Curcumin Attenuates Hydrogen Peroxide-Induced Cytotoxicity in Human Neuroblastoma SK-N-SH Cells

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ABSTRACT

Objective: Cellular damage induced by oxidative stress has been involved in the development of neurodegeneration. Curcumin, a dietary polyphenol found in the rhizome of *Curcuma longa*, has been shown, both *in vitro* and *in vivo*, to be an effective reactive oxygen scavenging molecule. We investigated an anti-oxidative effect of curcumin against H_2O_2-induced toxicity in human neuroblastoma cell line SK-N-SH.

Methods: The SK-N-SH cells were pre-treated with curcumin 2 hours prior to H_2O_2 treatment. We measured cell viability, intracellular reactive oxygen species (ROS) levels, expression of apoptotic-related proteins and caspase-3 activity 24 hours post H_2O_2-induced cytoxicity.

Results: Treatment with curcumin at concentrations ranging from 5 to 50 µg/mL was not cytotoxic. Pre-treatment with curcumin at the concentrations of 5 to 50 µg/mL prior to H_2O_2 exposure caused a significant decrease in intracellular ROS levels and a significant increase in cell viability in a dose-dependent manner. Expression of activated form of caspase-3 and BAX, a pro-apoptotic protein, measured by Western blotting were reduced when the SK-N-SH cell line was pre-incubated with curcumin. The curcumin pre-treated cells also exhibited less caspase-3 activity.

Conclusion: Curcumin has protective effects against H_2O_2-induced toxicity in a dose-dependent manner through its anti-apoptotic and anti-oxidative properties in an *in vitro* H_2O_2-treated SK-N-SH model.

Keywords: Curcumin; apoptosis; anti-oxidant; oxidative stress; SK-N-SH cells (Siriraj Med J 2018;70: 184-190)

INTRODUCTION

Oxidative stress-mediated cellular damage has long been recognized to be a crucial factor in pathophysiology of several neurological disorders, for instance, Alzheimer's disease, stroke and Parkinson's disease.[1-3] The damage is a result of an imbalance between anti-oxidants and pro-oxidants, which are known as reactive oxygen species (ROS). Super-oxide radical (O_2-) and hydrogen peroxide (H_2O_2) are examples of ROS. Excess ROS reacts with DNA, lipids and proteins leading to DNA damage, lipid peroxidation and protein oxidation, respectively. Consequently, cell death via apoptosis is unavoidable.[4-5]

Curcumin (diferuloylmethane), the most common form of curcuminoids found in the rhizome of *Curcuma longa* or turmeric, has long been commonly used for culinary purposes.[6-8] Beneficial biological properties of curcumin include anti-neoplastic, anti-inflammation, anti-oxidative, and anti-angiogenic properties.[6-9] Among these biological properties, its potent anti-oxidative effect is well known. Several investigators have reported the protective effects of curcumin against oxidative stress both *in vitro* and *in vivo*. Curcumin reduces rat pheochomocytoma PC12 cell death induced by β-amyloid via a reduction of oxidative stress and DNA damage.[10] In the *in vitro* model of mouse neuroblastoma Neuro-2A cell line, curcumin protects the cells against H_2O_2- induced oxidative stress as determined by a decrease in intracellular ROS and apoptosis.[11] These studies indicated that curcumin could

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be used for treatment or prevention of oxidative stress-related diseases. Here we used H_2O_2-induced cytotoxicity model in human neuroblastoma cell line, the SK-N-SH, to investigate the neuroprotective activity of curcumin.

MATERIALS AND METHODS

Chemicals

Curcumin was purchased from Government Pharmaceutical Organization (GPO), Bangkok, Thailand. Dimethyl sulfoxide (DMSO), H_2O_2 and DCFH-DA were from Sigma. Apo-ONE® homogeneous caspase-3/-7 assay and CellTiter-Blue® cell viability assay were obtained from Promega. Clarity™ Western ECL Blotting substrate kits were purchased from Bio-Rad. Stock solution of curcumin was prepared by dissolving curcumin powder in DMSO at a concentration of 10 mg/mL. Stock curcumin was kept at -20°C until diluted before used.

Cell culture and treatment of curcumin/H_2O_2

The human neuroblastoma cell line SK-N-SH was purchased from the ATCC (Rockville) and cultivated in complete fibroblast medium (DMEM with 2 mM L-glutamine, 10% v/v fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 100 units/mL penicillin and 100 µg/mL streptomycin, and all were from Gibco) in a 37°C incubator with 5% CO_2. Passage numbers of the SK-N-SH cells used in this study were between 9 and 39. Cells were maintained for 24 hours before an addition of curcumin at seven different concentrations (5, 10, 15, 20, 25, 50 and 100 µg/mL) for 2 hours. The cells were then treated with 300 µM H_2O_2 and incubated for 24 hours. The control cells were cultured in complete fibroblast medium without $H_2O_2$and curcumin treatment.

Cell viability

Measurement of cell viability was performed by CellTiter-Blue® cell viability assay. In brief, 25,000 cells were cultured in each well of a 96-well plate and incubated overnight. Curcumin/H_2O_2 treatment was done as described. Twenty four hours after H_2O_2 treatment, the medium was then carefully discarded and replaced with 100 µL of fresh complete fibroblast medium. 10 µL CellTiter-Blue® reagent was then added into each well. The SK-N-SH cells were kept in a 37˚C incubator with 5% CO_2 for an hour. Fluorescence was measured with a microplate reader (Synergy HT, BioTek Instrument) using 540 nm and 590 nm as the excitation and emission wavelength, respectively. The fluorescent signal was directly proportional to the percentage of cell viability.

Intracellular ROS production

25,000 cells were cultured in each well of a 96-well plate and incubated overnight at 37°C with 5% CO_2. Curcumin/H_2O_2 treatment was done as described. 24 hours after H_2O_2 treatment, 5 µL DCFH-DA (a final concentration of 50 µM) was added to each well and incubated for 1 hour, and fluorescence intensity was measured by microplate reader (Synergy HT, BioTek Instrument) using 485 nm and 530 nm as the excitation and emission wavelength, respectively. The fluorescence intensity was proportional to the levels of ROS produced.

Assessment of DNA fragmentation by gel electrophoresis

To determine DNA fragmentation in apoptotic cells, 1 x 10^6 cells were cultured overnight in each well of a 6-well plate. Curcumin/H_2O_2 treatment was done as described. After 24 hours of incubation, cells were collected and isolation of genomic DNA was done with genomic DNA mini kit (Geneaid) as per the manufacturer's instruction. Extracted genomic DNA was subjected to gel electrophoresis, and the image was obtained with GelDoc™ EQ (Bio-Rad).

Western blot analysis

1 x 10^6 cells were cultured overnight in each well of a 6-well plate. Curcumin/H_2O_2 treatment was done as described. Twenty four hours after H_2O_2 treatment, medium was then carefully removed and washed once with fresh medium. Cells were lysed in a lysis buffer. After centrifugation of the lysate, protein concentrations were measured by Nanophotometer (Implen). 10% SDS-PAGE was then done with 30 µg protein samples. Proteins were blotted onto a polyvinylidede fluoride membrane (Bio-Rad). Membranes were blocked with 5% non-fat dried milk in TBS for 1 hour before incubating with primary antibodies (rabbit anti-human actin antibody at 1:1,000 dilution, Cell Signaling; rabbit anti-activated human caspase-3 antibody at 1:500 dilution, Cell Signaling; mouse anti-human BAX antibody at 1:500 dilution, Santa Cruz) in 0.1% Tween-20 in TBS at 4°C overnight. The membrane was incubated with secondary antibodies, conjugated with horseradish peroxidase (HRP), against rabbit or mouse (1:1,000, both from Cell Signaling) at room temperature for 1 hour. Membranes were then incubated with Clarity™ Western ECL Blotting substrate (Bio-Rad). Blots were visualized by ImageQuant LAS 4010 (GE Healthcare). Density of bands was quantified using densitometric analysis of ImageJ software (National Institutes of Health).

Measurement of caspase-3/-7 activity

25,000 cells were cultured in each well of a 96-well plate and incubated overnight at 37˚C with 5% CO_2. Curcumin/H_2O_2 treatment was done as described. After 24 hours of incubation, medium was discarded and 100 µL of fresh complete fibroblast medium was added into each well. Activity of caspase-3 was assessed by Apo-ONE® homogeneous caspase-3/-7 assay as per manufacturer's instructions. Briefly, 50 µL caspase-3/-7 reagent was added into the culture and incubated for 1 hour at 37°C and 5% CO_2. Fluorescent signal was then obtained at 521 nm with micro-plate reader (Synergy HT, BioTek Instrument) with the excitation wavelength of 499 nm.

Statistical analysis

Data is presented as the mean ± SEM (n=3). Statistical analysis was by one-way ANOVA, followed by Dennett's test for comparing between control/H_2O_2-treated and curcumin pre-treated experimental groups which was done with Graphpad Prism version 5 (GraphPad Software). P-value of less than 0.05 was used for determining significant differences.

RESULTS

Cytotoxicity of curcumin on the SK-N-SH cells

We investigated whether curcumin is toxic to the SK-N-SH cells by treating the cells with different concentrations of curcumin (5-100 µg/mL) for 24 hours. Cell viability after treatment with curcumin at the concentration of 5, 10, 15, 20, 25, 50 and 100 µg/mL were 99.1±2.23%, 95.13±2.08%, 97.81±1.37%, 98.33±0.53%, 97.43±2.25%, 94.7±3.35% and 38.88±10.98%, respectively (Fig 1A). The data showed that exposure to curcumin at the concentrations ranging from 5 to 50 µg/mL for 24 hours did not alter cell viability. However, curcumin was toxic at the concentration of 100 µg/mL as demonstrated by a significant reduction of cell viability (one-way ANOVA: F=23.34, p<0.0001; Dunnett's post-hoc test: p<0.0001 control vs 100 µg/mL curcumin; n=3 for each group). The cytotoxic concentration of curcumin that caused 50% reduction of cell viability (CC50) was 92.045 µg/mL (Fig 1B).

Curcumin protects SK-N-SH cells against H_2O_2-induced toxicity

To test whether curcumin exhibits protective effect against H_2O_2-induced cytotoxicity, we first identified the H_2O_2 concentration that could reduce cell viability by 50% by treating the SK-N-SH cells with various concentrations

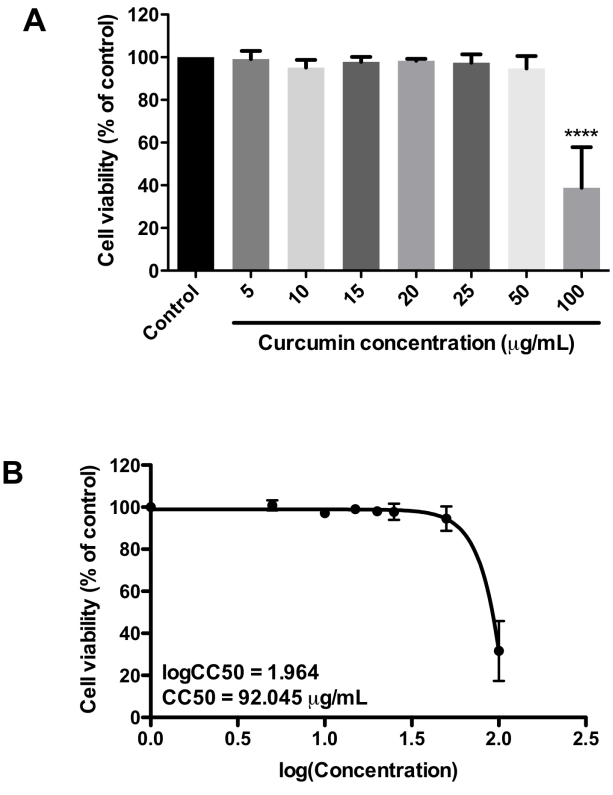


Fig 1. The effect of curcumin on cell viability. Cells were incubated at 37°C with several concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 µg/mL) for 24 hours. The cytotoxicity of curcumin was measured using CellTiter-Blue® reagent. ****p<0.0001 vs control group. Values are mean±S.E.M. from three independent experiments.

of H_2O_2. Treatment of H_2O_2 at all tested concentrations showed significantly decreased cell viability (one-way ANOVA: F=68.68, p<0.0001; Dunnett's post-hoc test: p<0.0001 control vs all H_2O_2 concentrations; n=3 for each group). The reduction of cell viability was in a dose-dependent manner (Table 1 and Fig 2A). From the data, we chose 300 µM H_2O_2, which induced approximately 50% reduction in cell viability, for assessing the protective effect of curcumin.

We then pre-treated the SK-N-SH with different concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 µg/mL) 2 hours prior to H_2O_2 treatment at a concentration of 300 µM. We found a significant increase in cell viability in curcumin pre-treated cells at all concentrations except 100 µg/mL as compared to the H_2O_2 treated group (Fig 2B, one-way ANOVA: F=30.15, p<0.0001: Dunnett's post-hoc test: p<0.001 300 µM H_2O_2 vs 5, 10, 15, 20, 25 and 50 µg/mL curcumin; n=3 for each group). The data indicated that pre-treatment with curcumin at the concentrations rangilng from 5 to 50 µg/mL rescued H_2O_2-induced cytotoxicity.

TABLE 1. Concentration-dependent cytotoxicity of H_2O_2 in SK-N-SH cells.

H_2O_2 concentration (μM)	Cell viability (% of control)
37.5	62.87±2.17%
75	59.60±3.95%
150	56.64±1.92%
300	52.48±1.21%
600	49.28±1.32%
1,200	37.4±4.48%
2,400	25.56±2.27%

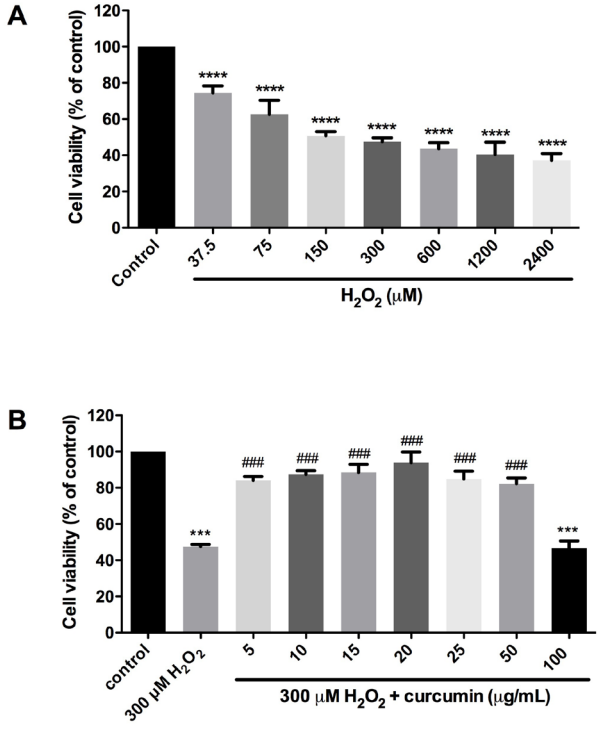


Fig 2. Effect of H_2O_2 on cells viability. (A) Cells were treated with different concentrationsof H_2O_2 (37.5, 75, 150, 300, 600, 1,200 and 2400 μM) for 24 hours. Percentage of cell viability was inversely correlated with H_2O_2 concentrations. (B) Protective effect of curcumin on H_2O_2- induced cytotoxicity was demonstrated at 5, 10, 15, 20, 25 and 50 μg/mL concentrations. Pre-treatment of curcumin at 100 μg/mL did not rescue cytotoxicity induced by H_2O_2. ***p<0.001 and ****p<0.0001 compared to the control group, ###p<0.0001 compared to the H_2O_2-treated group. Values are mean±S.E.M. from three independent experiments.

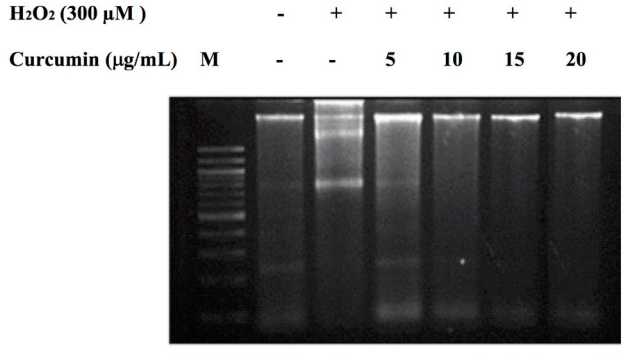


Fig 3. Protective effect of curcumin (5, 10, 15 and 20 μg/mL) on H_2O_2-induced apoptotic DNA fragmentation in SK-N-SH cells. M; DNA ladder.

Curcumin attenuates H_2O_2-induced apoptosis in SK-N-SH cells

DNA fragmentation is one of the observed features in apoptotic cells. If curcumin could potentially inhibit apoptosis in H_2O_2-treated cells, reduction of DNA fragmentation would be evident in cells pre-treated with curcumin prior to H_2O_2 addition. We, thus, performed a gel electrophoresis to see whether the DNA fragmentation could be ameliorated by curcumin (Fig 3). Cells treated with 300 μM H_2O_2 showed a more intense smear pattern compared to the control group. Interestingly, pre-treatment with curcumin at the concentration of 5, 10, 15 and 20 μg/mL dose-dependently decreased the smear. The findings support that H_2O_2-treated cells underwent apoptosis and this could be prevented by pre-treatment with curcumin.

To further investigate the anti-apoptotic property of curcumin, we checked the expression levels of apoptotic-related proteins. Western blotting demonstrated that H_2O_2treatment triggered an up-regulation of the pro-apoptotic protein BAX to be 166.45±11.99% of the control (Fig 4A; one-way ANOVA: F=16.99, p=0.0002; Dunnett's post-hoc test: p<0.001; n=3 for each group). Interestingly, pre-treatment of curcumin at the concentration of 5,

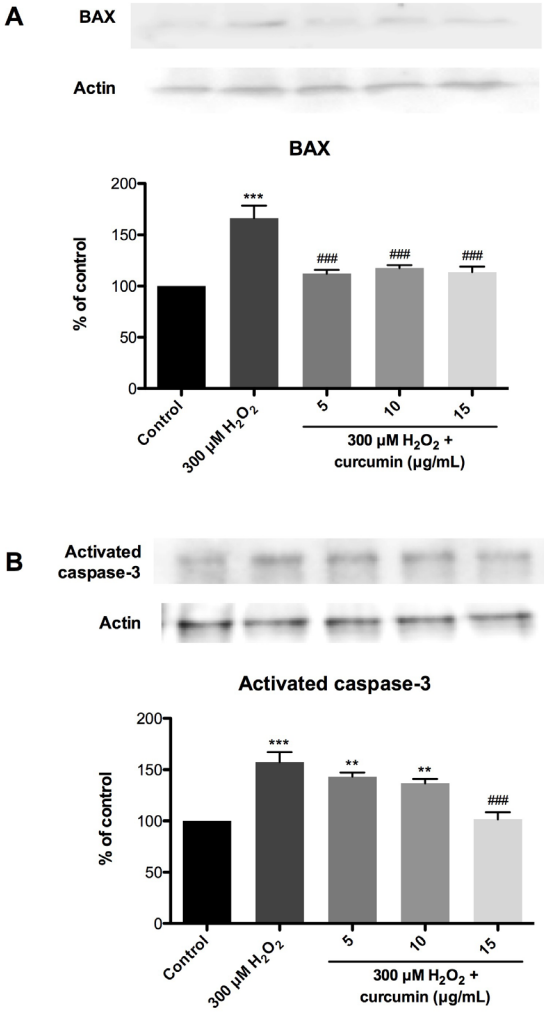


Fig 4. Pre-treatment of curcumin decreased levels of pro-apoptotic protein BAX (A) and activated caspase-3 (B). The SK-N-SH cells were pre-treated with 5, 10 or 15 μg/mL curcumin for 2 hours before $H_2O_2$was being added. Western blot analysis of samples 24 hours after H_2O_2treatment showed a significant decrease in BAX levels when the cells were pre-treated with curcumin at all concentrations. Activated caspase-3 levels were downregulated only when cells were pretreated with 15 μg/mL curcumin. **p<0.01 and ***p<0.001 compared to the control group, ###p< 0.001 compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

10 and 15 μg/mL prior to H_2O_2 addition effectively reversed the increase in BAX expression induced by H_2O_2 (Dunnett's post-hoc test: p<0.001; n=3 for each group). Likewise, H_2O_2 treatment induced a significant increase in activated caspase-3 levels compared to control (157.41±9.66%; Fig 4B; one-way ANOVA: F=19.05, p=0.0001; Dunnett's post-hoc test: p<0.001; n=3 for each group). The increase in activated caspase-3 levels mediated by H_2O_2 was inhibited by pre-treatment of curcumin at the concentration of 15 μg/mL (Dunnett's post-hoc test: p<0.001; n=3 for each group).

In addition, we checked whether a reduction in activated caspase-3 levels by curcumin resulted in a

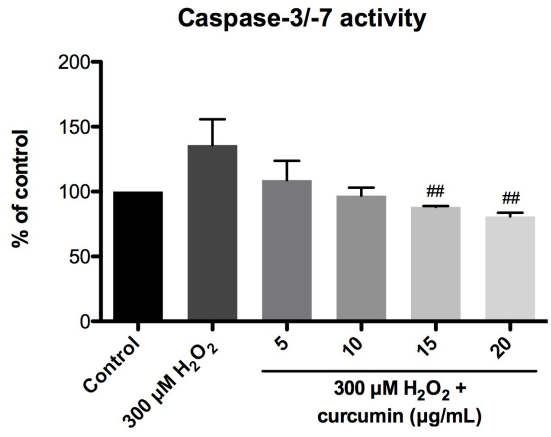


Fig 5. Reduction of caspase-3/-7 activity in samples pre-treated with curcumin. SK-N-SH cells were pre-treated with curcumin (5, 10, 15 and 20 μg/mL) prior to inducing apoptosis by H_2O_2. Inhibition of apoptosis by curcumin was demonstrated by a reduction in caspase-3/-7 activity in samples pre-treated with 15 and 20 μg/mL curcumin. ##p< 0.01 compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

decrease in H_2O_2-induced apoptosis. Thus, the activity of caspase-3/-7 was assessed by Apo-ONE® homogeneous caspase-3/-7 assay. We found that H_2O_2 treatment caused a rise in caspase-3/-7 activity (135.84±19.85%; Fig 5). Pre-treatment of curcumin at the concentrations of 15 and 20 μg/mL prior to an addition of H_2O_2 significantly reduced caspase-3/-7 activity to 88.17±0.78% and 80.83±2.96%, respectively (Fig 5, one-way ANOVA: F=3.379, p=0.039; Dunnett's post-hoc test: p<0.01; n=3 for each group). All of the data demonstrated that pre-treatment of curcumin reduced H_2O_2-induced apoptosis in SK-N-SK cells.

ROS induction by H_2O_2 can be scavenged by curcumin

To determine whether anti-apoptotic property of curcumin is mediated via a reduction in ROS levels, we measured intracellular ROS levels by DCFH-DA assay. We found that the percentage of ROS levels when the cells were pre-treated with curcumin was significantly decreased compared to H_2O_2-treated group (Fig 6, one-way ANOVA: F=32.1, p<0.0001; Dunnett's multiple comparison test: p<0.001; n=3 for each group). The results suggested that the anti-oxidative property of curcumin contributed, in part, to its anti-apoptotic property.

DISCUSSION

Oxidative stress is caused by excessive levels of ROS. Excess ROS leads to damages of cellular components including lipids, proteins and DNAs. Consequently, the damages contribute to pathogenesis of many age-associated and neurodegenerative diseases. Among a great variety of ROS, H_2O_2 is a major ROS found since it is a

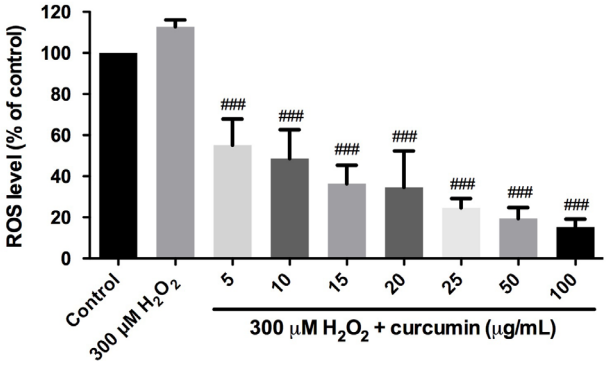


Fig 6. Intracellular ROS levels were reduced in samples pre-treated with curcumin. Cells were pre-treated with various concentrations of curcumin (5, 10, 15, 20, 25, 50 and 100 μg/mL) 2 hours prior to H_2O_2 addition. ROS levels were measured by DCFH-DA assay 24 hours after H_2O_2-induced cytotoxicity. ###p<0.001 as compared to the H_2O_2-treated group. Values are mean ± S.E.M. from three independent experiments.

by-product of aerobic metabolism in mitochondria and peroxisome.[5,12] Oxidative damage by H_2O_2 is normally prevented by catalase, an enzyme that converts H_2O_2 to water and oxygen.[5]

Curcumin has long been shown to exhibit a wide range of pharmacological activities, such as anti-inflamation,[13] anti-oxidant[10-11] and anti-cancer.[14] Several *in vitro* studies reported protective effects of curcumin against oxidative agents in various cell types. For instance, curcumin at a concentration of 10 μg/mL significantly reduced oxidative stress triggered by beta-amyloid in the rat pheochromocytoma PC12 cell line and led to an increase of cell viability.[10] An increase in cell viability and an attenuation of apoptosis, a consequence of intracellular ROS reduction, were found when the H_2O_2-treated mouse neuroblastoma cell line Neuro-2A was pre-treated with curcumin at 20 and 25 μg/mL.[11] Moreover, simultaneously addition of 25-100 μM curcumin and H_2O_2 protected H_2O_2-induced cell damage in the ng108-15 cell culture, a hybrid line between mouse neuroblastoma and rat glioma.[15] In our study, we assessed the protective effect of curcumin on H_2O_2-induced cytotoxicity in the human neuroblastoma SK-N-SH cell line. Our studies demonstrated that treating the cells with H_2O_2 caused a reduction in cell viability in a dose-dependent manner. This reduction can be significantly ameliorated by pre-treating the cells with curcumin at six different concentrations (5, 10, 15, 20, 25 and 50 μg/mL). Additional data revealed that pre-treatment of curcumin at the concentration of 5, 10, 15, 20, 25, 50 and 100 μg/mL prior to cytotoxicity induction by H_2O_2 led to a significant decrease in intracellular ROS levels. Our data was consistent with other studies,[10-11] which showed that pre-treatment of curcumin at 10 μg/mL,[10] 20 μg/mL[11] and 25 μg/mL[11] prior to an addition of oxidative agents into rodent neural lineage cancer cell lines protected against oxidative agents-induced cytotoxicity.

Reduction of cell viability can be caused by various mechanisms including apoptosis. Previous study reported that curcumin inhibited H_2O_2-induced apoptosis in Neuro-2A cells by reducing intracellular ROS levels,[11] a finding which we also observed in our study. Thus, we hypothesized that curcumin prevents H_2O_2-induced cytotoxicity in part via a reduction in apoptosis. Indeed, we found that curcumin at the concentrations of 5, 10, 15 and 20 μg/mL reduced DNA fragmentation, a characteristic of apoptosis. Expression of the pro-apoptotic protein BAX, which was induced by H_2O_2 treatment, was significantly decreased when the cells were pre-treated with 5, 10 and 15 μg/mL curcumin. Furthermore, pre-treatment by 15 μg/mL curcumin resulted in a reduction in both the levels of activated caspase-3 and its activity, confirming the anti-apoptotic effect of curcumin. Although caspase-3 activity in cells pre-treated with 5 and 10 μg/mL curcumin was not reduced significantly, viability of cells pre-treated with curcumin at those concentrations was significantly higher than that of H_2O_2-treated cells. This indicates that curcumin could also potentially inhibit a reduction in cell viability induced by H_2O_2 via another mechanism in addition to its anti-apoptotic activity.

It is known that curcumin, at a high concentration, is toxic to the cells.[16-18] In the PC12 cell culture model, curcumin concentration of at least 50 μg/mL was able to reduce cell viability by half.[10,18] In the present study, we found that addition of 100 μg/mL curcumin dramatically decreased cell viability to 38.88±10.98% compared to untreated control. Several pieces of evidence stated that the cytotoxicity of curcumin at high concentration is mediated via various mechanisms including cell proliferation inhibition and apoptosis induction.[19] For instance, curcumin, at high concentration, exhibited negative effect on cell proliferation in a glioma cell line U251.[20] High dose curcumin, especially in a presence of transition metal ions such as copper,[21-22] triggered oxidative stress and DNA damage, conditions that caused apoptosis, in human hepatoma HepG2 cells.[16] Thus, curcumin exerts either anti-apoptotic or pro-apoptotic effects, depending on its concentrations. However, *In vivo* toxicities of curcumin, would be unlikely since its toxicity levels would never be reached via oral administration.[9]

CONCLUSION

To summarize, our study showed that curcumin at an optimal concentration (15-20 µg/mL) possessed potent anti-oxidative and anti-apoptotic properties. H_2O_2-induced cytotoxicity in SK-N-SH cell line can be reversed by pre-treating cells with curcumin. Neuroprotective effect of curcumin is mediated in part via an inhibition of intracellular ROS accumulation, which consequently, leads to a reduction in H_2O_2-induced apoptosis as evident by a decrease in levels of pro-apoptotic protein BAX and activated caspase-3 as well as reduced caspase-3 activity. We, therefore, propose that curcumin has great potential for ameliorating neurocytotoxicity induced by H_2O_2. However, further studies such as *in vitro* study in primary rodent brain slices and human induced pluripotent stem cell-derived neurons are needed for validating the findings in neuroblastoma cell line. Additional *in vivo* preclinical research, e.g. study in Alzheimer's disease mouse model, would also be required for addressing pharmacokinetic properties, cytotoxicity, protective effect and, more importantly, neurobehavioral benefits of curcumin in a more relevant context.

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