RESEARCH PAPER

Protective effects of nanomicelle curcumin on the phosphine-induced oxidative stress and apoptosis in human liver HepG2 cells

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ABSTRACT

Objective(s): Aluminium phosphide is used worldwide as a rodenticide and fumigant against sorted grain. Accidental or intentional exposure to aluminium phosphide (determined as PH3) results in extreme toxic effects on human. Due to the lack of a specific antidote, management of PH3 poisoning remains supportive and symptomatic. Curcumin, the main compounds of turmeric, has been reported to possess strong antioxidant and anti-apoptotic properties. This study was conducted to evaluate the effect of nanocurcumin on PH3 toxicity in HepG2 cell line.

Materials and Methods: For this study, the cells pretreated with nanocurcumin (nCUR) and then exposed to PH3 for 6 hr. The cytotoxic and apoptotic effects of PH3 and nCUR were evaluated by MTT and propidium iodide flow cytometry. Indeed, the level of reactive oxygen species and glutathione were determined by fluorometric and colorimetric method. The oxidative DNA damage (8-OHdG) marker was also measured by ELISA kit.

Results: Pretreatment with nCUR elevated the cell viability in PH3-treated cells and antagonized the PH3-induced glutathione depletion at high doses. Indeed, a significant decrease in the level of ROS and 8-OHdG as well as apoptotic activities were observed following exposure to nCUR.

Conclusion: These results indicated that nCUR could protect HepG2 cells against PH3 induced cell injury by attenuation of ROS and increasing GSH level. The nCUR efficiently suppressed increased apoptosis activity and formation of 8-OHdG and ultimately improved cell viability. Therefore, nCUR can be considered as promising therapeutic agents in treatment of aluminium phosphide poisoning.

Keywords: *Aluminium phosphide; Curcumin; Nanomicelle; Phosphin; Toxicity*

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INTRODUCTION

Aluminium phosphide (ALP) is used worldwide as a rodenticide and fumigant against sorted grain. Easily availability of ALP in different form including tablet, dust, pellet and grains makes it an important public health challenge especially in developing countries. In Iran, the tablet form of AlP is used for protection of rice and known as 'rice tablet' and consists of a combination

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of 56% AlP and 44% ammonium carbonate (1). Accidental or intentional exposure to ALP results in extreme toxic effects on human. High death rate has been reported following significant exposures to phosphide salts (aluminium, calcium or zinc phosphides) over the past 35 years (2-4). Following the ingestion, AlP is chemically react with hydrochloric acid and water in the stomach and releases the phosphine (PH3) gas that is rapidly absorbed after ingestion, inhalation or upon contact with mucous membranes and skin. The colorless PH3 gas with an odor of decaying fish induces very toxic effects by inhibition of cytochrome c oxidase and generation of lipid peroxidation products and reactive oxygen species (ROS)(5, 6). PH3 is a non-specific metabolic poison that affects many organ including respiratory, cardiovascular, gastrointestinal, nervous, hepatic and musculoskeletal systems (5, 7). The common manifestations of ALP toxicity are nausea, drowsiness, labored breathing, chest tightness, pulmonary irritation, pulmonary edema and intense gastric pain, coma and death in severe cases(8). Due to the lack of a specific antidote, management of PH3 poisoning remains supportive and symptomatic (9).

Curcuma longa L. (Turmeric) is a perennial rhizomatous herb of the ginger family that is cultivated in many parts of the tropics and subtropics (10, 11). The orange-yellow powder known as turmeric from dried rhizomes of the plant is used traditionally as spice, dye, cosmetic and as medicinal Purposes(12). Curcuminoids and essential oils are the main compounds of turmeric. Curcumin (CUR), the most active curcuminoids, possess a wide range of beneficial effects for cardiovascular, alzheimer's diseases, metabolic syndrome, inflammation and cancer (13, 14). Most therapeutic benefits to CUR are attributed to its anti-inflammatory and antioxidant (15). Despite many benefits health reported for CUR, it has low oral bioavailability, mainly due to poor absorption and rapid metabolism and elimination (16). It is well documented that CUR in the form of nanoparticle (micellar curcumin) is associated with an 60-fold increase in the biological half-life of CUR (17, 18). This study was conducted to evaluation the effect of nanocurcumin (nCUR) on PH3 toxicity in HepG2 cell line.

MATERIALS AND METHODS

Chemicals and reagents

The fluorescent probe propidium iodide (PI), RPMI1640 medium 3-(4, 5-dimethylthiazol-2-yl)-2, fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) and 5-diphenyl tetrazolium (MTT) were obtained from Sigma Chemicals Co. The 8-hydroxy-20-deoxyguanosine (8-OHdG) ELISA kit was purchased from (CA: ab201734; Abcam, Cambridge, MA, USA).

Preparation of curcumin nano-micelle

Nanocurcumin (curcumin complex-loaded nanomicelles) and native CUR were purchased from Exir Nano Sina Co., Tehran, Iran. The mean diameter of nanomicelles is around 10 nm, according to dynamic light scattering analysis. The encapsulation efficiency of curcuminoids in nanomicelles is almost 100%. The curcuminoid content and size distribution of nanomicelles remains constant for at least 24 months. The oral absorption of Sina CUR is at least 59 times more than the conventional powder of CUR in mice (Novel nanomicelle formulation to enhance bioavailability and stability of curcuminoids (19).

Cell culture

The human liver cancer cell line (HepG2) was obtained from Pasteur Institute (Tehran, Iran). The cells were grown in RPMI medium supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2 in 95% humidified atmosphere.

Cytotoxicity assessment

The nCUR and PH3 cytotoxicity in HepG2 cells was determined using MTT assay. Briefly, cells were cultured in a 96-well plate at a density of 5000 cell/well. To determine appropriate concentrations, the cells were incubated with fresh medium containing 2.5-80 µM of nCUR for 24, 48 hr, or 1-10 mM of PH3 for 6 hr. PH3 was generated by adding the AlP to the aqueous medium. AlP was added to PBS at 0.58 mg/ml (10 mM PH3 equivalent), the mixture was shaken and the aqueous PH3 solution was achieved by aspiration after3–5 min. The target concentrations of PH3 were made immediatelybefore adding to the culture(6).At the next step, cells were exposed to selected concentration of nCUR (2.5, 5, 10 or 20 µM) or CUR (10 or 20 µM) for 24 hr. Then PH3 at final concentration of 1 mM was added to each well. After incubation for 6 hr, MTT solution at final concentration 0.5 mg/mL was added to the well. Then, the media was removed, and the formazan crystals were dissolved in dimethylsulfoxide

(DMSO). The absorbance of purple solution was measured at 545 nm (630 nm as a reference) in an ELISA reader (Start Fax-2100, UK) (20, 21).

Propidium iodide staining

Apoptotic cells were detected by PI staining and flow cytometry, aiming to detect the sub-G1 peak on the DNA content histogram. In brief, HepG2 cells were seeded at 2×10^4 cells per well in a 6-well plate. The cells after 24 hr pretreatment with nCUR at concentration of 2.5, 5, 10 and 20 µM were incubated with 1 mM PH3for 6 hr. After then, suspensions of single cells were prepared by trypsinization and washed twice with cold phosphate-buffered saline (PBS). The pellet cells were suspended in PBS containing 50 µg/ml PI and 150 U/ml RNAse for 45 min at 37 °C. The fluorescence of cells was analyzed with an FCM (BECKMAN-COULE, USA). The apoptosis percentage were analyzed with WinMDI version 2.8 software (22).

Measurement of intracellular ROS

A fluorometric microplate assay was used to evaluate the production of reactive oxygen species. The 2,7-dichlorofluorescin diacetate (DCFH2-DA) is enzymatically hydrolyzed by intracellular esterases to a non-fluorescent compound (H2DCF), which is oxidized by ROS to highly fluorescent DCF. As explained before, the hepG2 cells were exposed to nCUR (2.5, 5, 10 and 20 µM) in a 96 well plate for 24 hr, then 1 mM PH3 was added to each well for exposure times of 6 hr. At the end of experiment, the medium was replaced with RPMI containing 10 µM of DCFH-DA for 30 min in the dark at room temperature. The intensity of fluorescence from DCF was measured by Partec TM cytometry (Germany) (excitation wavelength 480 nm; emission wavelength 530 nm) (23).

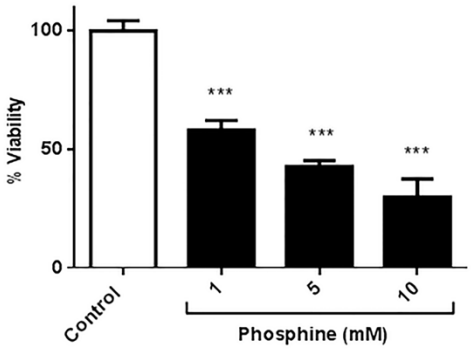

Fig 1. Effect of PH3 on cell viability of HePG2 cells. Cells were treated with different concentrations of PH3 for 6 h. Results are the mean ± SD of three independent experiments. ***P< 0.001 show significant differences as compared to the control group

Reduced gluthathion (GSH) assay

GSH was measured via the formation of yellow color in the presence of DTNB [5,50 di thiobis-(2-nitrobenzoic acid)] (24). Briefly, 6hr after PH3 treatment, cells were washed twice with PBS and then treated with 5% trichloroacetic acid incubated for 30 min at 37 °C. Following centrifugation, the supernatant mixed again with 1 ml of a reaction mixture containing 0.1 M sodium phosphate buffer (pH 7.5), 0.6 mM DTNB at the room temperature for 5 min. The absorbance of solution was measured at 412 nm using spectyrophotometer (21).

DNA damage assay

The amount of biomarker of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG), was quantified with an ELISA kit (CA: ab201734; Abcam, Cambridge, MA, USA). Measurements were performed according to the manufacturer's guidelines. Microplates were read at 450 nm for 8-OHdG using an automated reader (Biohit- BP800, Finland). The levels of 8-OHdG was expressed as units/mg protein or pg/ml (25).

Statistical analysis

Results are expressed as mean ± SD. One-way ANOVA followed by Tukey-Kramer was performed to compare the results. Differences were considered statistically significant when P<0.05.

RESULTS

Effects on cell viability

The cells were incubated with various concentrations of PH3 (1, 5, and 10 mM) for 6 h. PH3 significantly decreased cell viability in a concentration dependent manner. Cell viability decreased to 56% at 1mM exposure when compared with controls (P<0.001) (Fig. 1).

At the next step, HepG2 cells were exposed to different concentrations of nCUR (2.5, 5, 10, 20, 40, and 80 µM) for 24 and 48 hr to determine the non-toxic concentration. As shown in Fig. 2, nCUR reduced the cell viability at concentration higher than 40 µM in both time (Fig. 2).

As shown in Fig. 3, treatment of cells with PH3 at 1 mM significantly decreased the cell viability in comparison with control group (P<0.001). Pretreatment of cells with nCUR (2.5-10 µM), 24 hr before exposure to PH3 (1 mM) for 6 hr, significantly increase cell viability (P<0.05),as compared to PH3-treated cells. CUR exposure also recovered the

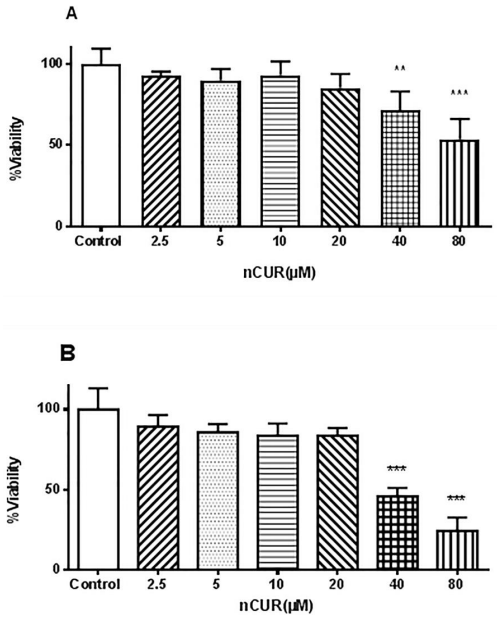

Fig 2. Effect of nanocurcumin (nCUR) on cell viability of HePG2 cells. Cells were treated with different concentrations of nCUR for 24 (A) and 48 (B) hr. Results are the mean ± SD of three independent experiments. **P<0.01 and ***P< 0.001 show significant differences as compared to the control group

viability of HepG2 cells to % 56-57 (P<0.05) (Fig. 3).

Role of apoptosis

The results showed that exposure of HepG2cells to PH3, significantly increased cell apoptosis compared with control cells (P<0.001). A significant decrease in PH3-inducedapoptosis was observed following pretreatment with nCUR at concentrations of 5 and 10 µM (P<0.001 vs. PH3 group). CUR could only reduce the rate of apoptosis at 10 µM (P<0.05). Results of apoptosis assay are illustrated in Figs 4A and B.

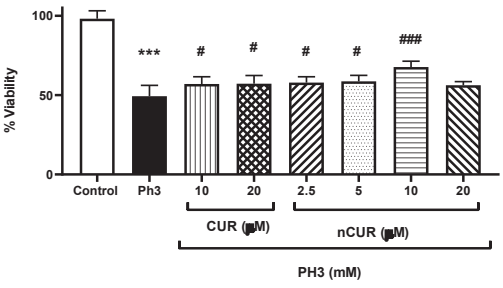

Fig 3. HepG2 cells were pretreated with different concentrations of CUR (10 and 20 µM) or nCUR (2.5-20 µM) for 24 hr before exposure to 1 mM of PH3 for 6 hr. Data are expressed as the mean ± SD of three separate experiments; ***P< 0.001 vs. control, #P<0.05 and ###P< 0.001 vs, PH3. PH3, Phophine; CUR, Curcumin; nCUR, Nanocurcumin

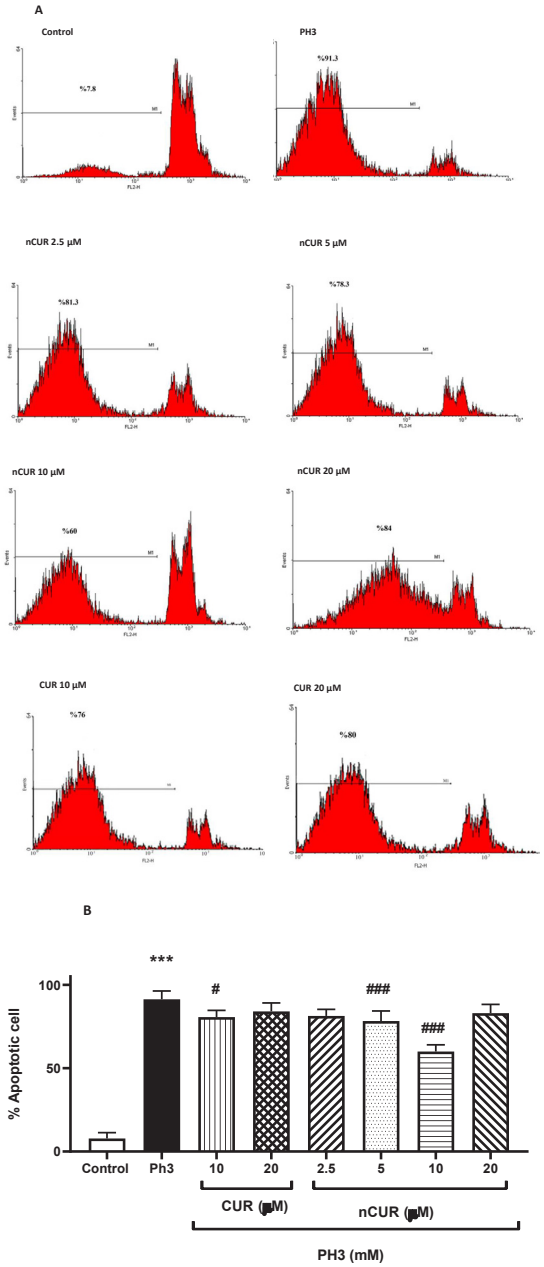

Fig 4. Flow cytometry histograms of apoptosis assays by PI method in HepG2 cells (A). Cells were treated with CUR (10 and 20 µM) or nCUR (2.5-20 µM) for 24 h before exposure to of PH3 (1 mM) for 6 h. Bar chart illustrates data as mean ± SEM of six separate experiments (B). *** P< 0.001 vs. Control group, #P<0.05 and ###P<0.001 vs. PH3 treated group. PH3. PH3, Phophine; CUR, Curcumin; nCUR, Nanocurcumin

Determining the level of cellular ROS

Treatment of cells with PH3 for 6 hr elevated intracellular ROS production up to %46.41 ± 3.2 (P<0.001 vs control). This effect was decreased through incubation of cells with nCUR 24 hr before

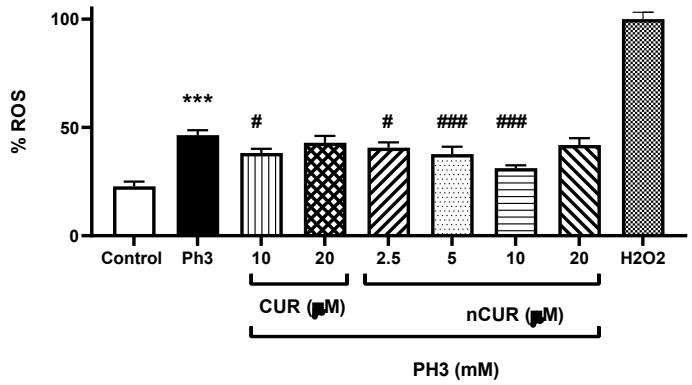

Fig. 5. Effect of nCUR on PH3-induced ROS generation in HepG2 cells. The cells were pretreated with different concentrations of CUR (10 and 20 μM) or nCUR (2.5-20 μM) for 24 hr before exposure to 1 mM of PH3 for 6 hr. Data are expressed as the mean ± SD of three independent experiments. ***P<0.001 vs. control, ###P<0.001 vs. Ph3. PH3, Phophine; CUR, Curcumin; nCUR, Nanocurcumin

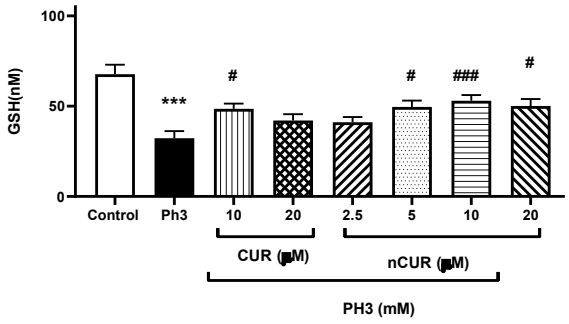

Fig. 6. GSH content of HepG2 cells that were pretreated with different concentrations of CUR (10 and 20 μM) or nCUR (2.5-20 μM) for 24 hr before exposure to 1 mM of PH3 for 6 h. Data are expressed as the mean ± SD of three independent experiments. ***P<0.001 vs. control, #P<0.05 and ##P<0.05 vs. Ph3. PH3, Phophine; CUR, Curcumin; nCUR, Nanocurcumin

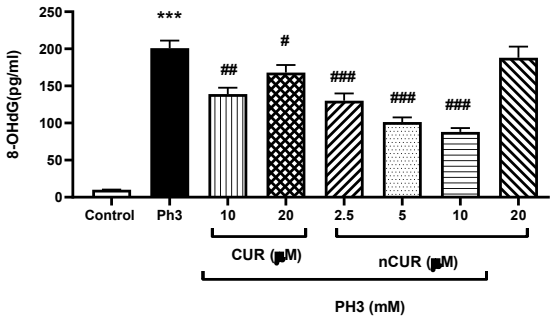

Fig. 7. Effect of PH3 and nCUR on 8-OHdG formation in HepG2 cells. The cells were pretreated with different concentrations of CUR (10 and 20 μM) or nCUR (2.5-20 μM) for 24 hr before exposure to 1 mM of PH3 for 6 hr. Data are expressed as the mean ± SD of three independent experiments. ***P<0.001 vs. control, ###P<0.001 vs. Ph3. PH3, Phophine; CUR, Curcumin; nCUR, Nanocurcumin

adding PH3. A significant reduction in ROS production was observed at 2.5, 5 and 10 μM of nCUR (P<0.01 and P<0.001 vs PH3 group). Hydrogen peroxide (H_2O_2) was used as a positive control. CUR at dose of 10 and not at 20 μM, could significantly decrease the ROS level (P<0.05) (Fig. 5).

Determining the levels of glutathione

Our data demonstrated that HepG2 cells exposed to PH3 (1mM) exhibited elevated GSH levels(P<0.001) that was reversed by administration of nCUR at higher doses (5, 10 and 20 μM, P<0.05, P<0.001 and P<0.05, respectively). The nCUR at 2.5 μM could not increase the GSH content, compared with PH3 group. The protective effect of CUR on GSH level, also observed at 10 μM concentration (P<0.05) (Fig. 6).

8-OHdG

Our results showed that the amounts of 8-OHdG in PH3-treated HepG2 cells increased (P<0.001, compared with the control group), while it was reversed by nCUR (2.5-10 μM) (P<0.001, compared with PH3 group). No difference was found between PH3 and nCUR at concentration of 20 μM. CUR at both concentrations could significantly decrease the 8-OHdG level (P<0.01 and P<0.05) (Fig. 7).

DISCUSSION

Generation of reactive oxygen species and disturbance in cellular homeostasis play key roles in PH3-induced toxicity that ultimately result in cell death (5). The results showed that exposure to PH3 induced significant cell toxicity mediated by ROS generation and glutathione depletion. It was well documented that the effect of ALP and its off-gas product PH3 was tied to the ROS generation as well as disturbance of antioxidant protection. PH3

especially increased the level of H_2O_2 as the most significant ROS and decreased the GSH content as the strongest protective antioxidant (6, 7). GSH not only protected the cell from oxidative stress, but also increased the cell survival as well (7). Consequently, over generation of ROS can damage biomolecules such as proteins, DNA, and lipids and resulting in the release of apoptotic factors and cell death (26). Previous studies demonstrated that PH3 could cause mitochondrial dysfunction through releasing of cytochrome c. activation of caspase 3 and 9 and ultimately DNA damage (27).

CUR exhibited a high antioxidant activity in various *in vitro* and *in vivo* studies. It can act from directly scavenging reactive species or indirectly enhance the antioxidant defense (28). CUR is able to scavenge superoxide anion, singlet oxygen, H_2O_2, peroxylradicals, hydroxylradicals, nitricoxide and peroxynitrite (29). The phenolic compounds with hydroxyl groups in the structure of CUR are capable of scavenging ROS and RNS (reactive nitrogen species) (30). CUR also indirectly upregulates the expression of antioxidant enzymes as well as increasing concentration of GSH (31, 32). The results showed that nCUR significantly increased the cell viability and the level of GSH and decreased the ROS generation in HepG2 cells exposed to PH3. The anti-apoptosis properties of nCUR has also been observed especially at 10 µM. Several studies indicated that PH3 could trigger apoptosis in several tissues such as kidney, heart and liver. Oxidative stress and mitochondrial pathway play a pivotal role on PH3-induced DNA damage and apoptosis (33). On the other hand, numerous in vitro and in vivo studies have confirmed the anti-apoptotic action of CUR. It was able to reduce apoptosis in intestinal epithelial cells treated with IFNγ (34). Curcuma longa L. extract its main active compounds, provided significant protection against blue light-mediated cytotoxicity and apoptosis in retinal pigment epithelium through regulation of c-Abl and p53 proteins (35). Indeed, in a rat model of acetic acid-induced colitis, oral CUR administration prevented programmed cell death, decreased lipid peroxidation, inflammatory reactionsby modulating MAPK pathways (36). The result of different in vitro studies have been shown that cCUR at concentrations lower than 20 µM could not inhibit the cell viability and induce apoptosis (37-39). It was demonstrated that a 10 µM dose of CUR could promote the proliferation of neural stem cell (39). Therefore, a dose of 10 and 20 µM CUR were selected for evaluation in our study. However, CUR was not effective at any doses as nCUR at 10 µM and CUR even failed to prevent the effects of Ph3 on increased apoptosis and GSH and reduced ROS levels. According to our results and others, CUR exerts its protective effect in a dose-dependent manner(40).

8-OHdG is one of the main forms of free radical damages that produces by oxidation of deoxyguanosine and is therefore considered as a defined biomarker of oxidative DNA damage (23). In accordance with our results, CUR also prevented DNA damage through suppression the 8-OHdG level and increasing the DNA repair enzymes in patients who had been chronically exposed to arsenic (41).

However, CUR has a dual effect on oxidative events and apoptosis, which can act as an antioxidant and anti-apoptotic or pro-oxidant and pro-apoptotic. CUR treatment alone can induce ROS generation and apoptosis in normal and cancerous cell lines (42, 43). It seems that dual effect of CUR is related to ROS concentration. When the cell reacts to stressful situationssuch as metal or PH3 (according to the result of this study) exposure and the level of ROS exceeds specific threshold, CUR attenuates oxidation and apoptosis. Low levels of ROS generation are required to maintain apoptosis and oxidative functions of CUR (42). The antioxidant/pro-oxidant activity of CUR is also concentration-dependent. The higher concentration of CUR results in a reduction of GSH levels and increase of ROS production, whereas in low concentrations possess antioxidant properties (44). In this study, nCUR at narrow concentration range, around 10 µM, exerted its maximum protective effects.

Low oral bioavailability of CUR is one of the major problems that limits its clinical application. CUR is absorbed poorly within the intestine, rapidly metabolized and mainly excreted into feces (16). Several strategies have been developed to overcome the challenges of low oral bioavailability (45). CUR in the form of nano micelle increases the solubility and decreases the rate of hydrolyzing that result in improved bioavailability (46). Although in the presence of PH3, the ROS concentration in the cell is extremely high and according to the above hypothesis, the CUR exerts its antioxidant effects. However, nanomicelle formulation increase the CUR bioavailability and therefore ROS production due to increase in CUR concentration at higher

doses may be happened.

CONCLUSION

These results indicated that nCUR could protect HepG2 cells against PH3 induced cell injury by attenuation of ROS and increasing GSH level. The nCUR efficiently suppressed increased apoptosis activity and formation of 8-OHdG and ultimately improved cell viability. Therefore, nCUR can be considered as promising therapeutic agents in treatment of aluminium phosphide poisoning.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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