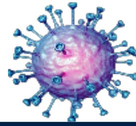

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Curcumin Mitigated TCE-induced Toxicity of A549 cell by Regulating Oxidative Stress and Apoptosis

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Abstract

Trichloroethylene (TCE), as a known cytotoxic substance, causes oxidative stress in cells. Curcumin exhibits extensive pharmacological effects, especially antioxidant and antitumor activities. The purpose of this study is to investigate the effect and underlying mechanism that curcumin alleviates the toxicity induced by TCE in human lung cancer A549 cells. As the results, curcumin alleviated the inhibitory effect of TCE on cell proliferation by clone formation and migration by wound healing and expression level of MMP2 in A549 cells. Curcumin also inhibited intracellular ROS levels by decreasing gp91-phox and increasing Nrf2 and protects membrane integrity and reduced apoptosis through enhancing anti-oxidative intracellular defense system. These results, taken together, suggest that curcumin can alleviate TCE-induced A549 cytotoxicity by regulating oxidative stress and apoptosis.

Keywords: Trichloroethylene; A549 cell; Oxidative stress; Apoptosis; Curcumin.

Introduction

Trichloroethylene (TCE, Figure 1A) is a mass-produced chemical that is widely used in many industrial applications, such as in degreasing and dry cleaning of metal parts worldwide [1,2]. Although its widespread use, this chemical is a ubiquitous environmental pollutant [3]. TCE was classified in the list of toxic and hazardous water pollutants in the year 2019. Despite the recent regulations limit the use of TCE, its use has not been completely banned [4]. The risk of TCE on human health aroused widespread concern and its toxicity has been recognized and well-documented [5]. TCE exposure can lead to numerous types of diseases, such as renal tubular injury, renal tubular epithelial cell necrosis, liver damage, especially respiratory disease (asthma, chronic bronchitis, rhinitis) [6-8].

The toxicity of TCE has been considered to be caused by generating excessive Reactive Oxygen Species (ROS) in the cellular systems [9]. Under normal physiological conditions, oxidation and reduction in the cells are in a stable state. When stimulated by external toxic and harmful substances, free radicals will be generated and the antioxidant system of cells will be activated. When excess free radicals exceed the scavenging capacity of cells, the homeostasis of cellular redox is unbalanced, resulting in oxidative stress and oxidative damage to cells. Studies have shown that oxidative stress is the mechanism of action for TCE-induced liver damage [10,11]. TCE induces the generation of oxygen free radicals through the metabolites of the CYP450 oxidation pathway, which affects the activity of the body's antioxidant enzymes, and ultimately causes the body's antioxidant system to be disordered [9,12].

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Curcumin (Cur, Figure 1A) is a polyphenolic compound extracted from the edible spice turmeric with various pharmacological effects [13]. It plays an important role in Indian and Chinese traditional medicine for thousands of years [14]. In addition, experiments have shown that curcumin is safe in humans even at high doses (12 g/day) [13]. In recent years, curcumin has received more and more attention from scientists due to its antioxidant, anti-inflammatory and anti-tumor properties [15]. Studies have demonstrated promising therapeutic outcomes in cancer, cardiovascular disease, and immune disease including arthritis, uveitis, ulcerative proctitis, and tropical pancreatitis [16]. And there is evidence that cellular superoxide dismutase (SOD) activity is enhanced by curcumin treatment and can eliminate intracellular superoxide anion, reduce the level of ROS in cells, and reduce the damage of oxidative stress to cells [17].

However, the protective effect and potential mechanisms of curcumin after TCE exposure have not been fully investigated. Therefore, we speculate that curcumin might have a protective effect against TCE-induced A549 cell cytotoxicity. Cytotoxicity assessments of TCE exposure (eg: cell viability, cell migration, ROS characterization, mitochondrial membrane potential, apoptosis levels) were performed on A549 cells pretreated or untreated with curcumin.

Results

Effects of trichloroethylene on the viability of A549 cells

To assess the effect of TCE on the viability of A549 cells, A549 cells were treated with TCE at different concentrations of 0, 3.125, 6.25, 12.5, 25, 50, 100 mM for 24 h using an MTT assay. The obtained results are shown in Figure 1B. TCE decreased the viability of A549 cells in a dose-dependent manner. When the concentration of TCE was 3.125 mM, there was no obvious toxic effect of TCE on A549 cells. The viability of A549 cells treated with 25 mM and 50 mM TCE was significantly lower than that of control (Ctrl) A549 cells. The results of cell viability assay showed that TCE could inhibit the cell viability of A549 cells. The IC_{50} of TCE on A549 cells was calculated to be 21.74 mM. In further experiments, a TCE concentration of 5 mM was chosen as the lowest concentration for A549 cells. For curcumin, it has been experimentally proved that the IC_{50} value of curcumin on A549 cells is around 20 μM, and the final selected concentration is 20 μM [18].

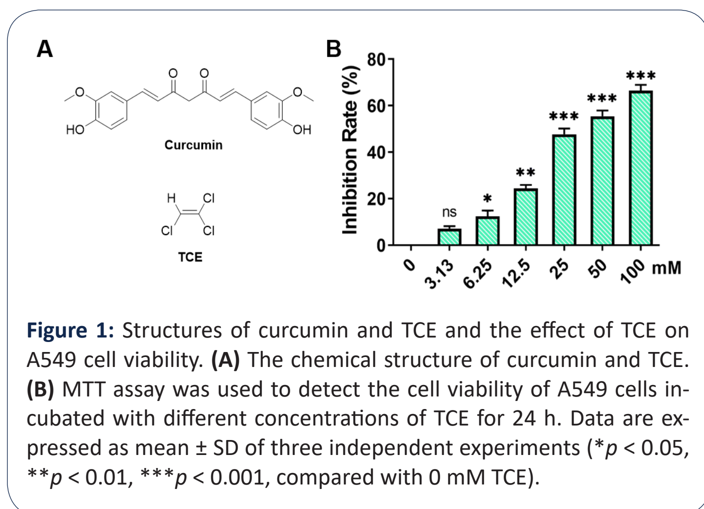

Figure 1: Structures of curcumin and TCE and the effect of TCE on A549 cell viability. **(A)** The chemical structure of curcumin and TCE. **(B)** MTT assay was used to detect the cell viability of A549 cells incubated with different concentrations of TCE for 24 h. Data are expressed as mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with 0 mM TCE).

Curcumin alleviates the inhibitory effect of TCE on cell proliferation and migration

To investigate the effect of curcumin (20 μM) and/or TCE (5 mM) on the proliferation of A549 cells, a clone formation assay was performed. After two weeks, the number of clones formed in the control and curcumin-only groups was 181±6.9 and 176±4.1, respectively (Figure 2A). While the TCE group was 75 ± 11.5, the curcumin + TCE group was 132 ± 1.0. The results indicated that TCE could significantly inhibit the proliferation of A549 cells with an inhibition rate of 58.56% and the inhibition rate of TCE+curcumin group was 27.07%. Curcumin reduced TCE-induced A549 cell proliferation by 31.49%, indicating that curcumin could alleviate the inhibitory effect of TCE on A549 cells (p<0.05).

To verify the effect of curcumin (20 μM) and/or TCE (5 mM) on the migration ability of A549 cells, a wound healing test was carried out for 24 h and 48 h. The result is shown in Figure 2B. The cell migration rates of the control group and curcumin-treated group were 32.93% and 30.67% at 24 hours, and 43.82% and 42.98% at 48 hours, respectively. However, the migration rates in the TCE only treatment group at 24h and 48h were 10.15% and 12.12%, respectively. The migration rate of curcumin+TCE treatment group was 27.24% at 24h and 33.54% at 48h, which was significantly higher than that of TCE only treatment group. The results indicated that TCE exposure inhibited the migration ability of A549 cells and curcumin could alleviate the inhibition of migration ability caused by TCE, which was in agreement with western blot analysis (Figure 2C). The expression of MMP2 in A549 cells in curcumin+TCE group was significantly higher than that in TCE-only group. These results suggest that curcumin alleviates the inhibitory effect of TCE on A549 cell migration.

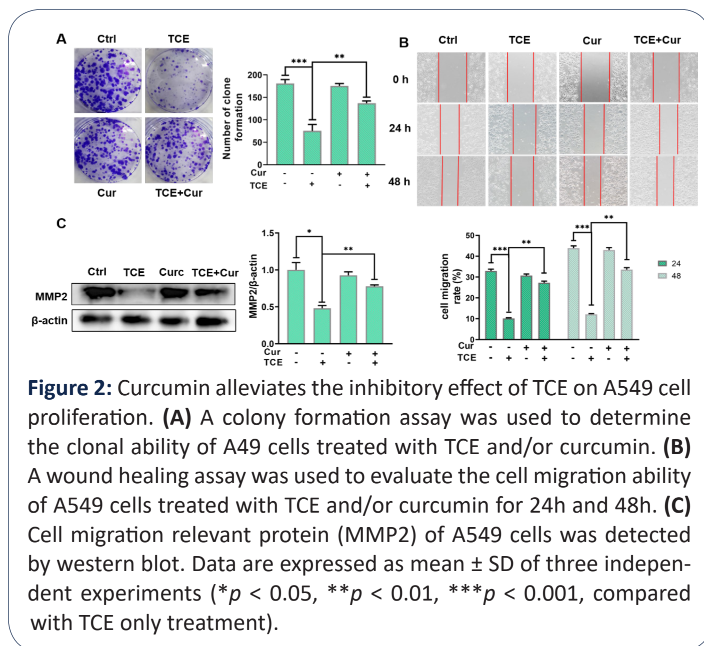

Figure 2: Curcumin alleviates the inhibitory effect of TCE on A549 cell proliferation. **(A)** A colony formation assay was used to determine the clonal ability of A49 cells treated with TCE and/or curcumin. **(B)** A wound healing assay was used to evaluate the cell migration ability of A549 cells treated with TCE and/or curcumin for 24h and 48h. **(C)** Cell migration relevant protein (MMP2) of A549 cells was detected by western blot. Data are expressed as mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with TCE only treatment).

Curcumin inhibits intracellular ROS levels in A549 cells

The change of intracellular ROS level was observed and analyzed by ROS assay. As shown in Figure 3A, A549 cells were loaded with ROS fluorescent probes and the green color represented elevated intracellular ROS levels. Curcumin (20 μM)-treated cells showed no significant change compared with the control group, while TCE (5 mM) treated cells showed a significant increase in

green fluorescence, which was very similar to the Rosup positive control. However, the green fluorescence of curcumin+TCE group was significantly decreased. The expressions of ROS-related proteins were demonstrated by western blot in Figure 3B. The expression of the oxidation marker gp91-phox in the curcumin+TCE group was decreased compared with the TCE only exposure group, while Nrf2 was significantly increased. Nrf2 is an essential transcription factor that regulates an array of detoxifying and antioxidant defense gene expression in the liver. The results of ROS assay and western blot indicated that the antioxidant system was activated to inhibit the occurrence of ROS. In conclusion, curcumin can inhibit the increase of ROS level in A549 cells induced by TCE (5 mM).

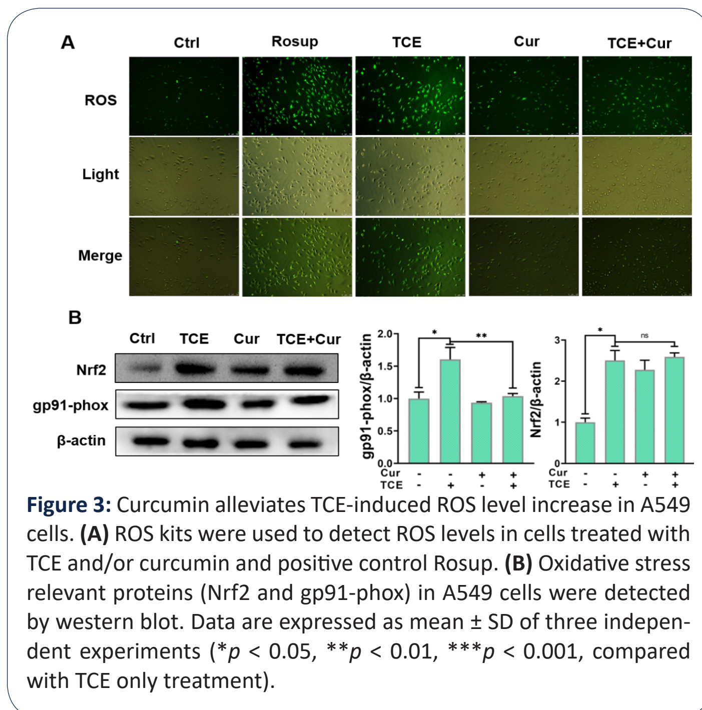

Figure 3: Curcumin alleviates TCE-induced ROS level increase in A549 cells. **(A)** ROS kits were used to detect ROS levels in cells treated with TCE and/or curcumin and positive control Rosup. **(B)** Oxidative stress relevant proteins (Nrf2 and gp91-phox) in A549 cells were detected by western blot. Data are expressed as mean ± SD of three independent experiments (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with TCE only treatment).

Curcumin protects membrane integrity and reduces apoptosis against TCE in A549 cells

The degree of cell membrane damage was detected with the mitochondrial membrane potential detection kit. In normal mitochondria, JC-1 forms aggregate in the mitochondrial matrix and emits strong red fluorescence. In lesions, JC-1 can only exist in the cytoplasm as a monomer, producing green fluorescence [19]. A549 cells treated with curcumin (20 μM) and/or TCE (5 mM) were stained with JC-1 staining solution. The results are shown in Figure 4A. Mitochondrial changes were not significantly different between the control group and the curcumin-only group. In contrast, the green fluorescence in the TCE exposure group was significantly increased, which suggests that the mitochondrial membrane of the cell is damaged. The green fluorescence of cells in the curcumin + TCE group was significantly reduced compared with the TCE alone treatment group, which indicated that curcumin could alleviate the membrane potential damage caused by TCE.

To investigate the effect on the apoptosis ability of A549 cells, the apoptosis rate of A549 cells after TCE (5 mM) and/or curcumin (20 μM) treatment for 24 h was determined using Annexin V-FITC/PI staining (Figure 4B). The results showed that the apoptosis rates of A549 cells in the control and curcumin-treated groups were 19.4 ± 3.23% and 23 ± 4.93%, respectively. The apoptosis rate of A549 cells in TCE treatment group was 78.4 ± 4.78%, and the apoptosis rate of cells treated with curcumin (20 μM) and TCE (5 mM) was significantly reduced by 46.6 ± 4.49%. These results suggest that curcumin can effectively protect A549 cells from TCE-induced apoptosis. The results of western bolt in Figure 4C showed that the expression of the pro-apoptotic protein cleaved caspase 3 in the TCE treatment group was significantly increased, and the expression of the anti-apoptotic protein Bcl2 was decreased. However, the curcumin + TCE treatment group decreased the expression of cleaved caspase 3 and increased Bcl2, which indicated that curcumin could induce apoptosis against TCE in A549 cells.

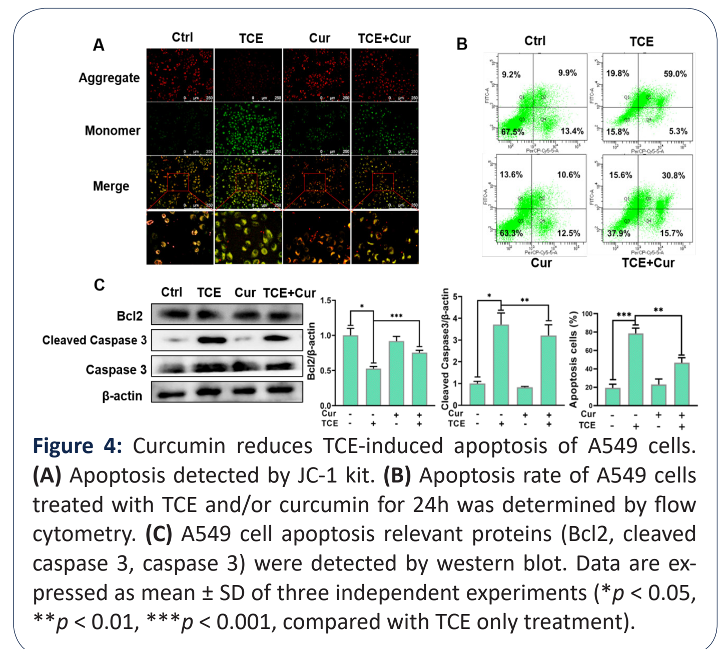

Figure 4: Curcumin reduces TCE-induced apoptosis of A549 cells. **(A)** Apoptosis detected by JC-1 kit. **(B)** Apoptosis rate of A549 cells treated with TCE and/or curcumin for 24h was determined by flow cytometry. **(C)** A549 cell apoptosis relevant proteins (Bcl2, cleaved caspase 3, caspase 3) were detected by western blot. Data are expressed as mean ± SD of three independent experiments (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with TCE only treatment).

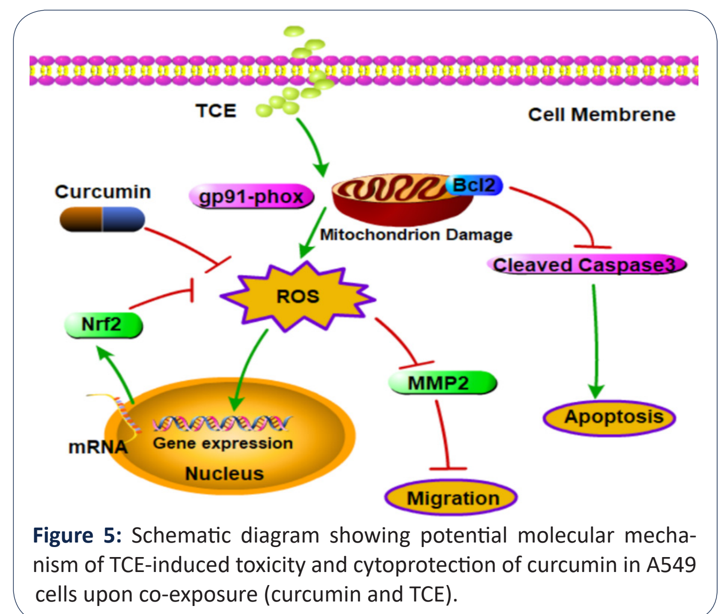

Figure 5: Schematic diagram showing potential molecular mechanism of TCE-induced toxicity and cytoprotection of curcumin in A549 cells upon co-exposure (curcumin and TCE).

Discussion

Trichloroethylene (TCE) is an occupational high-risk poison and many workers die from TCE occupational hazards every year due to its widespread use [20]. The toxicity of TCE to cells is mainly caused by intracellular oxidative stress, which in turn leads to

oxidative damage to cells and eventually leads to cell death [8]. Curcumin, as a natural compound, has been confirmed to have various pharmacological activities such as anti-inflammatory, antioxidant, and anti-tumor [21,22]. This study mainly explores that curcumin reduces TCE-induced cytotoxicity through its antioxidant capacity. Curcumin works by enhancing anti-oxidative intracellular defense system, alleviating the inhibitory effect of TCE on cell proliferation and migration, improving cell membrane integrity and regulating apoptosis.

Our study showed that 5mM TCE decreased the viability of A549 cells. In cell cloning experiments and scratch healing experiments, TCE exhibited inhibitory effects on the proliferation and migration of A549 cells (Figures 2A, 2B). Compared with the TCE group, the proliferation and migration ability of the cells in the curcumin+TCE group were significantly restored. We also detected the cell migration-related protein MMP2 by western bolt. MMP2 is involved in angiogenesis by disrupting basal layer molecules and remodeling the extracellular matrix during angiogenesis. Involved in cell migration activities, high expression of MMP2 makes it easier for tumor cells to invade surrounding tissues and metastasize to secondary sites [23]. Therefore, the detection of MMP2 protein allows us to effectively evaluate the migration ability of cells. In the TCE exposure group, the expression of MMP2 protein was significantly decreased (Figure 2C). Significant recovery of MMP was obtained in the curcumin+TCE group. That is, the migratory ability of the cells is restored. The experimental results indicated that curcumin could relieve the inhibition of A549 cell proliferation and migration ability induced by TCE.

Many researchers have reported that the toxic effects of TCE on various cells are mainly inducing oxidative stress and leading to apoptosis [9]. When cells are exposed to toxic substances such as TCE, the antioxidant system in the cell is activated and can produce antioxidants (such as enzymes, vitamins and flavonoids) to neutralize intracellular superoxide anions and maintain a stable intracellular environment. When the level of intracellular oxidation is too high, there will be an imbalance between oxidation and anti-oxidation. As a result, the excess superoxide anion in the cell cannot be removed, attacking the cell and causing damage to the cell [24]. While curcumin benefits from its antioxidant pharmacological action, it can effectively remove excess free radicals in cells, relieve intracellular oxidation levels and protect cells from damage [21].

In this study, we measured the level of oxidation inside the A549 cell. The reactive oxygen species detection kit is a commonly used reactive oxygen species detection method and intracellular oxidative stress can be detected by the kit in the form of green fluorescence. The results of this study showed that the fluorescence level of A549 cells exposed to TCE was significantly increased under microscope, and close to the positive control group (Figure 3A). The green fluorescence level of A549 cells in the curcumin+TCE group was significantly lower than that in the TCE-treated group, which indicated elevated levels of oxidative stress in cells. In addition, gp91-phox protein, as a marker of oxidative stress, was detected by western bolt analysis (Figure 3B). Compared with the increase in the expression of gp91-phox in the TCE group, it could be proved that the co-exposure of curcumin and TCE could significantly reduce the level of intracellular oxidative stress.

Recently, many scientific researchers reported that curcumin exhibited strong protective effects through the Keap1/Nrf2 signaling pathway. Nrf2 is considered to be an emerging and important regulator of cellular antioxidants [25]. Our results showed that the expression of Nrf2 in cells was significantly increased in the TCE exposure group, while the expression of Nrf2 in the curcumin + TCE treatment group was higher than that in the control group. This means that the antioxidant system in the cell is activated and plays an important role in scavenging peroxides in cells. Oxidative stress induced by TCE is alleviated by the antioxidant effect of curcumin (Figure 3B). Our findings are similar to those reported by previous research [26]. They demonstrated that curcumin protected PC12 cells from arsenic-induced oxidative damage by activating Nrf2. Therefore, curcumin protected A549 cells from TCE-induced oxidative stress.

TCE can cause cell death and the way is through damaging and destroying the mitochondrial membrane of cells. The apoptosis rate of A549 cells in the TCE treatment group was significantly increased by JC-1 experiments and flow cytometry in Figure 4A, 4B. The apoptosis rate of A549 cells in the curcumin + TCE group was significantly lower than that in the TCE group. We also investigated proteins associated with apoptosis in Figure 4C. Members of the Bcl2 family play important regulatory roles in mitochondrial membrane permeability and can inhibit cell apoptosis [27]. Caspase 3 protein is a key protein in the apoptotic pathway, and cleaved caspase 3 can be activated to induce cell apoptosis [28]. The result showed that TCE exposure group significantly increased the expression of the pro-apoptotic protein cleaved caspase 3 and decreased the expression of the anti-apoptotic protein Bcl2. Similar findings were also reported [26]. In the curcumin + TCE group, the decreased expression of cleaved caspase 3 and the increase of Bcl2 indicated that curcumin successfully reduced TCE-induced apoptosis in A549 cells.

In conclusion, we believe that cell apoptosis induced by TCE is closely related to oxidative stress. Curcumin works by scavenging excess free radicals and reducing oxidative stress levels in cells.

Materials and methods

Chemicals and materials

Trichloroethylene (CAS No. 79-01-6) (Aladdin, Shanghai, China) and curcumin (CAS No. 458-37-7) (Beijing Lujia Technology Co., Ltd, Beijing, China) were dissolved by dimethyl sulfoxide (DMSO) (St. Louis, Mo, USA). The second antibody was purchased from cell signaling technology (CST) (Danvers, Ma, USA). Antibodies to MMP2 (ab92536), Nrf2 (ab62352), gp91-phox (ab129068), Bcl2 (ab182858), cleaved caspase 3 (ab32042) were purchased from Abcam Biological Technology (USA). Antibodies to caspase 3 (#14220) were purchased from Cell Signaling Technology (USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazole bromide (MTT) was purchased from Sigma (USA).

Cell culture and treatment

The human lung cancer cell line A549 was purchased from ATCC. The cell lines were cultured in DMEM medium (Biosharp, Lanjieke, Beijing) supplemented with 10% fetal bovine serum (Sijiqing Bioengineering Materials, Hangzhou, China). The cells were placed in a 37°C incubator with 5% of CO_2.

Cell viability analysis

Cell viability assay of TCE to A549 cell was carried out using 96-well plate cultures and MTT staining [29]. Briefly, the A549 cell line (10000 cells/well) was treated with TCE at different concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100mM) in 96-well culture plates for 24 h. Then 10 μL of MTT (5 mg/mL MTT in PBS) solution was added to each well, and the microplates were further incubated at 37°C for 4 h. After carefully removing the medium, the precipitates were dissolved in 200 μL of DMSO in the dark, shaken mechanically for 10 min, and then absorbance values at a wavelength of 490 nm were taken on a Super Microplate Reader (MQX2OO) (BioTek, the United States). Cell inhibition rate (%) = 1- [(Experimental group - Blank group) / (Control group - Blank group)] × 100%. IC_{50} values were calculated through nonlinear fitting in GraphPad Prism 8 software. The results were determined from replicates of 96 wells from at least three independent experiments.

Clone formation assay

Cell clone formation assay is an important technical method used to detect cell proliferation ability [30]. A549 cells were seeded in 6-well plates (approximately 50 cells/well). They were treated with curcumin and/or TCE and placed in a cell incubator. When a single cell proliferates for more than 6 passages, it can form a cell population that is visible to the naked eye. The former colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cell colonies was counted and compared with the control group.

Wound-healing assay

A wound healing test was used to evaluate the effects of curcumin on tumor cell motility by TCE-induced A549 tumor cell lines [31]. A549 cells (2×10^5 cells/well) were seeded in 6-well plates containing 15% fetal bovine serum medium. Treated with curcumin (20 μM) and/or TCE (5 mM), cultured in a cell incubator at 37°C, 5% CO_2. When the cells grow to about 80%, use a 200 μM pipette tip to draw a straight line in the 6-well plate, and use PBS to remove unattached or damaged cells. The width of the scratched area was then photographed with an optical microscope at 0h, 24h and 48h.

Intracellular ROS levels measurement

The ROS detection kit was used to analyze the changes of ROS levels in A549 cells exposed to TCE. Briefly, A549 cells were loaded with probes first and then treated with curcumin (20 μM) and/or TCE (5 mM). Rosup was added to new A549 wells as a positive control for 1.5 hours of dosing stimulation. After 2 hours, the cell culture medium was changed to 1 × PBS, and the ROS level of A549 cells was quantitatively observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Mitochondrial membrane damage detection

Cells were seeded in 24-well plates at a cell density of 2×10^5 cells/well. The cells were pretreated with curcumin and/or TCE and then stained with the pre-configured JC-1 dye pair. After shaking gently, the 24-well plate was placed in an incubator for 20 minutes. The plate was then washed and 1 × PBS was added. Mitochondrial membrane damage was observed under a fluorescence microscope.

Flow cytometry analysis

Annexin V-FITC Apoptosis Detection Kit (BioVision, USA) was used. A549 cell death was analyzed using flow cytometry. Briefly, A549 cells treated with curcumin (20 μM) and/or TCE (5 mM) were cultured for 24 h and washed with 1 × PBS. After washing, cells were suspended in 500 μL of 1× binding buffer containing 5 μL Annexin V-FITC and propidium iodide (PI). The resulting solution was then inhibited for 5 minutes in the dark. Finally, A549 cell samples were analyzed by flow cytometry (BD FACSVerse, BD Biosciences).

Western blot

Western blot analysis was performed according to the previous reference [32]. Proteins from A549 cells that had been treated with curcumin and/or TCE for 24 hours were extracted. They were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Incubate with primary antibodies against MMP2, gp91-phox, caspase 3, cleaved caspase 3, Bcl2, Nrf2 for 12 hours at 4°C. Membranes were then washed three times with TBST and incubated with HP-conjugated goat anti-rabbit or anti-mouse IgG for 1.5 h at room temperature. After washing with TBST to remove excess antibody, the expressions of the target proteins were detected by a chemiluminescence detection system according to the instructions.

Statistical analysis

All experiments were performed three times independently under the same conditions and were expressed as the mean standard error (SEM) of the mean. The T test (p=0.05) was used to determine the statistical significance between the two groups. SPSS software was used for all statistical analyses. Differences were considered statistically significant if the p-value was less than 0.05 (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Conclusion

In conclusion, curcumin may regulate the expression of apoptosis-related proteins and enhance the antioxidant defense system to inhibit the generation and accumulation of ROS. Thus, A549 cells are effectively protected from TCE-induced oxidative damage and cytotoxicity. It can be seen that the natural compound curcumin has strong antioxidant and anti-apoptotic effects. These findings suggest that curcumin would be a potential and safe therapeutic agent to combat TCE toxicity. Further studies on the interaction and protective mechanisms of curcumin against TCE-induced toxicity are warranted.

Declarations

Conflicts of interest: The authors declare that they have no conflicts of interest.

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