in the expression of *catalase, Cu,Zn-SOD, HO-1* and *Mn-SOD* genes at 6 and 18 h as compared to curcumin treatment alone (Fig. 2A–D). These results indicated a differential effect of GSH and NAC on curcumin induced gene expression in RAW cells. The group treated with GSH alone did not show changes in the expression of all the five antioxidant genes at 6 and 18 h compared to control levels (supplementary data). However, the group treated with NAC showed significant upregulation of *GPx* at 18 h, repression of *Mn-SOD* and *HO-1* at both timepoints. However, the expression of *catalase* and *Cu,Zn-SOD* was comparable to control levels (Supplementary data).

3.3. Effect of curcumin on cell viability

To investigate, how changes in the redox environment induced by curcumin affect cell viability, cells were incubated with curcumin (5 and 25 μM) for 24 h. The % cell viability under different treatment conditions has been shown in Fig. 3A. The figure clearly reveals that treatment at 5 μM curcumin resulted marginal decrease in cell viability while that at 25 μM showed significant reduction. Addition of GSH or NAC resulted in the complete recovery of viability in cells treated with 5 μM of curcumin. However, at 25 μM of curcumin, NAC offered better protection than GSH. Treatment with NAC or GSH alone did not induce loss of cell viability. Further mRNA expression levels of antiapoptotic genes such as *Bcl-2* and *Bcl-XL* were measured at 18 h after curcumin addition. The results showed that significant decrease in the mRNA expression levels of *Bcl-2* and *Bcl-XL* genes was seen only at 25 μM curcumin (Fig. 3B and C). However, treatment with 1 mM NAC or GSH prior to curcumin (5 and 25 μM) addition abrogated curcumin mediated decrease in expression of antiapoptotic genes and NAC was better than GSH in its action (Fig. 3B and C). The group treated with GSH alone did not change the mRNA expression levels of *Bcl-2* and *Bcl-XL*, while NAC showed significant upregulation of these genes (Fig. 3B and C).

3.4. Effect of GSH or NAC on cellular uptake of curcumin

In order to understand the mechanism responsible for differential effects of NAC or GSH on curcumin induced redox changes, uptake of curcumin in cells in presence of GSH or NAC was estimated. The cells were incubated with 20 μM of curcumin in presence or absence of 1 mM GSH or NAC for 4 h. Fig. 4A shows the absorption spectrum of intracellular curcumin and Fig. 4B shows relative uptakes in pmol/million cells under different treatment conditions. The basal level of cellular uptake of curcumin was about 17.5 ± 3.1 pmol/million cells. In the presence of NAC, the cellular uptake was marginally decreased to 14.7 ± 2.8 pmol/million cells. However, in the presence of GSH, it was significantly reduced to 8.75 ± 2.3 pmol/million cells. These results suggest that pretreatment with GSH inhibited the cellular uptake of curcumin, while NAC was less effective.

3.5. Concentration dependent response of curcumin to γ-irradiation

To investigate the concentration dependant differential antioxidant and pro-oxidant activity, the curcumin (5 and 25 μM) treated

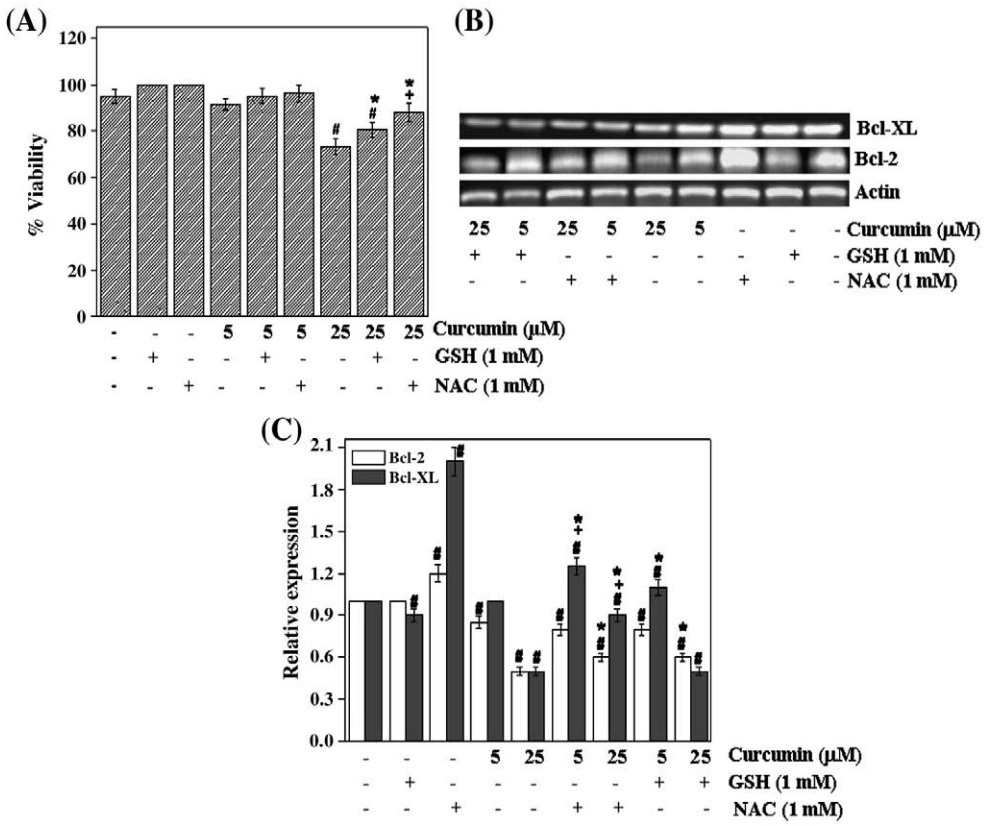

Fig. 3. Effect of curcumin (5 or 25 μM) in combination with NAC (1 mM) or GSH (1 mM) on cell viability. (A) Bar graph showing variation in the cell viability. (B) RT-PCR analysis showing comparative expression of antiapoptotic genes *Bcl-2* and *Bcl-XL* in cells. (C) Analysis of the relative expression of above genes under different treatment conditions. The GSH or NAC was added to cell culture 2 h prior to addition of curcumin. β-actin mRNA expression was used as an internal control. Results presented as mean ± S.E.M. for $n = 3$. $^{\#}P<0.05$ as compared to control cells. $^{*}P<0.05$ as compared to cells treated with curcumin. $^{+}P<0.05$ as compared to cells treated with curcumin in presence of GSH.

cells were exposed to γ-radiation (absorbed dose, 2 Gy) and examined for the reactive oxygen species levels at 2 h, expression of anti-apoptotic markers such as *Bcl-2* and *Bcl-XL* at 18 h and cell viability at 24 h. Fig. 5A shows the intracellular reactive oxygen species levels under different treatment conditions. Exposure of cells to γ-radiation elevated reactive oxygen species levels, which was marginally lowered in presence of 5 μM curcumin, while at 25 μM curcumin, the reactive oxygen species levels significantly increased. However, when cells were treated with curcumin (5 and 25 μM) in combination with 1 mM GSH or NAC, the radiation induced reactive oxygen species levels decreased significantly. NAC was again more effective than GSH. Further, the mRNA levels of *Bcl-2* and *Bcl-XL* and cell viability, assessed under the same conditions showed that γ-radiation suppressed the expression of anti-apoptotic genes and reduced the cell viability (Fig. 5B, C and D). Curcumin at 5 μM partially inhibited the radiation-mediated repression of *Bcl-2* and *Bcl-XL* genes while 25 μM of curcumin further suppressed radiation induced abrogation of these genes (Fig. 5B and C). Similar changes in cell viability were observed when cells treated with curcumin (5 and 25 μM) were exposed to radiation (Fig. 5D). The treatment with NAC or GSH prior to

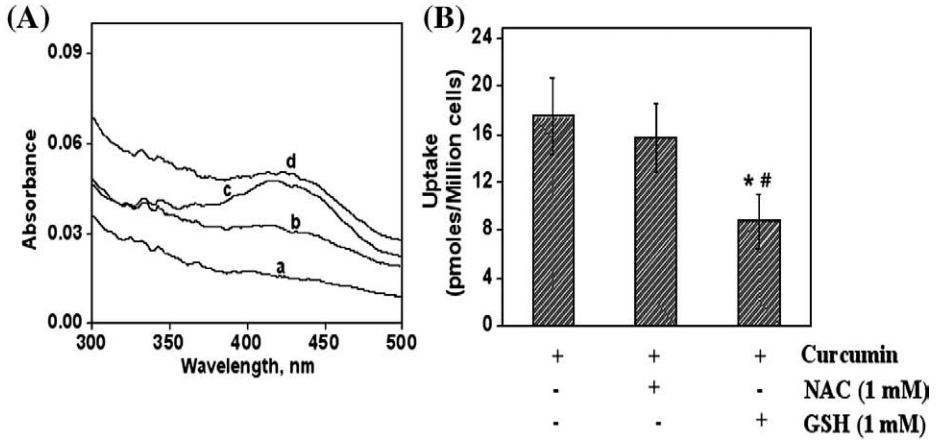

Fig. 4. Effect of NAC (1 mM) or GSH (1 mM) on cellular uptake of curcumin: (A) Absorption spectra of methanolic curcumin from cells (a) control cells (b) cells treated with 20 μM curcumin and GSH (c) cells treated with 20 μM curcumin and NAC (d) cells treated with 20 μM curcumin only. (B) Bar graph showing relative cellular uptake under various treatment conditions as mentioned above in pmol/million cells after normalization to 1 nmol of curcumin added/million cells/ml. Results presented as mean ± S.E.M. for $n = 3$. $^{*}P<0.05$ as compared to curcumin treated group. $^{\#}P<0.05$ as compared to curcumin and NAC treated group.

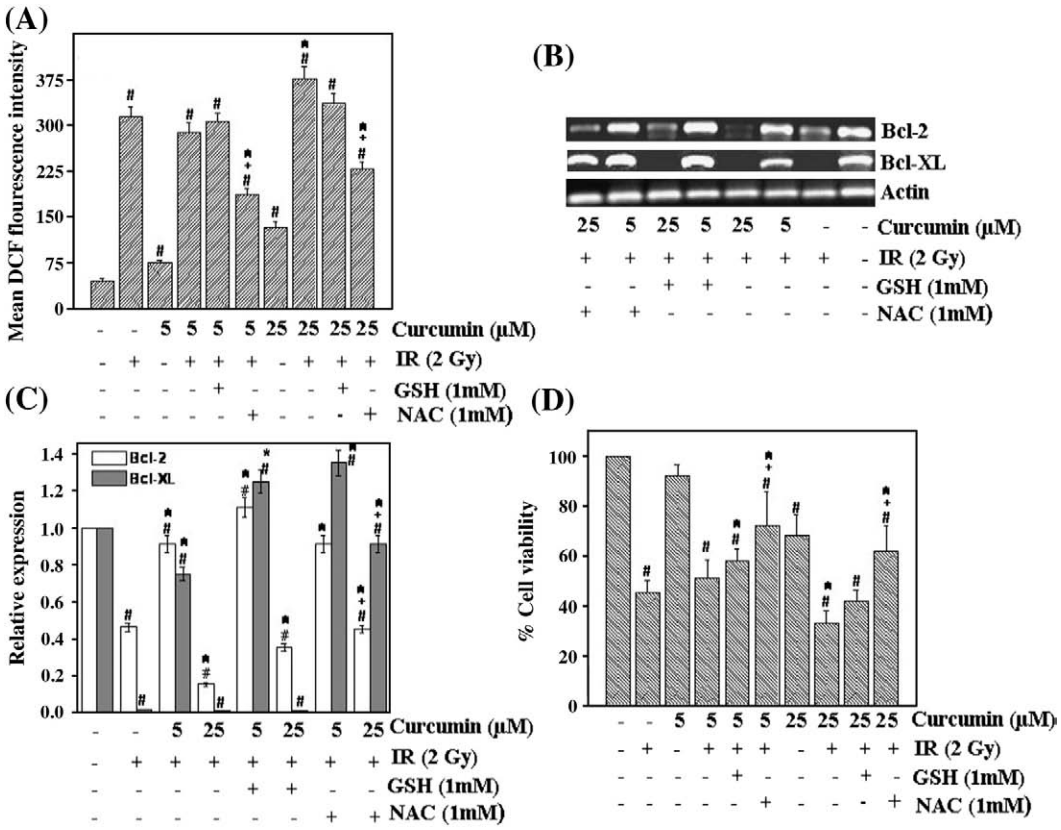

Fig. 5. Effect of curcumin pretreatment at 5 or 25 μM concentration in combination with NAC (1 mM) or GSH (1 mM) on γ-radiation (absorbed dose, 2 Gy) induced oxidative stress in cells. (A) reactive oxygen species levels. (B) RT-PCR analysis showing comparative expression of antiapoptotic genes *Bcl-2* and *Bcl-XL*. (C) Analysis of the relative expression of the above genes under different treatment conditions. (D) Bar graph showing variation in the cell viability. β-actin mRNA expression was used as an internal control. Results presented as mean ± S.E.M. for $n = 3$. $^{\#}P<0.05$ as compared to control cells. $^{*}P<0.05$ as compared cells treated with curcumin. $^{+}P<0.05$ as compared to cells treated with curcumin in presence of GSH.

curcumin addition and exposure to γ-radiation partially inhibited the radiation-mediated decrease in expression of antiapoptotic genes and loss of viability in cells and between the two NAC was better than GSH.

4. Discussion

The circulating monocytes are continuously exposed to oxidative stress and play a key role in inflammation (Rushworth et al., 2006). Curcumin, a well-known antioxidant molecule from the rhizomes of *Curcuma longa* exerts antioxidative and anti-inflammatory effects in monocytes and other cell types and presently in different stages of clinical trials as antitumor agent (Aggarwal et al., 2003; Rushworth et al., 2006; Aggarwal et al., 2007). Besides its antioxidative effects, there is also an increasing evidence that curcumin exhibits pro-oxidative toxicity in proliferating or tumor cells (Bhaumik et al., 1999; Morin et al., 2001; Syng-Ai et al., 2004; Jung et al., 2005; Fang et al., 2005; Sandur et al., 2007a; Li et al., 2008). Therefore it is essential to understand the conditions under which curcumin acts as an antioxidant or pro-oxidant in macrophage cells. In order to address this problem, the present studies were performed by monitoring the changes in cellular redox environment in a concentration and time dependent manner in RAW 264.7 cells. GSH is the major intracellular reducing agent present in millimolar concentrations that protects cells from the oxidants. Exogenously added GSH is cell impermeable (Schafer and Buettner, 2001). Therefore to mimic intracellular concentrations of GSH and to investigate the influence of exogenous GSH on curcumin uptake by cells and its redox modulating ability, the cells were treated with 1 mM concentration of GSH prior to curcumin addition. Since NAC is another thiol containing reducing agent which is permeable to cell and exerts antioxidant effects by increasing intracellular GSH contents, the effect of NAC on curcumin induced modifications was also investigated at equimolar concentration (Hoffer et al., 1997).

Treatment of cells with curcumin (1–25 μM) elevated the DCF fluorescence (reactive oxygen species levels) at 2 h in a concentration dependent manner, similar to the earlier observations (Sandur et al., 2007b). This increase in reactive oxygen species levels at 2 h was also accompanied by decrease in the levels of intracellular non-protein thiols indicating that curcumin provoked oxidative stress early after its addition. Interestingly, we observed that reactive oxygen species levels decreased below basal levels at 18 h after curcumin addition suggesting that the pro-oxidant activity of curcumin was only transient. The mechanism by which curcumin induces reactive oxygen species is not clear. However, it has been proposed that curcumin binds to thioredoxin reductase, thus converting its activity to NADPH oxidase (Fang et al., 2005), which leads to the production of reactive oxygen species. The most important intracellular antioxidant GSH constitutes major non-protein thiols in cells (Sedlack and L'Hanus, 1982; Dickinson and Forman, 2002). The decreased levels of non-protein thiols at early time point (2 h) after curcumin addition may be due to oxidation of GSH to GSSG by reactive oxygen species or due to formation of Michael adduct between GSH and curcumin. However when low concentration of curcumin is available within cells due to poor cellular uptake, reactions of curcumin with GSH are not likely to play a prominent role in lowering non-protein thiols content. The incubation of cells with curcumin for 18 h led to significant increase in levels of non-protein thiols in a concentration dependent manner. The observed increase in non-protein thiols content also ruled out the possibility of formation of Michael adduct of curcumin with GSH in cells, since this reaction would also tend to decrease the non-protein

thiols content. One of the reasons responsible for these observations appear to be the oxidative stress mediated depletion of GSH leading to induction of its intracellular synthesis (Sedlack and L'Hanus, 1982). Additionally curcumin is also reported to increase the biosynthesis of GSH by stimulating the γ-glutamate–cysteine ligase activity (Biswas et al., 2005; Rushworth et al., 2006; Strasser et al., 2005).

Our investigations on the expression of antioxidant enzymes at 6 and 18 h after treatment with 5 or 25 μM curcumin, showed induction of *GPx*, *catalase*, *HO-1* and *Cu,Zn-SOD* genes with increasing time and concentration. Although the exact mechanism(s) by which curcumin activates the expression of these genes is not clearly understood, the observed increase in expression of some of the antioxidant genes like *GPx* and *HO-1* after curcumin addition to cells may be attributed to activation of transcription factor Nrf-2 as reported by Surh et al. (2008). These time course studies reveal that antioxidant effects precede pro-oxidant effects of curcumin. These studies also indicate that the reduced reactive oxygen species levels at 18 h after curcumin addition to cells may be due to elevated *GPx*, *Cu,Zn -SOD* expressions and non-protein thiols levels in the present study.

A striking observation of the present study is that curcumin reduced the *Mn-SOD* gene expression. The transcription factor NF-κB regulates the expression of *Mn-SOD* gene (Murley et al., 2006) and curcumin is a known suppressor of basal and induced NF-κB levels (Shishodia et al., 2005; Duvoix et al., 2005; Sandur et al., 2007a; Everett et al., 2007). Therefore the observed decrease in the *Mn-SOD* expression levels upon curcumin treatment can directly be attributed to inhibition of NF-κB.

The assessment of cell viability at 24 h revealed that curcumin did not induce loss of viability at 5 μM, however lower but significant loss of viability was observed at 25 μM. These results suggest that despite increased expression of antioxidant genes at 25 μM of curcumin, the cells did not survive. The loss of cell viability after treatment at 25 μM curcumin was also evident in terms of the decrease in the expression of antiapototic genes such as *Bcl-2* and *Bcl-XL*, which are required for cell survival. This observed loss of viability may be due to irreversible damage caused by increased production of reactive oxygen species at early time point (2 h), decreased *Mn-SOD* expression and induction of apoptosis. Earlier reports support this argument that increased reactive oxygen species levels leads to cell death and Mn-SOD is essential to protect the cells from mitochondrial oxidative stress (Lebovitz et al., 1996; Liu et al., 2005).

Further the effects of exogenously added reducing agents on curcumin-mediated cellular modifications were studied by pre-treating the cells with GSH or NAC. GSH inhibited curcumin induced oxidative stress and antioxidant effects at 2 and 18 h respectively. However, NAC inhibited the pro-oxidant effects of curcumin at early time point (2 h) and augmented the non-protein thiols levels at 18 h. NAC is a cell permeable reducing agent and acts as a precursor for the synthesis of intracellular GSH (Hoffer et al., 1997). This may be the reason for the additive effect of NAC on curcumin-mediated increase in non-protein thiols levels at 18 h. GSH also lowered the curcumin induced expressions of *GPx*, *Cu,Zn-SOD* and *HO-1* genes and increased the expression of *Mn-SOD* gene. While, NAC increased the curcumin induced expression of *GPx* and reduced the expression of all the other genes examined. The enhanced expression of *GPx* in the presence of NAC may be due to increased intracellular synthesis of GSH (Vivancos and Moreno, 2005). Pretreatment with equimolar (1 mM) concentration of GSH or NAC inhibited curcumin induced cell death however; NAC was more effective than GSH. This differential action could be due to their ability to inhibit cellular uptake of curcumin as GSH is impermeable to cells (Schafer and Buettner, 2001). Our experiments indeed showed that GSH significantly inhibited the cellular uptake of curcumin; however, pretreatment with NAC did not affect curcumin uptake by the cells. The mechanism(s) by which exogenously added GSH prevents the cellular uptake of curcumin is not known. Probably Michael addition reaction of curcumin with GSH in the cell culture medium may be responsible for this observation (Awasthi et al., 2000; Usta et al., 2007).

Studies on the effects of curcumin in combinations with GSH or NAC against γ-radiation induced oxidative stress in RAW cells showed interesting results. At 5 μM, curcumin decreased radiation induced reactive oxygen species production at 2 h and inhibited the loss of cell viability at 24 h only marginally. However, at 25 μM, curcumin significantly enhanced both radiation induced reactive oxygen species production and loss of cell viability. The protective effects at 5 μM were in correlation with the restoration of γ-irradiation induced decrease in *Bcl-2* and *Bcl-XL* expressions, while at 25 μM it completely abolished their expression. As expected, both GSH and NAC enhanced the radioprotective action of curcumin at 5 μM and inhibited the sensitizing activity at 25 μM.

In conclusion, the present studies confirm that both concentration and time of incubation with curcumin differentially modulate levels of reactive oxygen species, non-protein thiols and mRNA expression levels of antioxidant genes in RAW 264.7 cell. Curcumin mediated pro-oxidant action and decrease in *Mn-SOD* expression levels soon after its addition may be responsible for the loss of cell viability at higher concentrations. Reducing agents, GSH or NAC differentially modulated the activity of curcumin and its uptake. Thus, combination of low concentrations of curcumin with reducing agents is a better strategy to improve the antioxidant levels in cells. These studies would also help in the development of new curcumin treatment regimes for its therapeutic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.03.060.

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