Curcumin inhibits the proliferation and induces apoptosis in HT-29 cell lines through a reactive oxygen species (ROS)-dependent mechanism

Jinbo Wang, Lili Qi*, Lehe Mei and Zhige Wu
Ningbo Institute of Technology, Zhejiang University, Ningbo,China

Abstract: Curcumin, a natural pigment extracted from *Curcuma longa*, has anti-carcinogenic activities in many cancer cell lines. The molecular mechanism of apoptosis induced by curcumin are still unknown. In the current study, we investigated the roles of reactive oxygen species in curcumin stimulated apoptosis in HT-29 cells. Curcumin significantly reduced cell viability, induced apoptosis, activated caspase-3 activity and stimulated concentration-dependent release of ROS. Inhibition of ROS generation by scavengers suppressed apoptosis and Bcl-2 expression induced by curcumin, indicating the critical roles of ROS in the apoptotic process. However, caspase-3 inhibitor (z-VAD-FMK) couldn't completely inhibit the curcumin induced apoptosis, indicating ROS mediated apoptosis may be caspase-independent. Together, our findings showed that ROS played significant roles in the apoptosis induced by curcumin in HT-29 cells.

Keywords: curcumin, reactive oxygen species, apoptosis, HT-29 cells

INTRODUCTION

Curcumin is the principal active compound extracted from *Curcuma longa*, which is commonly used as a food additive in Southeast Asian countries. It has been used to treat inflammation and cancers in folk medicine for many years, particularly in China and India (Song *et al.,* 2012). Curcumin has been reported to have multiple biological effects including anti-oxidant, anti-bacterial, and anti-neoplastic properties (Fu *et al.,* 2014; Ghosh & Ryan 2014; Kloesch *et al.,* 2013; Packiavathy *et al.,* 2014). Importantly, we and others have observed that curcumin could stimulate apoptosis in cancer cell lines (Ghosh & Ryan, 2014; Ye *et al.,* 2015). The apoptotic mechanisms induced by curcumin are multiplex, including activation of p38 and caspase-3, decrease of the Bcl-XL and Bcl-2 levels, FasL-independent aggregation of Fas receptors, and increase of miR-7 expression (Wang *et al.,* 2013; Ma *et al.,* 2014). Up till now, several studies have been carried out to study the effects of curcumin on the apoptosis in human colon cancer cells (Bartik *et al.,* 2010; Kössler *et al.,* 2012), however, the cellular and molecular mechanisms have not been fully elucidated.

Reactive oxygen species (ROS) are normally generated in the cells by mitochondria and other sources. At low concentration, endogenous ROS are essential for proliferation of cells (Yoneyama *et al.,* 2010). But at high concentration, ROS result in serious damage to lipids, proteins, and DNA (Thayyullathil *et al.,* 2008). Many investigations have indicated that ROS could act as important regulators of the initiation of apoptosis (McManus *et al.,* 2014; Peng *et al.,* 2015). Curcumin has been indicated to have both anti-oxidative and pro-oxidative effects on cells. It could inhibit the oxidative stress and apoptosis in A431 cells, attenuate the apoptosis of HepG2 cells by reducing the ROS formation, and reduce the intracellular ROS generation in mastocytoma mast cells (Cao *et al.,* 2008; Chan *et al.,* 2003; Nishikawa *et al.,* 2013). However, curcumin has also been found to have pro-oxidative activities and could induce the apoptosis in some cell lines, including A549, Jurkat, RAW 264.7, and Molt-4 cancer cell line K1 (Kizhakkayil *et al.,* 2012; Kunwar *et al.,* 2009; Zhang *et al.,* 2010).

The present study was designed to determine whether curcumin induces apoptosis of HT-29 cells by potentiating intracellular ROS formation. The changes in intracellular ROS levels, capase-3 activities, and cell viabilities were evaluated. Moreover, we investigated the effect of curcumin on the expression of apoptosis-related protein Bcl-2.

MATERIALS AND METHODS

Cell culture

HT-29 cell line was propagated in RPMI-1640 supplemented with 10% foetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin in a humidified atmosphere of 95% air and 5% (v/v) CO_2 at 37°C.

Cell viability assay

Cell viability was determined by following the method of Liu *et al*. (2014) that employ MTT assay. Briefly, HT-29 cells were cultured in 96-well flat-bottom micro plates (5000 cells/well) and were treated with 0~80μM curcumin for 24 h in the presence or absence of NAC or catalase. Then media were removed and the cells were carefully

*Corresponding author: e-mail: qillacademy@126.com

washed three times with PBS (pH7.4). 25µl MTT (5mg/ml) was added to each well and incubated at 37°C for 4 h. The MTT formazan was dissolved with 200µl of dimethylsulfoxide (DMSO). Finally, the absorbance at 540 nm was determined. The absorbance of the untreated culture was set at 100%.

Hoechst 33258 staining

HT-29 cells were pre-incubated with NAC (5mM) or catalase (1000U/ml) for 1 h, and the cells were then treated with different molarities of curcumin (0~80µmol/L) for 24 h. The cells were washed thrice with PBS. Hoechst 33258 (10µg/ml) was added to stain the nucleus for 20 min at 37°C. Changes in nuclear morphology were observed by fluorescent microscopy (Wu *et al*., 2015).

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase 3 Activity Assay Kit (Zhu *et al*., 2012). After curcumin treatment for 24 h, HT-29 cells were lysed in the lysis buffer in ice water for 20min. The lysates were centrifuged at 20,000 g for 10 min at 4°C. A 10µl volume of the cell lysates were incubated with 80µl of the substrate (200 µM Ac-DEVD-pNA) for 4 h at 37°C. The absorbance in each well was determined at the wavelength of 405 nm.

Detection of intracellular ROS

The intracellular ROS levels were determined using the reactive oxygen species assay kit (Beyotime, Haimen, Jiangsu, China). Treated with 0~80µM curcumin for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 h, HT-29 cells were incubated with 10 µM DCFH-DA for 30 min at 37°C in the darkness. Then the cells were washed three times with PBS. The fluorescent intensity was measured at the wavelength of 488nm for excitation and at 525 nm for emission (Zhang *et al*., 2014).

The determination of the intracellular GSH levels

A commercial kit (Beyotime, Haimen, Jiangsu, China) was used to quantify the GSH levels (Diao *et al*., 2014). Briefly, HT-29 cells were exposed to different concentrations of curcumin for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 h. After incubation, the cells were collected and lysed in 400 µl sulfosalicylic acid(5%). The lysed cells were then centrifuged (10,000 g for 10 minutes, 4°C) and the supernatant was used to determine the level of GSH. The cellular protein was measured using the BCA Protein Assay Kit (Zheng *et al*., 2014).

Western blotting

HT-29 cells were treated as described above and Western blotting was performed to detect the expresion of BCl-2 (Li *et al*., 2015). Cell extracts were obtained by incubating the cells in lysis buffer [150mM NaCl, 50mM NaF, 30 mM $Na_4P_2O_7$, 10mM sodium vanadate , 1 mM phenylmethylsufonyl fluoride (PMSF), 1 mM EGTA, 0.5% NP-40, 20mM Tris-HCl, pH 7.5] containing additional protease inhibitors. The lysate was centrifugated at 15,000×g for 15min at 4°C, the protein content of the supernatant was determined with the BCA kit (Beyotime, Haimen, Jiangsu, China). 50µg of protein per sample was loaded and separated on 12.5% sodium dodecyl sulphate polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were blocked for 1.5 h with 5% (W/V) fat-free milk powder in TBS-T and incubated along with primary antibodies overnight at 4°C. After washing with TBS-T(3×5 min), the membranes were incubated with the horseradish peroxidase (HRP)-linked secondary antibody for 1.5 h. The bands were detected with enhanced chemiluminescence reagent (Millipore, USA). Densitometric analysis of immunoblots was performed with Quantity One (Bio-Rad, USA).

RESULTS

Curcumin decreased the viability of HT-29 cells

Curcumin has been indicated to decrease the viability of many tumor cell lines. We studied the effect of curcumin on HT-29 cell proliferation using MTT assay. After 24-h treatment, curcumin could induce HT-29 cells to shrink and partially detach at the high concentration (fig.1a). As shown in fig.1, curcumin significantly reduced the cell viability in time- and concentration-dependent manners.

Curcumin induced apoptosis and caspase-3 activation in HT-29 cells

It has been reported that curcumin could stimulate the apoptosis in many cancer cell lines. To determine whether curcumin induced the apoptosis in HT-29 cells, we performed Hoechst 33258 staining and determined Caspase-3 activities. As shown in fig. 2a, Hochest staining data indicated that curcumin treatment induced chromatin condensation of HT-29 cells. The numbers of apoptotic cells significantly increased at the concentrations equal to or greater than 40 µM following 24 h of treatment. Caspase-3 plays important roles in some of apoptosis signal transduction pathways. The

Table 1: Curcumin stimulated the release of ROS and reduced the glutathione levels in HT-29 cells

Time	Control (0h)	0.5h	1.0h	1.5h	2.0h	2.5h	3.0h
Relative DCF Fluorescence intensity	1.00±0.09	1.87±0.13	2.14±0.19	2.51±0.18	2.55±0.19	1.91±0.11	1.64±0.13
ROS content (% of the control)	100.00±8.58	37.65±3.25	25.11±2.26	20.07±1.93	17.17±1.45	36.57±3.29	45.97±4.62

effects of curcumin on the activities of caspase-3 were investigated, the results are shown in fig. 2b. The caspase-3 activities were significantly increased compared to those in control group.

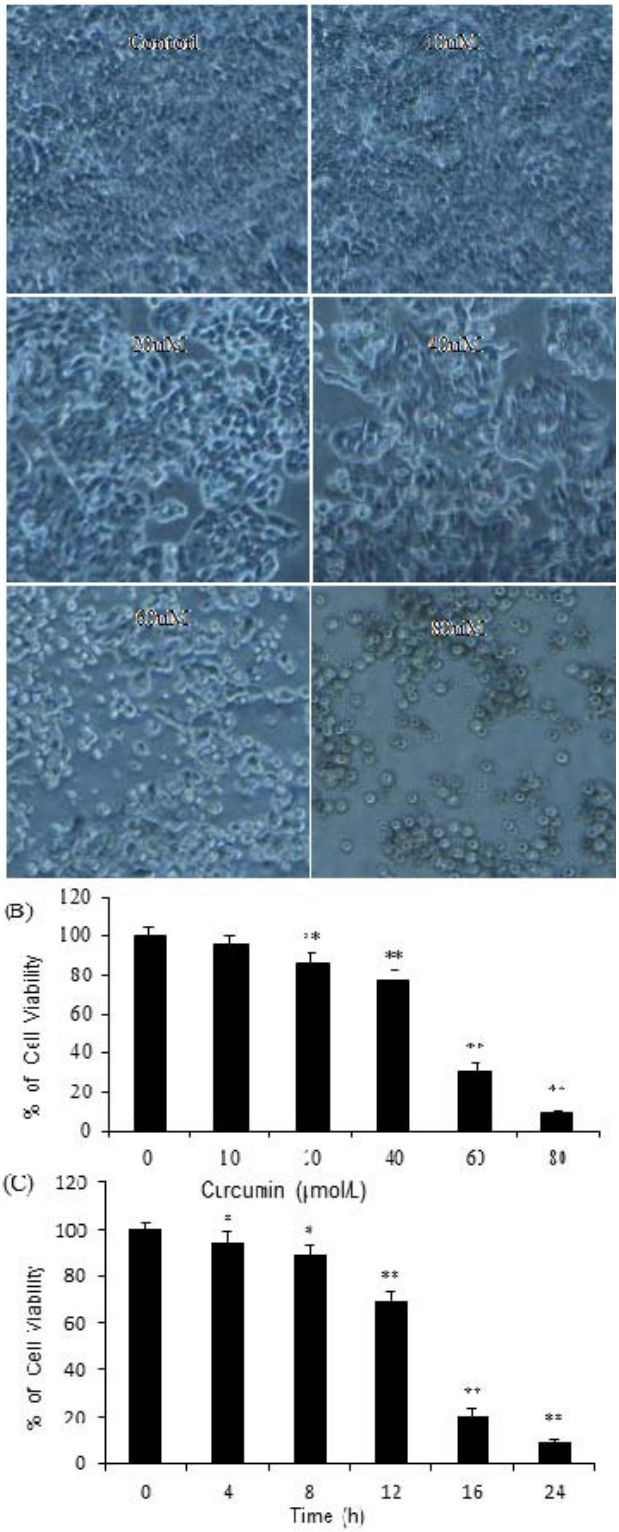

Fig. 1: Effects of curcumin on morphology and viability of HT-29 cells. A) HT-29 cells were exposed to 0-80μM curcumin for 24h and the cell morphological changes were observed in microscope . B) MTT assay was performed to determine the cell viability after curcumin treatment and incubation for 24 h. C) The cell viability was determined after treatment with curcumin(80μM) for 0-24h. * P<0.05 compared to the control, ** P <0.01 compared to control.

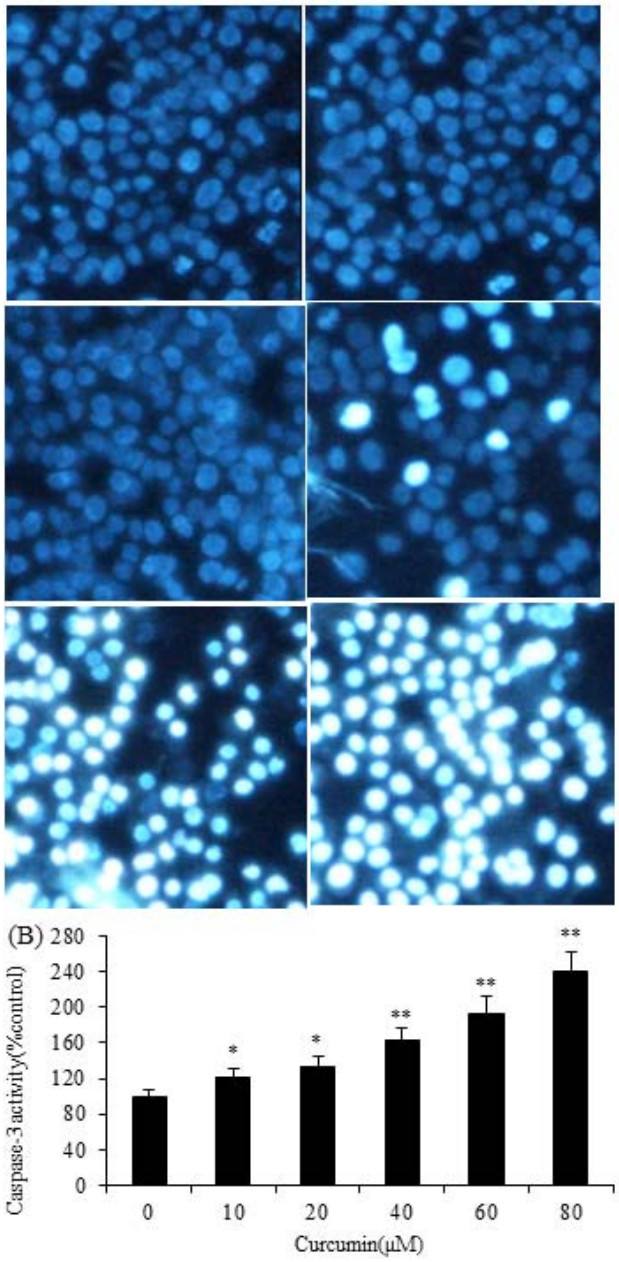

Fig. 2: Curcumin induced apoptosis and caspase-3 activation in HT-29 cells. A) After treatment with curcumin (0-80μM) for 24h, the cells were stained with Hochest 33342 (10μg/ml) and then observed under a fluorescent microscope. B) HT-29 cells were treated with curcumin for 24 h and caspase-3 activity was determined as described in materials and methods. Data shown represent the averages of three independent experiments(± SD). * P<0.05 compared to the control, ** P<0.01 compared to the control.

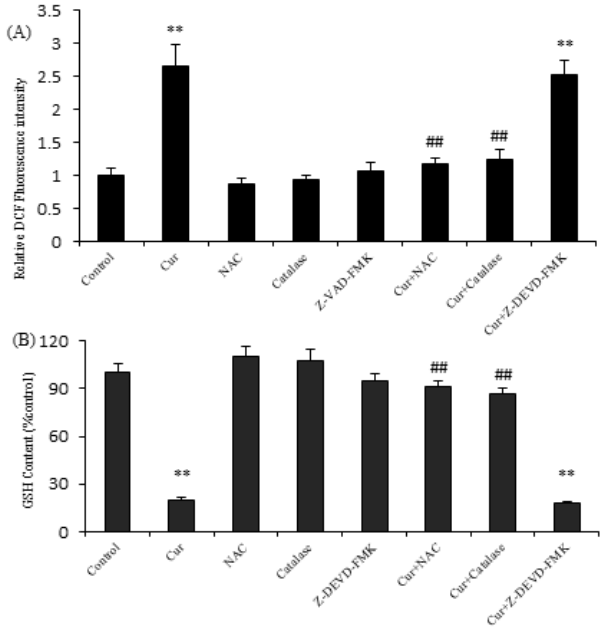

Fig. 3: Roles of antioxidants and caspase-3 inhibitor in the levels of ROS and glutathione in HT-29 cells stimulated by curcumin. HT-29 cells were pre-treated with NAC (5mM), catalase (1000U/ml), or Z-VAD-FMK (100μM) for 1 h before treating with curcumin (80μM) for 2h. Then the intracellular ROS levels (A) and GSH content (B) were determined. ***P*<0.01 compared to control, ## *P*<0.01 compared to the cells treated with curcumin.

Curumin stimulated ROS Release from HT-29 cells

Curcumin could stimulate ROS release and inhibit cell proliferation (Lundvig *et al.,* 2015). Therefore, we checked the levels of ROS and GSH in HT-29 cells treated with curcumin. The fluorescent probe DCF-DA was used to assess intracellular ROS levels. As shown in table 1, curcumin treatment resulted in 2.5-fold increase of DCF fluorescence intensity compared to the control within 2 h. After 2-h treatment, the ROS levels gradually decreased. Glutathione is an intracellular antioxidant, which can scavenge ROS (Xu *et al.,* 2012). We assessed the glutathione content in the cells. The data showed that curcumin stimulation significantly reduced glutathione concentration in HT-29 cells compared to the control group within 2 h and then the glutathione concentration steadily increased.

Antioxidants inhibited ROS generation and the apoptosis induced by curcumin

To study the roles of ROS in the apoptotic process induced by curcumin, the effects of antioxidants (NAC or catalase) and specific inhibitor of caspase-3 (z-VAD-FMK) were examined. Pretreatment with NAC (5mM) or catalase (1000U/ml) significantly reduced intracellular ROS levels and GSH concentration (fig. 3). However, z-VAD-FMK pretreatment could not attenuated ROS release in HT-29 cells (fig. 3). In the presence of the antioxidants, cell viability significantly increased after curcumin (80μM) exposure compared to the cells untreated with antioxidants (fig. 4a). The Caspase-3 activity was also decreased by pretreatment with antioxidants (fig. 4b). To determine whether antioxidants affect the curcumin induced apoptosis, we performed Hochest 33342 staining. As shown in fig. 5, antioxidants pretreatment significantly inhibited curcumin induced chromatin condensation, whereas Z-VAD-FMK pretreatment couldn't prevent curcumin induced apoptosis. These data showed that curcumin induced apoptosis might be mediated by ROS.

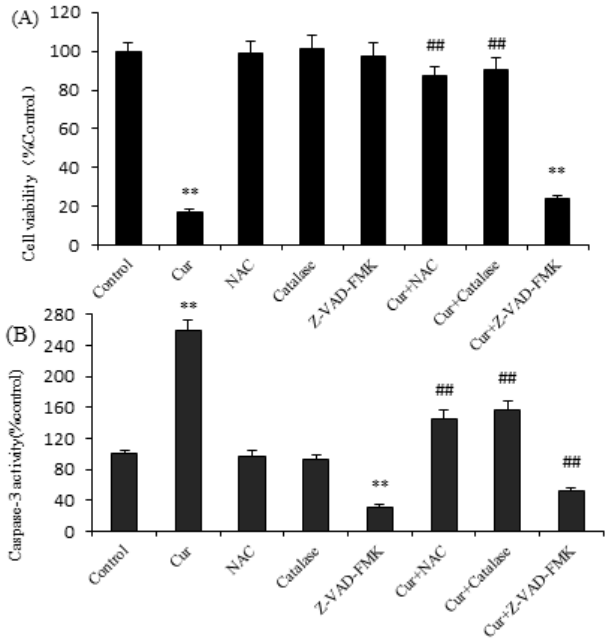

Fig. 4: Effects of antioxidants and caspase-3 inhibitor on curcumin induced cell apoptosis and caspase-3 activation. Cells were treated with NAC, catalase or Z-VAD-FMK before being stimulated by curcumin(80μM) for 24 h. Cell viability was assessed by MTT assay. Caspase-3 activity was determined as described in materials and methods. ***P*<0.01 compared to control, ## *P*<0.01 compared to the cells treated with curcumin.

Glutathione in HT-29 cells stimulated by curcumin. HT-29 cells were pre-treated with NAC(5mM), catalase (1000U/ml), or Z-VAD-FMK (100μM) for 1 h before treating with curcumin (80μM) for 2h. Then the intracellular ROS levels (A) and GSH content (B) were determined. ***P*<0.01 compared to control, ## *P*<0.01 compared to the cells treated with curcumin.

Curcumin induced Bcl-2 expression is attenuated by antioxidants

Bcl-2 plays important roles in mitochondrial apoptotic pathway as an anti-apoptotic factor (Brunelle & Letai, 2009). We investigated the expression levels of Bcl-2 in the presence or absence of antioxidants or z-VAD-FMK.

The results showed that curcumin could significantly reduce the expression of Bcl-2 in HT-29 cells, while this effect was blocked by pretreatment with antioxidants (fig. 6).

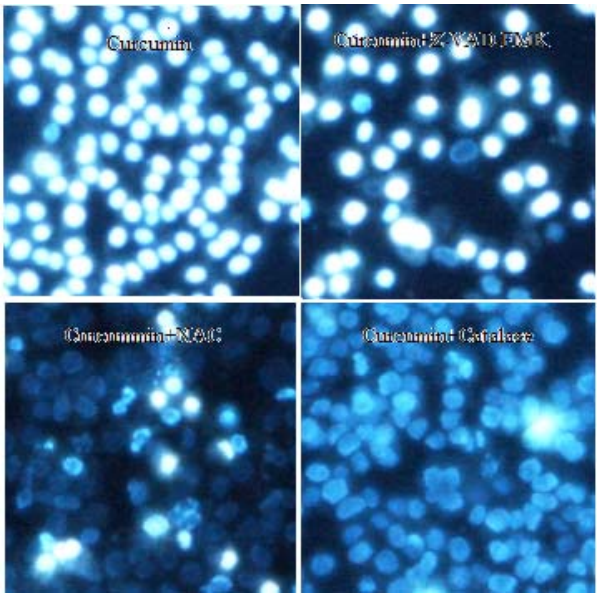

Fig. 5: Effects of antioxidants and caspase-3 inhibitor on the apoptosis induced by curcumin. HT-29 cells were pretreated with NAC, catalase and Z-DEVD-FMK for 1 h. Then the cells were stimulated with 80μM curcumin for 24 h. Hochest 33342 staining were performed and the cell changes were observed in fluorescent microscope.

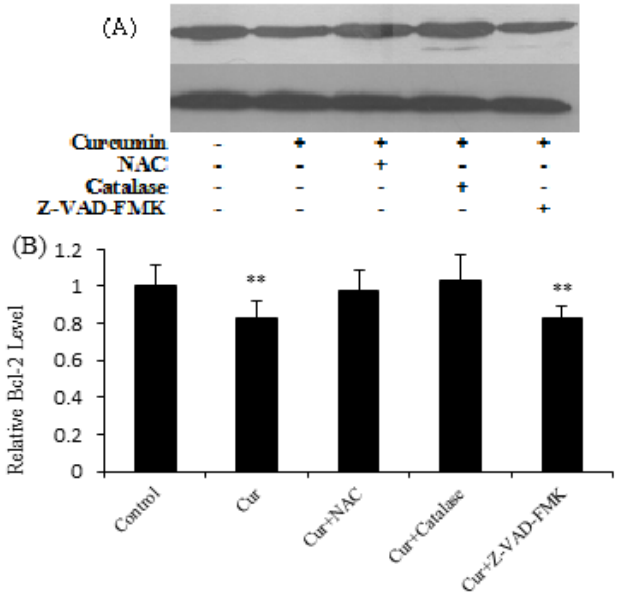

Fig. 6: Effects of curcumin on the expression of Bcl-2 in HT-29 cells. After incubation with antioxidants or Z-VAD-FMK for 1h, HT-29 cells were treated with curcumin for 24 h. Cellular lysates (50μg/lane) were analyzed by Western blotting using anti-Bcl-2 antibody. β-actin was used as internal control. ***P*<0.01 compared to control.

DISCUSSION

It has been shown that curcumin could induce apoptosis and inhibit the proliferation of many tumor cells (Prasad *et al.,* 2014; Ravindran *et al.,* 2009). Curcumin has been found to induce the apoptosis via ROS-dependent mechanism (Ibrahim *et al.,* 2011). We have reported that curcumin could reduce the viability and stimulate the apoptosis in HT-29 cells (Wang *et al.,* 2009). In this study, the roles of ROS in the apoptotic process induced by curcumin in HT-29 cells were investigated. Curcumin stimulated the generation of ROS, increased the caspase-3 activity, decreased the level of Bcl-2, inhibited the cell growth and induced apoptosis. The apoptosis induced by curcumin was suppressed by pretreatment with antioxidants, indicating the important roles of ROS in curcumin induced apoptosis.

Consistent with previous findings, this study confirmed that curcumin resulted in morphological changes and reduced the viability of HT-29 cells in dose- and time-dependent manners (fig. 1a,b,c). At high concentrations, curcumin activated the capase-3 and caused apoptosis (fig. 2a and b).

ROS are mediators of intracellular signaling cascade, which could regulate diverse function of the cells (Yoneyama *et al.,* 2010). Curcumin could act as a pro-oxidant to stimulate ROS generation in many tumor cell lines (Hail, 2008; Mohankumar *et al*., 2014). The antioxidant status of HT-29 cells is low, which results in the cells more sensitive to ROS (Wenzel *et al.,* 2005). Therefore, we measured the levels of ROS and GSH in HT-29 cells treated with curcumin. The results showed curcumin markedly simulated the rapid release of ROS and decreased the level of GSH within 2 h (fig. 3a,b). These findings were consistent with the previous reports indicating that curcumin induced ROS generation in some cells (Mohankumar *et al.,* 2014; Nishikawa *et al.,* 2013). However, other studies have indicated that curcumin could act as an anti-oxidant to inhibit ROS production (Rong *et al.,* 2012; Yang *et al.,* 2013). Curcumin exhibits antioxidant or pro-oxidant activities, which might depend on cell type, cellular oxidative status and treatment duration during the studies.

ROS has been shown to play critical roles in initiating apoptosis (Peng *et al.,* 2015). To determine whether ROS mediated the HT-29 cell apoptosis induced by curcumin, we assessed the impact of antioxidants on the apoptosis and viability of HT-29 cells. Pretreatment with antioxidants significantly decreased the levels of intracellular ROS, inhibited apoptosis and elevated the cell viability (fig.4, 5a). These results demonstrated that ROS were important mediators in curcumin induced apoptosis of HT-29 cells. On the other hand, Z-VAD-FMK pretreatment suppressed the activation of caspase-3

(fig. 5), but couldn't inhibit the apoptosis induced by curcumin. This result showed that ROS could also mediate caspase-independent apoptosis in HT-29 cells. These results were consistent with the previous study using L929 cells (Thayyullathil *et al.,* 2008).

As a key anti-apoptotic protein, Bcl-2 could suppress cytochrome c leakage from mitochondria and prevent apoptosis (Motaghinejad M *et al.,* 2015). We found that curcumin incubation reduced the level of Bcl-2, whereas the antioxidants pretreatment could inhibit this down-regulation (fig.6). These results demonstrated that curcumin induced down-regulation was ROS-dependent.

In summary, we have demonstrated that ROS play critical roles in the apoptosis induced by curcumin in HT-29 cells. Curcumin induces rapid production of ROS, which may be a initiating signaling candidate. ROS mediated apoptosis induced by curcumin is both caspase-dependent and caspase-independent. Further studies should be performed to decipher the roles of ROS in curcumin induced apoptosis.

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