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Article

Curcumin Ameliorates Furazolidone Induced DNA Damage and Apoptosis in Human Hepatocyte L02 Cells via Inhibiting ROS Production and Mitochondrial Pathway

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Abstract: Furazolidone (FZD), a synthetic nitrofuran derivative, has been widely used as an antibacterial and antiprotozoal agent. Recently, the potential toxicity of FZD has raised concerns, but its mechanism is still unclear. This study aimed to investigate the protective effect of curcumin on FZD induced cytotoxicity and the underlying mechanism in human hepatocyte L02 cells. The results showed that curcumin pre-treatment significantly ameliorated FZD induced oxidative stress, characterized by decreased reactive oxygen species (ROS) and malondialdehyde formations, and increased superoxide dismutase, catalase activities and glutathione contents. In addition, curcumin pre-treatment significantly ameliorated the loss of mitochondrial membrane potential, the activation of caspase-9 and -3, and apoptosis caused by FZD. Alkaline comet assay showed that curcumin markedly reduced FZD-induced DNA damage in a dose-dependent manner. Consistently, curcumin pre-treatment markedly down-regulated the mRNA expression levels of p53, Bax, caspase-9 and -3 and up-regulated the mRNA expression level of Bcl-2. Taken together, these results revealed that curcumin protects against FZD induced DNA damage and apoptosis via inhibiting oxidative stress and mitochondrial pathway. Our study indicated that curcumin may be a promising combiner with FZD to reduce FZD-related toxicity in the clinical applications.

Keywords: curcumin; furazolidone; oxidative stress; DNA damage; mitochondrial pathway

1. Introduction

Furazolidone (FZD), a synthetic nitrofuran drug, has been used to treat the infections caused by bacteria and protozoa in human and animals [1,2]. Recent literatures reported that FZD had the novel applications in treating leukemia [3] or leishmaniasis [4]. Especially, FZD is usually used as a low-cost and effective drug to treat the infections caused by *Helicobacter pylori* in human in developing countries including China [5]. However, FZD is limited in the clinic due to potential side effects, such as genotoxicity, hepatotoxicity, and carcinogenicity [5-7]. A pooled-data analysis reported that FZD-based regimens achieved low eradication rates for *Helicobacter pylori* infections at the current dosage regimen, but the incidence of severe side effects was observed when increasing the dose [8]. As a result, development of agents against FZD-related adverse effect is very urgent and it is a crucial strategy for optimizing potential antimicrobial activity and clinical usage of FZD.

Previous study showed that oxidative stress may play a major role in FZD induced cytotoxicity and genotoxicity in human hepatoma (HepG2) cells [9]. The liver was suggested as the primary target organ of FZD metabolic using the pig model; in addition, its metabolites 3-amino-2-oxazolidinone could be cumulative in liver [10]. The *in vitro* studies showed that FZD at the concentrations of 4–10 μg/mL could increases the frequency of sister chromatid exchanges (SCE) in human lymphocyte and

increased SCE was detected when mice were exposed to FZD at the dose of 30 mg/kg [11]. Reactive oxygen species (ROS) are mainly generated from mitochondria [12, 13]. Mitochondrion is the major mediator of oxidative stress and it is considered as the most vulnerable target in the process of FZD induced cytotoxicity [9, 13]. Very recently, Deng et al demonstrated that mitochondrial pathway and phosphatidylinositol-3-kinase (PI3K)/Akt pathway played the critical roles in FZD induced apoptotic cell death in HepG2 cells [14].

Curcumin, a natural polyphenol in the spice turmeric, has many biological functions, such as anti-inflammatory, anti-oxidative, anti-carcinogenic and immuno-regulatory abilities [15]. Many studies have demonstrated that curcumin could protect against DNA damage and oxidative stress caused by some drugs or environmental mutagens including arsenic [16], acrylamide [17] and cisplatin [18] via scavenging ROS and improving the anti-oxidative ability. Curcumin administration showed immense therapeutic effect against *Helicobacter pylori* infection in mice and reduced the gastric damage due to infection [19]. In addition, curcumin also showed antiparasitic potential, including trypanocidal and leishmanicidal activity, in several *in vitro* and *in vivo* models [20, 21]. Human clinical trials showed that healthy human volunteers orally administrating 500 mg of curcumin per day for 7 days significantly decreased the level of serum lipid peroxide, a biomarker of oxidative stress [22]. Curcumin combination with some antibiotics and chemotherapy agents showed the better therapeutic effect for infections and cancer compared to either one alone, which have raised wide concern in clinical practice [23-25]. Therefore, the present study investigated the protective role of curcumin on FZD induced cytotoxicity and DNA damage using hepatocyte L02 cells, aimed to provide a promising combination of FZD with curcumin in the toxicology aspect *in vitro*.

2. Results

2.1 Curcumin attenuates FZD induced cytotoxicity in L02 cells

FZD treatment for 24h reduced the cell viability of L02 cells in a dose-dependent manner. Compared to the negative control group (0.2% DMSO), FZD treatment at 10, 20, 40 and 60 μg/mL for 24h significantly decreased the cell viabilities to 89.4%($p<0.05$), 73.2% ($p<0.01$), 53.4%($p<0.01$) and 42.6%($p<0.01$), respectively (**Figure 1**). However, curcumin pre-treatment, especially at the final concentrations of 2.5 and 5 μM, markedly attenuated FZD induced cytotoxicity, compared to the FZD alone treatment groups (**Figure 1**). There was no significant change in cell viability in curcumin alone treatment (at 1.25, 2.5 and 5 μM, respectively) groups (**Figure 1**).

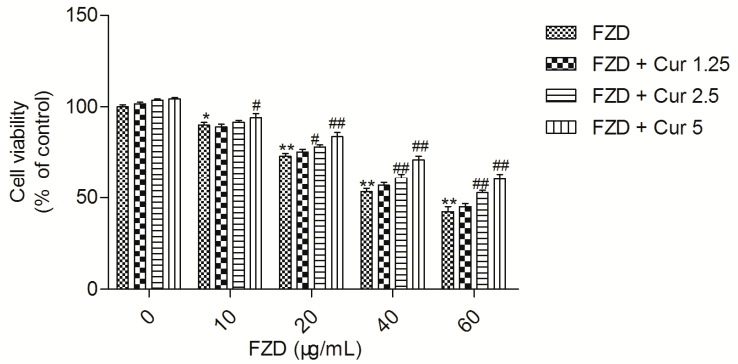

Figure 1. Curcumin protects against FZD induced cytotoxicity in L02 cells. Values were presented as mean ± SD, from five independent experiments. *$p<0.05$, **$p<0.01$, compared to the negative control group (0.2% DMSO); #$p<0.05$, ##$p<0.01$, compared to the FZD alone group. FZD, furazolidone.

2.2 Curcumin suppresses FZD induced oxidative stress in L02 Cells

To investigate the protective effect of curcumin, the intracellular ROS and the levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) were measured. As shown in **Figure 2**, compared to the negative control group, FZD treatment at 40 μg/mL for 24h significantly increased the production of intracellular ROS to approximate 4.5 folds ($p<0.01$) (**Figure 2A**). Meanwhile, after FZD treatment, MDA level increased to 3.1 nmol/mg protein (equal to 194% of control), and the SOD, CAT activities and GSH levels decreased to 21.3 U/mg protein, 41.1 U/mg protein and 84.5 mg/g protein (equal to 56.2 %, 58.7% and 61.4% of control, respectively) (all $p<0.01$), compared to the control group(**Figure 2B-E**). Moreover, curcumin pretreatment, especially at 5μM could effectively inhibit the ROS production, decrease the levels of MDA, and increase the activities of SOD and CAT and GSH levels (all $p< 0.01$), respectively, compared to the FZD alone group (**Figure 2**).

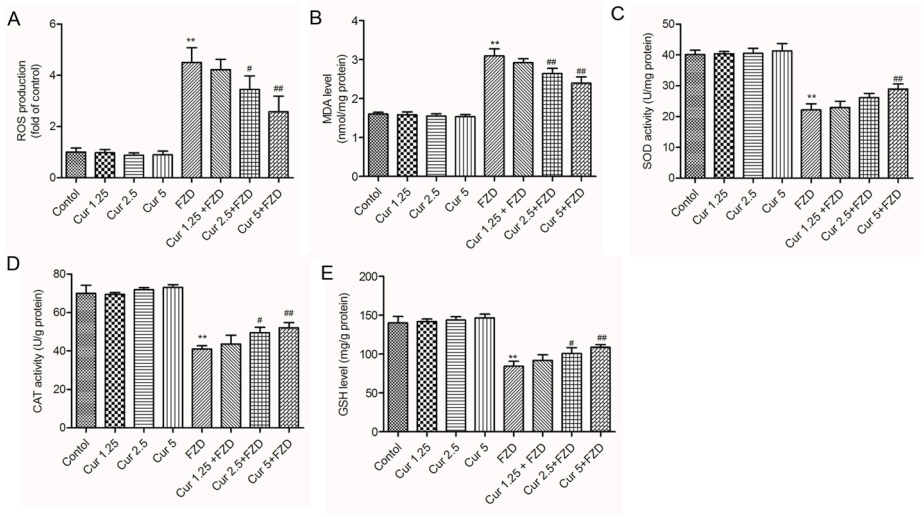

Figure 2. Curcumin protects against FZD induced oxidative stress in L02 cells. (A) Intracellular ROS levels were detected using the ROS sensitive dye 2, 7-dichlorofluorescein diacetate staining. (B)-(E) The effects of curcumin pretreatment at 1.25, 2.5 and 5 μM on the cellular MDA level, SOD, CAT activities and GSH level. Values were presented as mean ± SD, from three independent experiments. **$p<0.01$, compared to the negative control group; #$p<0.05$, ##$p<0.01$, compared to the FZD alone group.

2.3 Curcumin protects against FZD induced the loss of mitochondrial membrane potential ($\Delta\psi_m$) in L02 cells

As shown in **Figure 3**, FZD treatment at 40 μg/mL for 24h significantly decreased $\Delta\psi_m$ to 49.6 % ($p<0.01$), compared to the negative control group. Curcumin treatment at 1.25, 2.5 and 5 μM had no effect on the changes of $\Delta\psi_m$, but significantly reduced the loss of $\Delta\psi_m$ caused by FZD in a dose – dependent manner (increased to 53.8%, 62.6%, 69.8%,respectively), compared to FZD alone group (**Figure 3B**).

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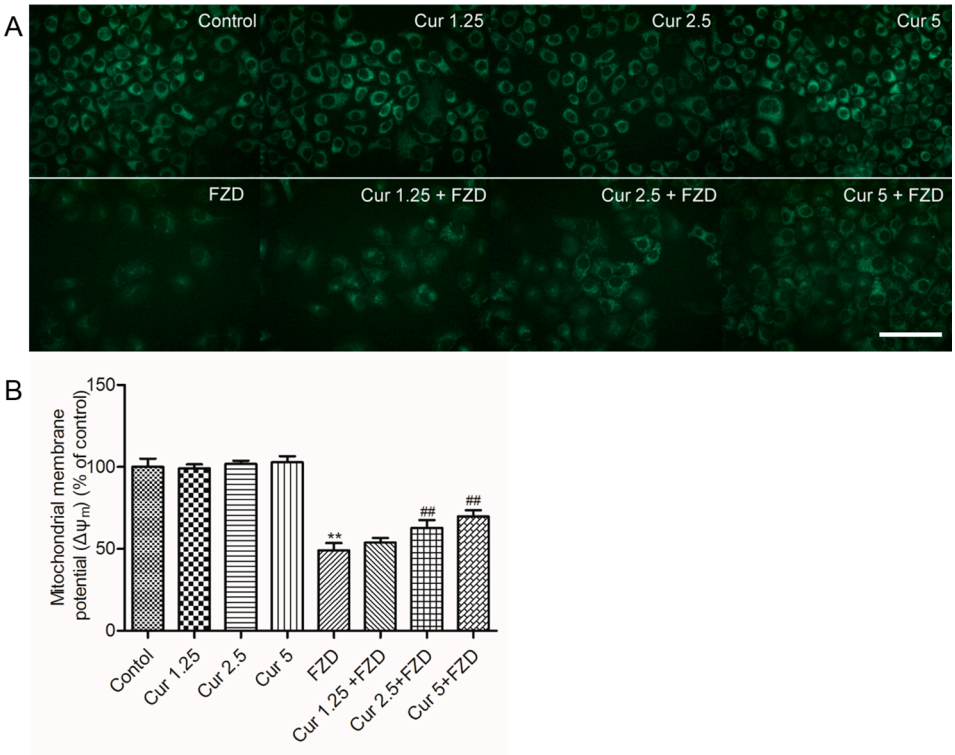

Figure 3. Curcumin reduces FZD induced the loss of mitochondrial membrane potential ($\Delta\psi_m$) in L02 cells. (A) The changes of $\Delta\psi$m were observed under a fluorescence microscope after stained with 10 μg/mL Rhodamine 123. Bar=50 μm. (B) The average fluorescent intensities from for five independent microscopic fields were analyzed using Image Pro Plus 5.0 software. The reduction of Rhodamine 123 fluorescence indicated loss of mitochondrial membrane potential. $^{**}p<0.01$, compared to the negative control group; $^{\#\#}p<0.01$, compared to the FZD alone treatment group.

2.4 Curcumin inhibits FZD induced the activations of caspase-9 and -3

As shown in Fig.4, FZD treatment of L02 cells at 40 μg/mL for 24h significantly increased the activities of caspase-9 and -3 to 3884.3 and 5623.4 units/mg protein (4.2 and 4.7 folds of control), respectively, compared to the negative control group. Curcumin pretreatment at 2.5 and 5 μM significantly deceased the caspase-9 activity to 2951.2 and 2112.5 units/mg protein (equal to 3.2 and 2.3 folds of control) (**Figure 4A**), and decreased the caspase-3 activity to 4498.6 and 3334.1 units/mg protein (**Figure 4B**), respectively (all $p<0.05$ or 0.01). There was no marked change of caspase-9 and -3 activities in curcumin alone treatment groups (**Figure 4 A and B**).

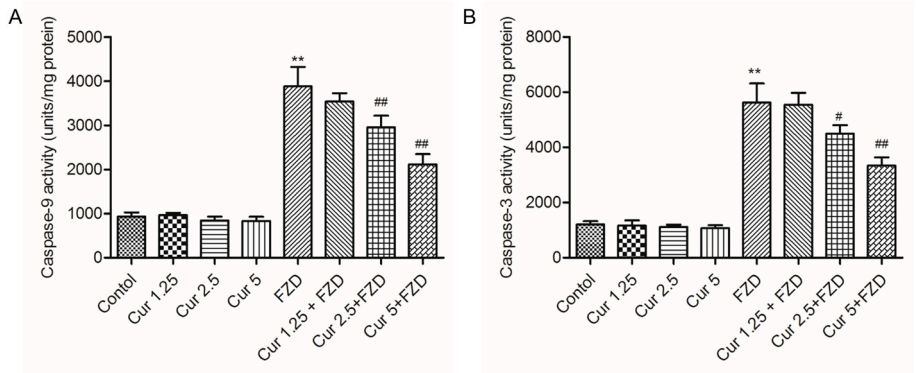

Figure 4. Curcumin treatment attenuated the increases of caspase-9 and -3 activities caused by FZD exposure. L02 cells were pretreated with curcumin at 1.25, 2.5 and 5μM for 2h, followed to wash with PBS twice, then exposed with or without FZD at 40 μg/mL for additional 24h, caspase-9 (A) and 3 (B) activities were measured, respectively. Values were presented as mean ± SD, from three

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independent experiments. **p<0.01, compared to the negative control group; #p<0.05, ##p<0.01, compared to the FZD alone treatment group.

2.5 Curcumin reduces FZD induced apoptosis in L02 cells

After FZD treatment, L02 cells displayed condensed and fragmented chromatin and bright blue nuclei, which are indicative of apoptosis (**Figure 5A**). Furthermore, the apoptosis ratios were investigated by flow cytometry. Compared to the negative control group, FZD treatment increased the apoptotic rates to 43.7% (p<0.01). Curcumin pretreatment at 2.5 and 5 μM significantly attenuated FZD induced nuclear condensation and fragmentation, and the apoptotic rates decreased to 33.2% and 22.6 % (both p<0.01) (**Figure 5B**), respectively, compared to the FZD treatment group. The nuclear morphology and apoptotic rate had no marked change in the curcumin alone treatment groups (Figure 5), compared to the negative control group.

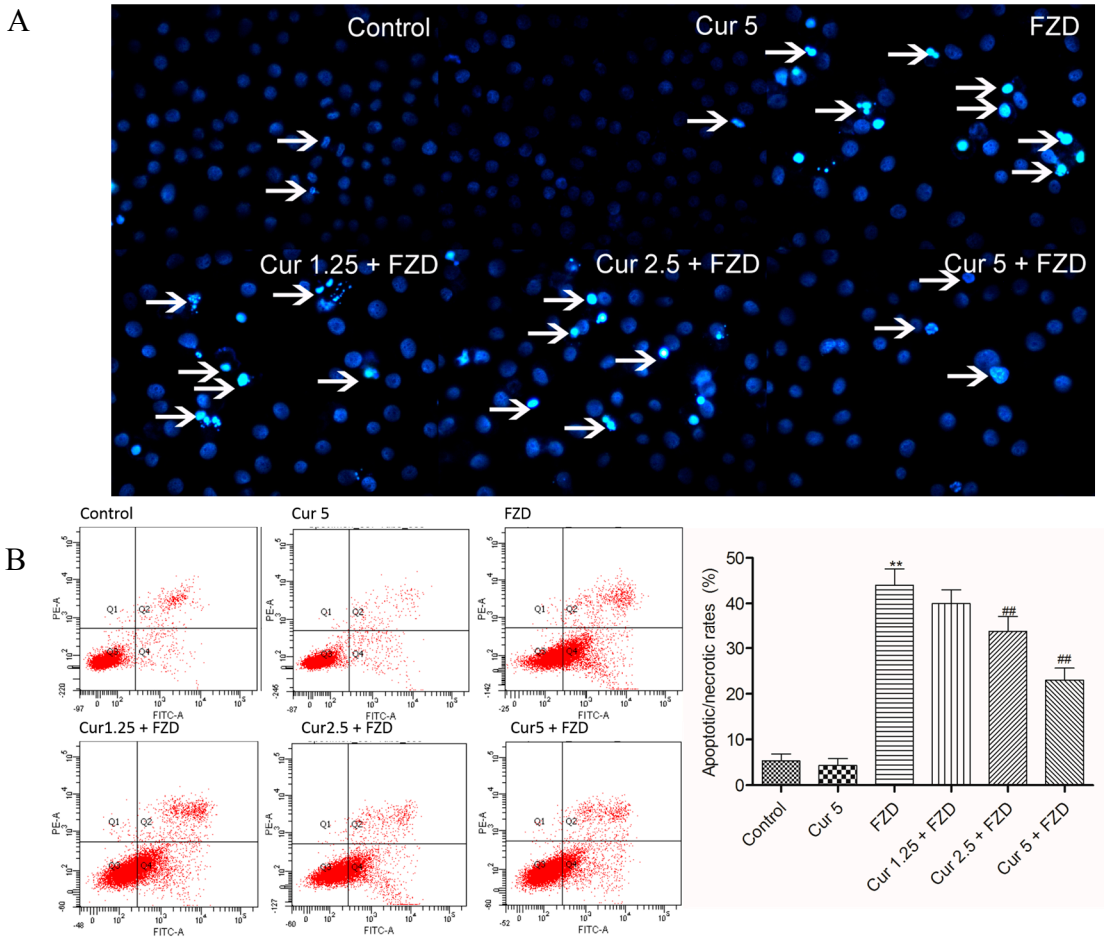

Figure 5. Curcumin treatment reduces FZD induced the apoptosis in L02 cells. L02 cells were pretreated with curcumin at 1.25, 2.5 and 5μM for 2h, followed to wash with PBS twice and expose with FZD at 40 μg/mL for additional 24h. (A) Nuclear morphology of L02 cell was observed and photographed by fluorescence microscopy following Hoechst 33342 staining. The white arrows indicated apoptotic cells. (B) Cellular apoptosis were quantified using flow cytometry following annexin V-FITV/PI staining. Q1 necrosis cells, Q2 later apoptotic cells, Q3 survival cells, Q4 early apoptotic cells. Values were presented as mean ± SD, from three independent experiments. **p<0.01, compared to the negative control group; ##p<0.01, compared to the FZD alone treatment group.

2.6 Curcumin reduces FZD induced DNA damage in L02 cells

FZD exposure at 40 μg/mL for 3 h caused L02 cells the marked DNA damage, as indicated by the greater migration of DNA fragments on the agarose gel (**Figure 6A**). The tail length and tail DNA

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(%) were both significant higher (both $p < 0.01$), compared with the negative control group (**Figure 6 B and C**). The tail moment significantly increased to 30.2 μm, exceeding over 29 folds (**Figure 6D**). The pretreatment with 1.25, 2.5 and 5 μM prevented FZD-induced DNA damage in a dose-dependent manner, the tail moment decreased to 23.2, 13.5 and 5.4 μm (all $p<0.05$ or 0.01) (**Figure 6D**), respectively, as well as the decreases of tail length and tail DNA (%).Curcumin treatment at 5 μM did not cause the significant increases in tail length, tail DNA (%) and tail moment, compared to the negative control group.

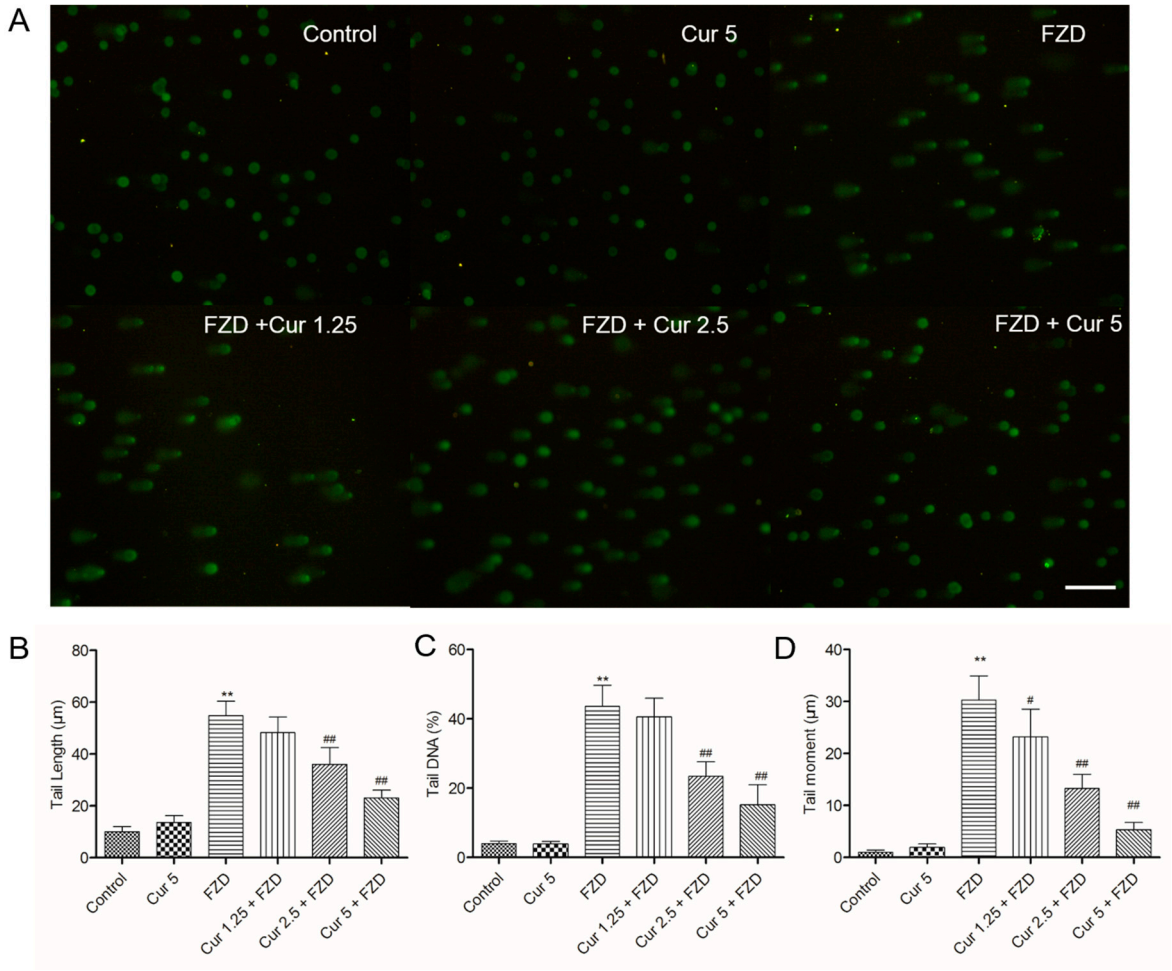

Figure 6. Curcumin treatment reduces FZD induced DNA injury in L02 cells. (A) L02 cells were pretreated with curcumin at 1.25, 2.5 and 5μM for 2h, followed to wash with PBS twice and treat with or without FZD at 40 μg/mL for additional 3h, alkaline comet assay was carried out to evaluate the DNA injury. (A) The typical DNA comet images were showed. Bar=100 μm (B)-(D) represented the analysis results of tail length, tail DNA (%) and Tail moment (μm), respectively. Values were presented as mean ± SD from at least 100 cells. **p<0.01, compared to the negative control group; #p<0.05, ##p<0.01, compared to the FZD alone group.

2.7 Curcumin regulates the mRNA expression levels of apoptosis related factors

FZD treatment at 40 μg/mL for 24h significantly increased the mRNA expression levels of caspase -9, caspase-3, Bax and p53 to 3.6, 4.5, 2.4 and 2.7 folds (all $p<0.01$), and decreased the mRNA expression level of Bcl-2 to 0.43 fold ($p<0.01$) (**Figure 7**), compared to the negative control group. However, curcumin pretreatment, especially at 2.5 and 5 μM, significantly ameliorated the expression levels of these genes, i.e., caspase-9 mRNA expression level decreased to 2.7 and 1.8 folds, caspase-3 mRNA expression level decreased to 3.2 and 2.6 folds, Bax mRNA expression level decreased to 1.8 and 1.5 folds, p53 mRNA expression level decreased to 2.0 and 1.5 folds, and Bcl-2 mRNA expression level increased to 0.64 and 0.76 folds (all $p<0.05$ or 0.01) (**Figure 7**), compared to

the FZD alone treatment group. In the curcumin control group, the expression levels of caspase-9, caspase-3, Bcl-2, Bax and p53 had no marked change, compared to the negative control group (**Figure 7**).

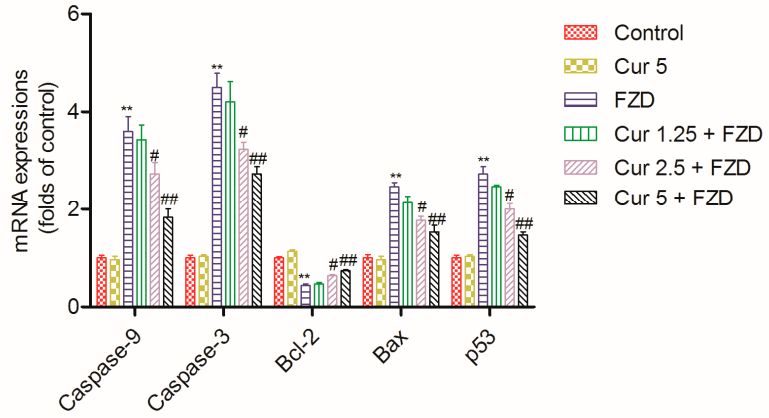

Figure 7. Curcumin treatment ameliorates the mRNA expression levels of apoptosis related factor caused by FZD exposure. Values were presented as mean ± SD, from three independent experiments. **p<0.01, compared to the negative control group; # p<0.05, ##p<0.01, compared to the FZD alone treatment group.

3. Discussion

FZD is widely used to treat the infections caused by gram-positive and -negative bacteria in human clinic due to its better effect and lower cost in some development countries including China [26]. FZD showed potential genotoxicity with a dose-dependent manner in a variety of test systems [9, 11, 27-30]. One previous study showed that oral administration of 40 mg/kg of FZD for 10 days could induce marked renal and hepatic toxicity in goat [31]. Gonzalez Borroto et al. demonstrated that FZD exposure at 20 μg/mL for 3h could induce marked DNA damage in human lymphoblastic TK6 cells [6]. The present study aimed to investigate the protective effect of curcumin on FZD induced DNA damage and apoptosis in human hepatocyte L02 cells.

In current study, FZD treatment at 10-60 μg/mL for 24h significantly decreased the cell viability of L02 cells in a dose dependent manner. Curcumin pretreatment at the range of 1.25-5 μM relieved the decrease of cell viability (**Figure 1**), indicating that curcumin could protect L02 cells against FZD-induced cytotoxicity. ROS commonly consists of the superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\bullet$) [32]. A disturbance between the production of ROS and the antioxidant system defense will result in excessive ROS production, leading to damaging effect on lipids, proteins, and DNA, and ultimately cause cell death [33, 34]. FZD is rapidly degraded in different tissues [2] and $O_2^{\bullet-}$ are produced during FZD metabolism [35]. MDA is used as a biomarker of oxidative stress to evaluate the degree of the peroxidation of membrane lipids [36]. In the present study, FZD exposure significantly increased the production of ROS and MDA content in L02 cells (**Figure 2 A and B**). Besides, we also observed a decrease in GSH levels and the activity of the antioxidant enzymes SOD and CAT (**Figure 2 C- E**). SOD can catalyze the dismutation of superoxide anion into oxygen and H_2O_2 [32]. CAT is a common enzyme and it can catalyze the decomposition of H_2O_2 to water and oxygen [32]. In addition, a previous study demonstrated that the rate of cellular H_2O_2 removal is partly dependent on GSH level [37]. These antioxidants' decrease might be due to FZD induced production of free radicals which in turn can impair the antioxidant defense system, finally resulting in exacerbating oxidative stress. Studies have demonstrated that curcumin can directly interact with $O_2^{\bullet-}$ and H_2O_2 and inhibit oxidative stress even stronger than that of vitamin E, which is an oxygen radical scavenger [38]. The previous studies had demonstrated that curcumin could effectively inhibit cytotoxicity and DNA injury induced by perfluorooctane sulfonate [39], quinocetone [40] and γ-radiation [41] via inhibiting ROS mediated oxidative damage. Consistently, our study found that curcumin pre-treatment could not only inhibit the over-production of ROS induced by FZD, but also enhance the total antioxidant capacity in L02 cells, i.e.,

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up-regulated the activities of SOD and CAT and GSH levels (**Figure 2**). This may be due to the antioxidant sparing action of curcumin. In addition, it has reported that curcumin can trigger the antioxidant response element (ARE) and induce some genes expression including SOD and CAT and GSH when the cells are under oxidative stress condition [41], which also contributed to partly explain the phenomenon (**Figure 2**) in the present study. Comet assay is the most popular method for measuring various types of DNA damage, including oxidative damage inflicted by ROS [40]. Our results further showed that L02 cells exposed to FZD for 3 h suffered severe DNA damage which chartered by increased comet length, tail moment and olive tail moment, but no significantly cell death and apoptosis was observed. Curcumin remarkably prevented the FZD-induced DNA damage in L02 cells in a dose-dependent manner (**Figure 6**). Taken together, these data revealed that curcumin protected against FZD induced cytotoxicity and DNA damage via inhibiting the ROS formation and up-regulation of intracellular antioxidant levels.

In the present study, FZD exposure led to the increase of apoptosis rates (**Figure 5**), characterized by cell shrinkage, chromatin condensation, nucleus condenses and the formation of apoptotic bodies [42]. Mitochondria is the most vulnerable target of ROS and mitochondrial dysfunction may contribute to and cause cell death by trigging endogenous apoptotic cascade reaction [13, 43]. As the data shown, FZD treatment caused significant loss of membrane potential ($\Delta\psi_m$) (**Figure 3**), an important indicative character of mitochondrial dysfunction [44]. Disrupted $\Delta\psi_m$ may influence the opening of mitochondrial permeability transition pore (MPTP), resulting in the release of cytochrome c (CytC) and cascading to the activation of caspase-9, -3 and apoptosome formation [45, 46]. In the present study, curcumin treatment significantly attenuated the loss of $\Delta\psi$m, and decreased the mRNA expression levels of Bax, caspase-9 and -3, and increased the mRNA expression of Bcl-2, then attenuated FZD induced apoptosis rates in L02 cells (**Figures 3, 4 and 7**). Bax and caspase-9 are also important markers of mitochondria apoptotic pathway [45, 46]. Bcl-2 is one of anti-apoptotic Bcl-2 family proteins, which regulates the mitochondrial pathway [33]. Several studies showed that curcumin can block the oxidative stress mediated apoptosis via suppressing the mitochondria apoptotic pathway against cisplatin, palmitate and 6-hydroxydopamin induced cytotoxicity [47-49]. These data indicated that curcumin protected L02 cells against FZD induced apoptosis via inhibiting mitochondrial pathway.

It had demonstrated that the direct activation of Bax by p53 mediated mitochondrial membrane permeabilization, caused mitochondrial CytC release and caspase activation, which triggers apoptosis [46]. The nuclear translocation and transactivation of p53 were usually implicated in apoptotic cell death in response to oxidative stress [50]. The previous study had demonstrated that FZD could significantly increase the p53 protein expression in acute myeloid leukemia cell [3]. In the present study, FZD treatment significantly increased the mRNA level of p53 (**Figure 7**). Our previous studies had showed that p38 MAPK and GADD45a, a downstream gene of p53, participated in FZD induced cell cycle arrest and apoptosis [51, 52]. Another study demonstrated that curcumin protected against 6-hydroxydopamine-induced neurotoxicity through attenuation of p53-mediated apoptosis in the dopaminergic cell line SH-SY5Y [47]. Consistently, the present study showed curcumin treatment markedly decreased the p53 gene expression caused by FZD (**Figure 7**), indicating that p53 pathway particulates in the protection role of curcumin. The proper mechanism need further *in vitro* and *in vivo* investigation.

In conclusion, the current study demonstrated that curcumin could protect L02 cells against FZD induced oxidative stress, DNA damage and cell apoptosis via inhibiting the ROS production, enhancing the intracellular anti-oxidative ability and inhibiting the mitochondrial apoptotic pathway (**Figure 8**). Meanwhile, curcumin could effectively inhibit the p53 gene expression, which may partly contribute to the protective role of cuurcumin on FZD exposure induced apoptosis (**Figure 8**). Importantly, the current study provided a promising combination of FZD with curcumin to prevent the adverse effect of FZD in human or animals.

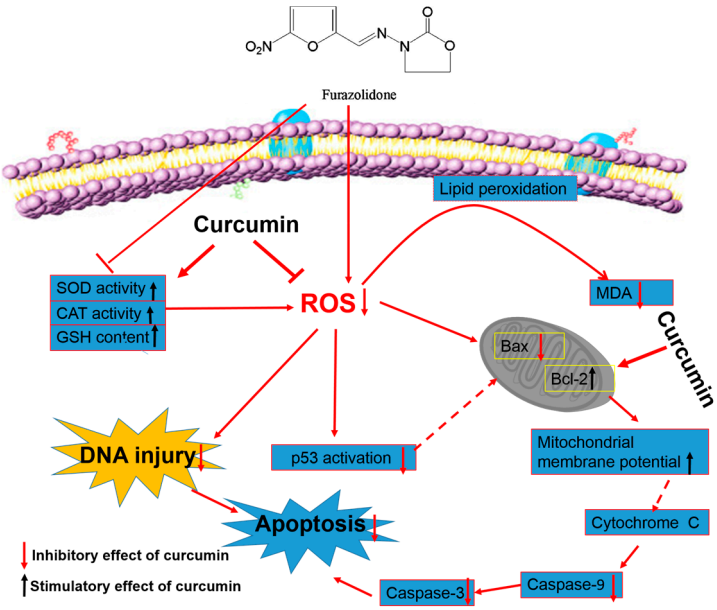

Figure 8. The schematic diagram of the possible mechanisms of curcumin protects against FZD-induced the cytotoxicity in L02 cells. There are several insights into the regulation mechanisms that contribute to the protective effect of curcumin on FZD induced cytotoxicity: (1) Inhibit the intracellular ROS formation and increase anti-oxidative enzyme activities to reduce DNA damage; (2) blockade of apoptosis by inhibiting mitochondrial pathway; (3) Inactivation of p53 may contribute to the protective role of cuurcumin.

4 Materials and Methods

4.1 Chemical and regents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Gibco, Grand Island, NY). FZD, 3-(4,5-Dimethyl-2-thiazolyl) -2,5-and diphenyl-2H-tetrazolium bromide (MTT), Triton X-100 were purchased from Sigma–Aldrich. Curcumin was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO, Sigma, Louis, MO) and stored at -20 °C for standby. All other reagents were of analytical reagent grade.

4.2 Cell culture

Human hepatocyte L02 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). L02 cells were cultured in DMEM containing with 2% L-glutamine, 10% FBS, penicillin (100 units/mL, Gibco), and streptomycin (100 μg/mL, Gibco) in a humidified incubator at 37 °C with 5% CO_2. The media was changed once per day.

4.3 Measurement of cell viability

Cell viability was examined using MTT assay method according to the previous study with modifications [34]. In brief, L02 cells (1 × 10^4cells/well) were seeded into 96-well plate and incubated overnight. The cells were treated with curcumin at the final concentration of 1.25, 2.5 and 5 μM, respectively for 2 h, then washed twice with PBS and treated with FZD at 10-60 μg/mL or replaced with 0.2% DMSO (as the curcumin control). Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, the medium was discarded and incubated with 100μL serum-free DMEM containing 10 μL MTT (5 mg/mL) for 4h at 37 °C. Then, the medium was discarded and replaced with 100μL DMSO. After incubation for 20 min at room temperature, the absorbance was read at 490 nm in a micro plate reader (Molecular Devices, Sunnyvale, CA, USA).

4.4 Measurement of intracellular ROS production

The production of intracellular ROS was measured using the ROS-specific fluorescent dye 2,7-dichlorofluorescein diacetate (DCFH-DA) (Beyotime, Haimen, China). In brief, L02 cells were plated into 96-well plate and pretreated with curcumin at 1.25 and 2.5 and 5 μM at 37°C for 2 h; then, the cells were washed twice with PBS and treated with FZD at 40 μg/mL or replaced with 0.2% DMSO (as the curcumin control) . Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, L02 cells were washed three times with PBS, followed to add 200 μL DMEM containing 10 μM DCFH-DA and incubate for 30 min at 37°C in the dark. After three washes with PBS, DCF fluorescence intensity was measured using a microplate fluorescence reader (excitation wavelength: 488 nm and emission wavelength: 530 nm) (MD, Spectramax M3, Geman).

4.5 Measurement of the activities of SOD, CAT and the levels of MDA and GSH

The activities of SOD, CAT and the levels of MDA and GSH were measured using their specific assay kits and the methods were according to the manufacturer's instructions (Nanjing Jiancheng Co., Ltd., Nanjing, China). In brief, L02 cells (2×10^5 cells) were pre-treated with or without curcumin at 1.25, 2.5 and 5μM for 2h; then, the cells were washed twice with PBS and treated with FZD at 40 μg/mL or replaced with 0.2% DMSO (as the curcumin control) . Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, L02 cells were washed with ice-cold PBS and lysed using the cell lysis buffer provided by the manufacturer. The collected lysates were centrifuged at 14,000×g for 10 min at 4 °C. Then, the supernatants were collected to measure the activities of SOD and CAT and the levels of MDA and GSH. Protein contents were examined using a BCA™ protein assay kit (Beyotime, Haimen, China). Values of the activities of SOD, CAT and the levels of MDA and GSH were corrected based on the protein contents.

4.6 Measurement of $\Delta\psi_m$

The changes of $\Delta\psi_m$ were determined using Rhodamin 123 (Rh123) method, according to our previous study [53] with some modifications. In brief, L02 cells (2×10^5 cells) were pre-treated with curcumin at 1.25 and 2.5 and 5 μM at 37 °C for 2 h; then, the cells were washed twice with PBS and treated with FZD at 40 μg/mL or replaced with 0.2% DMSO (as the curcumin control) . Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, L02 cells were incubated with 10 μg/mL Rh123 in the dark for 30 min at 37 °C. After washing twice with PBS, fluorescence image was observed using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and analyzed by the Image Pro Plus 5.0 software (Media Cybernetics, Inc., Silver Spring, MD).

4.7 Measurement of caspase-9 and -3 activities

The activities of caspase-9 and -3 were determined using the Caspase-9 and-3 assay kits according to the manufacturer's instructions (Beyotime, Haimen, China), respectively. In brief, L02 cells were plated into 6-well at a density of 5×10^5 cells per well and pretreated with curcumin at 1.25, 2.5 and 5 μM at 37 °C for 2 h; then, the cells were washed with PBS twice and treated FZD at 40 μg/mL or replaced with 0.2% DMSO (as the curcumin control) . Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, L02 cells were washed with ice-cold PBS and lysed using the cell lysis buffer provided by the manufacturer. The collected lysates were centrifuged at 14,000×g at 4°C for 10 min. The supernatants were used to measure the activities of caspase-9 and -3. Protein concentrations were examined using a BCA™ protein assay kit (Beyotime, Haimen, China). Values of the activities of caspase-9 and 3 were corrected based on the protein contents.

4.8 Measurement of apoptosis

Cell apoptosis were examined using flow cytometric analysis and Hoechst 33342 Staining. In brief, L02 cells (2×10^5 cells per well) were cultured on 6-well culture plates and pretreated with curcumin at 1.25, 2.5 and 5 μM at 37 °C for 2 h; then, the cells were washed with PBS twice and treated with FZD at 40 μg/mL for additional 24 h. Cells in the curcumin control group were treated with

curcumin at 5 μM for 2h, followed to wash with PBS twice and treat with 0.2% DMSO for additional 24h. Cells in the negative control group were treated with 0.2% DMSO for 24 h. After treatment, the cells were harvested and detected using an annexin V-FITC apoptosis detection kit (Vazyme Biotech Co., Ltd., Nanjing, China) according with the protocol described in the previous study [14].

For Hoechst 33342 staining, the treated L02 cells were stained with 1 μg/mL Hoechst 33342 (Vigorous Biotechnology, Beijing, China) for 20 min in the dark, then observed under a fluorescence microscope (excitation wavelength at 340 nm and emission wavelength at 510 nm).

4.9 Measurement of DNA damage by alkaline comet assay

To detect cellular DNA damage as single-strand breaks, alkaline comet assay was performed using Oxiselect Comet Assay® kit (Cell Biolabs, CA, USA) according to the manufacturer's instruction. In brief, L02 cells were pretreated with curcumin at 1.25, 2.5 and 5 μM for 2 h, then washed with PBS and exposed to FZD (40 μg/mL) or replaced with 0.2% DMSO (as the curcumin control) for 3 h. Cells in the negative control group were treated with 0.2% DMSO. To avoid artifacts resulting from necrotic and apoptotic cells, the cell suspensions (50 μL) were mixed with Hoechst 33342 (1 μg/mL) (Vigorous Biotechnology, Beijing, China). After incubation in the dark for 30 min, necrosis and apoptosis were identified under a fluorescent microscope. In all groups, the cell viability was more than 90%. Then, the harvested cells were mixed with low melting point agarose and then transferred onto the CometSlide™ following solidification of cell–agarose mix. Cells were then incubated in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris, pH 10 and 10% DMSO with 1% Triton X-100) in darkness at 4 °C for 1 h. After lysis, the slides were placed in alkaline solution (1mM Na2-EDTA and 300mM NaOH, pH 13) for 20 min at room temperature to allow DNA unwinding. Then, electrophoresis were performed in alkaline solution for 30 min at 25V. After electrophoresis, the slides were washed twice for 5 min each at 4 °C in a neutralizing buffer (0.4 M Tris, pH 7.5), dehydrated in 70% ethanol, stained with Vista Green DNA dye (provided by kit). Images were observed using fluorescent microscopy at an excitation wavelength of 490 nm and emission wavelength of 530 nm (Leica, Omachi,Japan). At least 100 randomly selected cells (30 or 40 cells from each of the three replicated slides) were analyzed using Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). The tail length is the length of the tail (in pixels); the tail DNA% is calculated as (tail DNA intensity/cell DNA intensity) ×100; the tail moment length is the length from the center of the head to the center of the tail; and the olive tail moment is calculated as the tail moment length × tail DNA%.

4.10 Measurement of mRNA expression of apoptosis factors by quantitative real-time PCR (qRT-PCR)

L02 cells were pretreated with curcumin at 1.25, 2.5 and 5 μM at 37°C for 2 h, followed to wash with PBS twice and treat with FZD or replaced with 0.2% DMSO (as the curcumin control) Cells in the negative control group were treated with 0.2% DMSO. After treatment for 24h, total RNAs from cells were extracted using TRIzol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The cDNA was synthesized from 2 μg of total RNA using the Prime Script RT-PCR kit (Takara, Dalian, China). The PCR primers were designed in Table 1. QRT-PCR was carried performed using an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). After the amplification phase, a melting curve analysis was conducted to eliminate the possibility of non -specific amplification or primer dimer formation. All reactions were conducted in triplicate. The fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ after normalizing to the expression level of β-actin.

Table 1. The primer sequences for β-actin and other target genes of apoptosis factors.

Gene	Primer sequences (5′-3′)	Product size (bp)
Caspase-9	Forward: 5′-gaggttctcagaccggaaacac-3′ Reverse: 5′-catttcccctcaaactctcaaga-3′	90
Caspase-3	Forward: 5′-gcgaatcaatggactctggaat -3′ Reverse: 5′-aggttgctgcatcgacatctg-3′	270
Bax	Forward: 5′-gatgcgtccaccaagaagct-3′ Reverse: 5′-cggccccagttgaagttg-3′	169
Bcl-2	Forward: 5′-gcggagttcacagctctatac-3′ Reverse: 5′-aaaaggcccctacagttacca-3′	136
p53	Forward: 5′-ccctcctcagcatcttatcc -3′ Reverse: 5′-gcacaaacacgcacctcaa -3′	260
β-actin	Forward: 5′-gggaaatcgtgcgtgac-3′ Reverse: 5′-ttgccaatggtgatgacctg-3′	138

4.11 Statistical analysis

Data were shown as mean ± standard deviation from at least three independent experiments performed in duplicate or triplicate. A one-way analysis of variance, followed by a Fisher's least significant difference (LSD) test, was employed to compare any two means when the variance was homogeneous; otherwise, Dunnett's T3 test was used (SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered significant difference.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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