

Anticancer effect of inactivated Sendai virus strain Tianjin on human osteosarcoma HOS cells

Qing Li^{1,*}, Huachong Ma^{2,*}, Shuya Sun¹ and Liying Shi¹

¹ Department of Pathogenic Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

² Department of General Surgery, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China

Abstract. Ultraviolet-inactivated Sendai virus strain Tianjin (UV-Tianjin) has been proved to have antitumor effects in many kinds of tumor cells. Here, we investigated the anticancer properties of UV-Tianjin on human osteosarcoma (HOS) cells and the underlying molecular mechanism. Apoptosis, intracellular reactive oxygen species (ROS) levels and mitochondrial membrane potential were determined by flow cytometry analysis. The expression levels of apoptosis-related proteins were tested by Western blotting. The results showed that UV-Tianjin concentration-dependently induced apoptosis in HOS cells. UV-Tianjin-induced apoptosis was mediated by the mitochondrial pathway, which was confirmed by mitochondrial dysfunction, downregulation of B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-xL (Bcl-xL) and myeloid cell leukemia-1 (Mcl-1), upregulation of Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), as well as the cleavage of caspase-9 and caspase-3. Further analysis showed that UV-Tianjin augmented the phosphorylation of c-Jun N-terminal kinase, the extracellular-regulated kinase and p38, the major components of mitogen-activated protein kinase (MAPK) pathways, as well as the generation of ROS. Moreover, UV-Tianjin-induced apoptosis was remarkably attenuated by MAPK inhibitors and ROS inhibitor. Taken together, our results indicated that UV-Tianjin exerts antitumor effects by inducing mitochondria-dependent apoptosis involving ROS generation and MAPK pathway in human osteosarcoma HOS cells.

Key words: Apoptosis — Human osteosarcoma cells — Mitogen-activated protein kinase pathway — Reactive oxygen species — Sendai virus strain Tianjin

Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in children and adolescents, and is characterized by poor prognosis and high mortality (Li et al. 2014). The current treatment options for patients with primary OS include neoadjuvant chemotherapy and surgical removal. However, side effects and drug resistance has limited the clinical application of chemotherapeutic agents (Li et al. 2015; Rhea and Oliveira 2018). Therefore, new

drugs with better tumor selectivity and fewer side effects are urgently needed.

Over the past decade, cancer-specific oncolytic viruses have received substantial attention for their potential as novel anticancer drugs (Ledford 2015; Varela-Guruceaga et al. 2018). Sendai virus (SeV) is a prototypic member of the family *Paramyxoviridae*. It has been demonstrated that ultraviolet (UV)-inactivated SeV may exert anticancer effects by inducing cancer-selective apoptosis, autophagy and anticancer immune responses (Kurooka and Kaneda 2007; Zhang et al. 2015; Li et al. 2017). However, the molecular mechanisms underlying its anti-tumor effects have not been fully elucidated.

Sendai virus strain Tianjin was isolated from the lungs of marmosets in 1999. Phylogenetic analysis of complete genome has confirmed that Tianjin strain belonged to *Paramyxoviridae* and was a new genotype of SeV (Shi et al. 2008). Our previous studies have demonstrated that UV-inactivated

* These authors contributed equally to this work.

Correspondence to: Liying Shi, Department of Pathogenic Biology, School of Basic Medical Sciences, Tianjin Medical University, No.22, Qixiangtai Road, Heping District, Tianjin 300070, China
E-mail: sly.good@126.com
shiliying@tmu.edu.cn

Tianjin strain (UV-Tianjin) may induce apoptosis or autophagy of certain cancer cell lines (Chen et al. 2014; Shi et al. 2015; Chen et al. 2016; Han et al. 2019). Whether UV-Tianjin elicits antitumor effect on osteosarcoma HOS cells through apoptosis or autophagy remains unknown.

Apoptosis induction is one of the main mechanisms of most anti-tumor drugs against cancer (Xiang et al. 2016; Dai et al. 2018). It is well known that there are two main signaling pathways to control apoptosis, the mitochondria-dependent pathway and death receptor-dependent pathway (Daniel 2000). In addition, many studies report mitogen-activated protein kinase (MAPK) pathways and reactive oxygen species (ROS) are involved in virus-induced apoptosis (Yang et al. 2010; Olavarria et al. 2015; Qian et al. 2018; Verma et al. 2018). MAPKs consist of growth factor-regulated extracellular signal-related kinases (ERKs), and the stress-activated MAPKs, c-jun NH2-terminal kinases (JNKs) and p38 MAPKs. MAPK pathways are known to be influenced not only by receptor ligand interactions, but also by different stressors placed on the cell. One type of stress that induces potential activation of MAPK pathways is the oxidative stress caused by ROS. Generally, increased ROS production in a cell leads to the activation of ERKs, JNKs, or p38 MAPKs (Son et al. 2011).

In the present study, we prepared UV-inactivated Tianjin strain and determine the antitumor effect and mechanism of UV-Tianjin on human osteosarcoma HOS cells for the first time.

Materials and Methods

Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, fetal bovine serum (FBS), phosphate buffer saline (PBS) and 0.25% trypsin were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), caspase-9 specific inhibitor (z-LEHD-fmk), Hoechst 33342 assay kit, annexin V-FITC/propidium iodide (PI) apoptosis detection kit, mitochondrial membrane potential (MMP) assay kit, and ROS assay kit were purchased from KeyGen Biotech (Nanjing, China). SP600125, SB203580, and PD98059 were purchased from Beyotime (Nanjing, China). Primary antibodies against JNK, p-JNK, p38, p-p38, procaspase-3, Bcl-2 homologous antagonist/killer (Bak), B-cell lymphoma-xL (Bcl-xL), myeloid cell leukemia-1 (Mcl-1) and secondly antibodies were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), cytochrome c (Cyt c), procaspase-9, extracellular signal-regulated kinase (ERK), p-ERK and β -actin were obtained from Santa Cruz Biotechnology,

Inc. (Dallas, TX, USA). Polyvinylidene difluoride (PVDF) membranes and enhanced chemiluminescence (ECL) detection kit were purchased from Millipore (Billerica, MA, USA).

Cell and viruses

The human osteosarcoma cell line HOS and human osteoblasts hFOB1.19 were obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM or DMEM/F12 supplemented with 10% FBS in a humidified atmosphere containing 5% CO₂. Sendai virus strain Tianjin (GenBank: EF679198.1) was propagated in 9 to 11-day-old specific pathogen free embryonated chicken eggs after inoculation through allantoic route. Inactivation of viral infectivity was achieved by UV irradiation (99 mJ/cm²), as previously described (Kaneda et al. 2002).

Cell viability assay

Cell viability was determined by using MTT assay. In brief, cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. Next, cells were treated with various concentration of UV-Tianjin [multiplicity of infection (MOI): 50-800]. After incubating for 24 h, 10 μ l of MTT reagent (5 mg/ml) was added to each well and followed by incubation at 37°C for 4 h in the dark. At the end of the incubation period, the medium was removed and DMSO solution (100 μ l) was added to each well. The optical density value of each well was measured using a micro-plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

Hoechst staining

HOS cells were seeded in 6-well plates at 2.0×10^5 cells/well. After reaching confluence, cells were treated with UV-Tianjin (MOI: 100, 200 and 400) for 24 h. Then the culture medium was removed and the cells were stained with Hoechst 33342 at 37°C for 15 min under dark condition. The cells were washed with PBS and observed with a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan).

Annexin V-FITC/PI analysis for cell apoptosis

The apoptosis induced by UV-Tianjin was measured with annexin V/PI double staining assay, according to the manufacturer's protocol. Briefly, HOS cells were seeded in a 6-well plate and then treated with UV-Tianjin (MOI 100, 200 and 400) for 24 h. At the end of the treatment, cells were digested, washed and then incubated with annexin V and PI at room temperature for 10 min in the dark. The stained cells were analyzed with a flow cytometry (Becton & Dickinson, Franklin Lakes, NJ, USA).

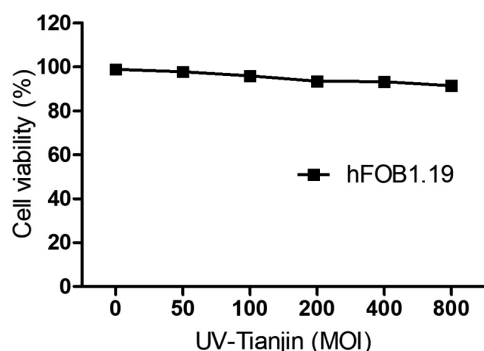


Figure 1. Effect of UV-Tianjin on the viability of hFOB 1.19 osteoblasts. Cells in a 96-well plate were treated with 50, 100, 200, 400 and 800 MOI of UV-Tianjin for 24 h. Cell viability was detected by MTT assay. The data are shown as mean \pm SEM of three independent experiments. MOI, multiplicity of infection; UV-Tianjin, ultraviolet-inactivated Sendai virus strain Tianjin.

Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

HOS cells were cultured in 6-well plates overnight and then exposed to UV-Tianjin (MOI 100, 200 and 400) for 24 h. Cells were harvested, washed and incubated with JC-1 dye for 30 min at 37°C, and then rinsed with JC-1 staining buffer twice. The $\Delta\Psi_m$ of HOS cells were detected by flow cytometry.

Western blot analysis

After treated with different doses of UV-Tianjin (MOI: 100, 200 and 400) for 24 h, the cells were collected and lysed in

radioimmunoprecipitation assay lysis buffer for 30 min on ice. After centrifugation, the concentration of protein was determined using a bicinchoninic acid protein assay kit. The samples were loaded to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and then transferred to a PVDF membrane. The membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline-Tween (TBST) and then incubated with the primary antibodies at 4°C overnight. After washing with TBST, membranes were incubated with a secondary antibody for 2 h. Protein bands were visualized using an ECL kit.

Measurement of reactive oxygen species

Intracellular ROS was detected with the fluorescent probe DCFH-DA according to the manufacturer's protocol. Briefly, following the treatment with UV-Tianjin (MOI: 100, 200 and 400) for 24 h, cells were incubated with DCFH-DA (10 μ M) for 20 min at 37°C in the dark and then washed three times with PBS. DCF fluorescence intensity was measured with flow cytometry.

Statistical analysis

All experiments were performed at least three times and data were expressed as mean \pm standard error of mean (SEM). Statistical analysis was performed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Comparisons between groups were made using one-way analysis of variance followed by Tukey's test. Results were considered statistically significant when the *p* value was less than 0.05.

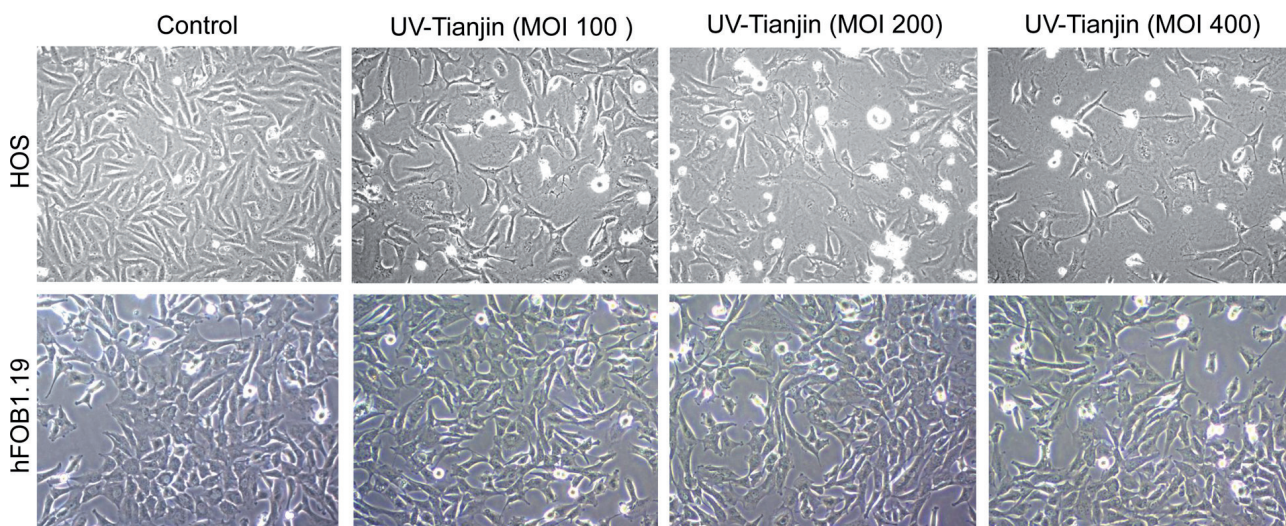


Figure 2. Effect of UV-Tianjin on the morphology of HOS cells and hFOB 1.19 osteoblasts. The morphological changes of HOS cells or hFOB 1.19 osteoblasts were examined by an inverted microscopy ($\times 100$) after 24 h of treatment with UV-Tianjin (MOI 100, 200 and 400). For abbreviations, see Fig. 1.

Results

UV-Tianjin had no significant effect on the viability of human osteoblasts hFOB1.19

Our previous study has shown that treatment with UV-Tianjin for 24 h resulted in a significant dose-dependent decrease in cell viability of HOS cells by MTT assay (Han et al. 2019). In the present study, the effect of UV-Tianjin on the viability of human osteoblasts hFOB1.19 was examined. As shown in Fig. 1, viability of human osteoblasts hFOB1.19 was not significantly affected by UV-Tianjin treatment, indicating

a strong selective effect of UV-Tianjin towards cancer cells. On the other hand, microscopic observation showed that UV-Tianjin induced significant cellular morphological changes in HOS cells, but not in hFOB1.19 osteoblasts (Fig. 2).

UV-Tianjin induced apoptosis in HOS cells

Firstly, the effect of UV-Tianjin on apoptosis of HOS cells was examined using Hoechst 33342 staining. We observed that, in a dose-dependent manner, the chromatin became condensed and marginalized following the treatment of UV-Tianjin (Fig. 3A). Subsequently, apoptosis was detected

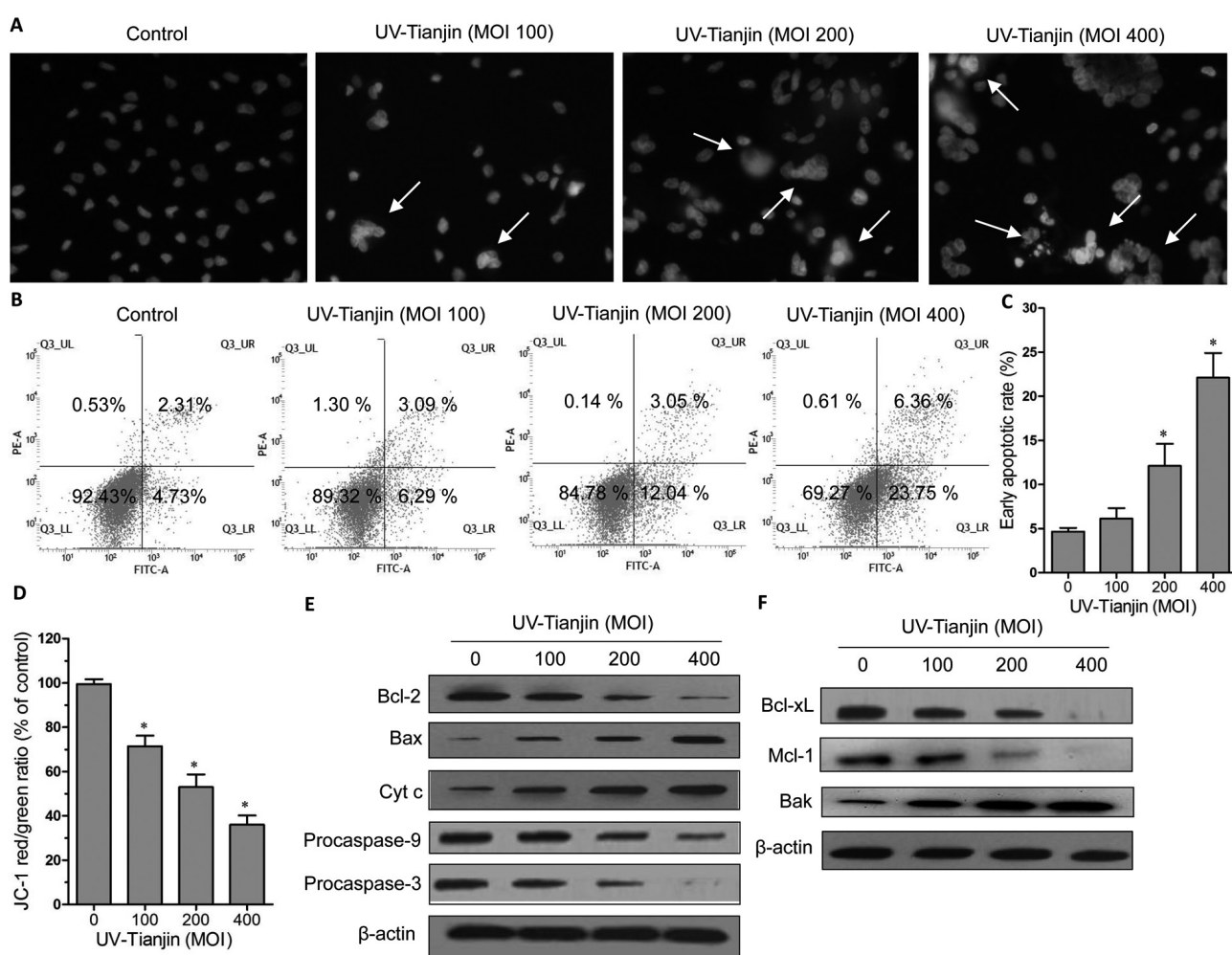


Figure 3. UV-Tianjin triggered apoptosis through the mitochondrial pathway in HOS cells. HOS cells were treated with UV-Tianjin at the indicated concentrations for 24 h. **A.** Nuclear condensation and fragmentation are detected by Hoechst 33342 staining ($\times 200$). Arrows indicate condensed chromatin and nuclear fragmentation. **B.** HOS cells were stained with annexin V-FITC/PI and determined by flow cytometry. Representative images were presented. **C.** The histogram illustrates the percentages of early apoptotic cells. **D.** The $\Delta\psi_m$ was assessed by flow cytometry using JC-1 staining. Quantitative analysis in histogram was presented. **E.** and **F.** Western blotting was used to analyze the expression of Bcl-2, Bax, Cyt c, procaspase-9, procaspase-3, Bcl-xL, Mcl-1 and Bak proteins. Results are expressed as the mean \pm SEM from three independent experiments. * $p < 0.05$ vs. untreated control. Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-xL; Cyt c, cytochrome c; Mcl-1, myeloid cell leukemia-1. For more abbreviations, see Fig. 1.

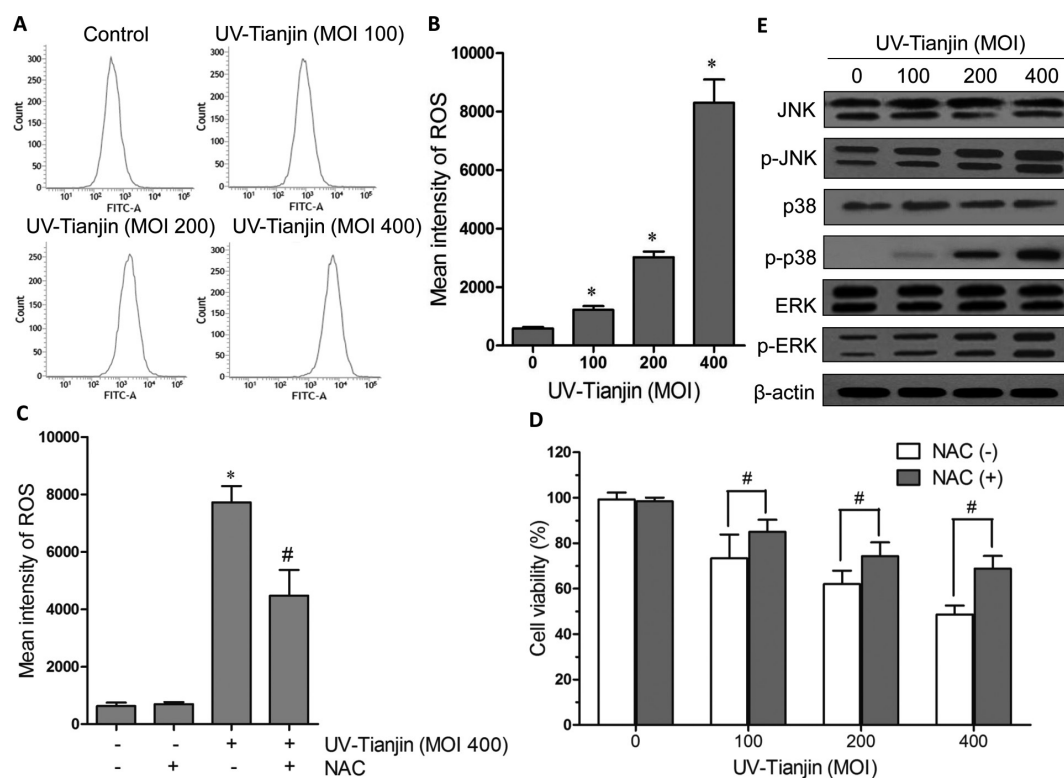


Figure 4. Effects of UV-Tianjin on intracellular ROS and MAPK pathway in HOS cells. HOS cells were treated with UV-Tianjin at the indicated concentrations for 24 h. **A.** The level of ROS was determined by flow cytometry. Representative images were presented. **B.** Quantitative analysis of ROS generation was shown in histogram. **C.** HOS cells were pretreated with or without NAC (5 mM) for 1 h, and then followed by UV-Tianjin at a MOI of 400 for 24 h. The ROS level was analyzed by flow cytometry. Quantitative analysis was shown in histogram. **D.** HOS cells were pretreated with or without NAC (5 mM) for 1 h, and then followed by UV-Tianjin (MOI 100, 200, 400) for 24 h. Cell viability was assessed by MTT assay. **E.** The expression of JNK, p-JNK, ERK, p-ERK, p38 and p-p38 were measured by Western blotting. Results are expressed as the mean \pm SEM from three independent experiments. * $p < 0.05$ vs. untreated control. # $p < 0.05$ vs. the single UV-Tianjin treatment groups. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases; NAC, N-acetylcysteine; ROS, reactive oxygen species. For more abbreviations, see Fig. 3.

by annexin V/PI double-staining using flow cytometry. UV-Tianjin treatment induced a dose-dependent increase in the percentage of early apoptotic cells (Fig. 3B and 3C). These results clearly indicate that UV-Tianjin provoked apoptosis in HOS cells.

UV-Tianjin induced apoptosis through mitochondrial pathway in HOS cells

Next, we studied the effect of UV-Tianjin on mitochondria. As shown in Fig. 3D, a loss of $\Delta\psi_m$ was indicated by a decreased in the ratio of red fluorescence to green fluorescence following UV-Tianjin treatment. Furthermore, we investigated the expression of proteins associated with mitochondria-mediated apoptosis. As shown in Fig. 3E and 3F, UV-Tianjin treatments dose-dependently decreased the expression of Bcl-2, Bcl-xL, Mcl-1, procaspase-9 and -3 and increased the levels of Bax, Bak and

Cyt c in HOS cells. To further confirm the findings, we investigated the role of caspase-9, a marker of the mitochondrial pathway, using the caspase-9 specific inhibitor z-LEHD-fmk. Expectedly, flow cytometry analysis showed that z-LEHD-fmk significantly inhibited the apoptosis induced by UV-Tianjin (Fig. 5A and 5B). These results clearly demonstrated that UV-Tianjin-induced apoptosis involves the mitochondrial pathway.

UV-Tianjin enhanced ROS levels in HOS cells

In order to explore the role of ROS in UV-Tianjin-mediated apoptosis, we examined the intracellular production of ROS. As shown in Fig. 4A and B, intracellular ROS production was significantly increased by UV-Tianjin, compared to control. To further confirm the involvement of ROS in UV-Tianjin-induced apoptosis, HOS cells were exposed to NAC (5 mM), the ROS scavenger, for 1 h and then treated with UV-Tianjin

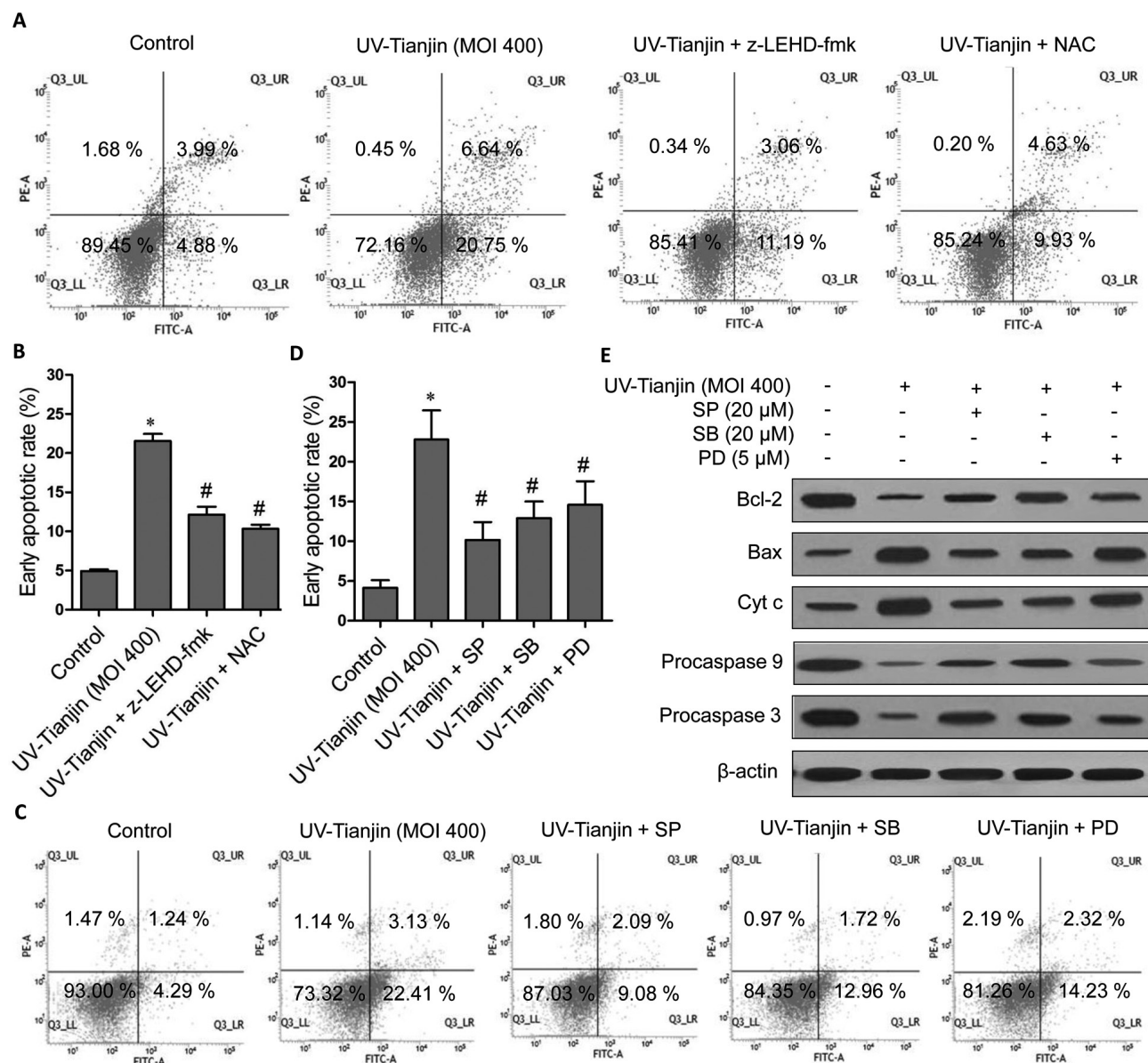


Figure 5. Inhibition of caspase, ROS production and MAPK pathway attenuated UV-Tianjin-induced apoptosis in HOS cells. HOS cells were preincubated with caspase-9 specific inhibitor z-LEHD-fmk (20 μM), ROS inhibitor NAC (5 mM), JNK inhibitor SP (20 μM), ERK inhibitor PD (5 μM) or p38 inhibitor SB (20 μM) for 1 h, and then treated with UV-Tianjin (MOI 400) for 24 h. **A–D.** Induction of apoptosis was assessed by flow cytometry using annexin V and PI staining. Quantitative analysis of early apoptosis rate was presented in histograms. **E.** The expressions of apoptosis-related proteins were detected by Western blotting. Representative images were presented. Results expressed as mean ± SEM of three independent assays. * $p < 0.05$ vs. control. # $p < 0.05$ vs. the single UV-Tianjin treatment groups. PD, PD98059; SB, SB203580; SP, SP600125. For more abbreviations, see Fig. 4.

for 24 h. As was expected, addition of NAC significantly decreased the level of ROS (Fig. 4C) and attenuated UV-Tianjin-induced cell viability loss (Fig. 4D) and apoptosis to a certain extent (Fig. 5A and 5B). These results suggest that increased ROS production in HOS cells plays an important role in UV-Tianjin-induced apoptosis.

UV-Tianjin induced apoptosis through MAPK pathway in HOS cells

To further elucidate the underlying mechanism of apoptosis induced by UV-Tianjin, we evaluated the activation of MAPK signaling pathway, which play critical roles in

apoptosis regulation. As shown in Fig. 4E, the phosphorylation of JNK, ERK and p38, the major components of MAPK pathway, was increased in a dose-dependent manner in HOS cells treated with UV-Tianjin. To further determine the role of MAPKs in UV-Tianjin-induced apoptosis in HOS cells, JNK inhibitor SP600125 (20 μ M), ERK inhibitor PD98059 (5 μ M) and p38 inhibitor SB203580 (20 μ M) were used to pretreat HOS cells before the treatment of UV-Tianjin. As shown in Fig. 5C, D and E, MAPK inhibitors could significantly reduce the apoptosis of HOS cells and attenuated the expression of apoptosis-related proteins triggered by UV-Tianjin. Overall, these results indicated that MAPK signaling pathway might mediate UV-Tianjin-induced apoptosis in HOS cells.

Discussion

Recently, SeV has been identified as a potential anticancer agent in many studies (Kurooka and Kaneda 2007; Zhang et al. 2015; Gao et al. 2016; Li et al. 2017). However, the molecular mechanisms for the anticancer activity of SeV are poorly understood. Our latest study showed that UV-Tianjin, a new genotype of SeV, significantly suppressed the proliferation of HOS cells in a dose-dependent manner (Han et al. 2019). In the present study, we found that UV-Tianjin had no significant effect on the viability of human osteoblasts hFOB1.19, indicating a strong selective effect of UV-Tianjin towards cancer cells. Moreover, the present study also showed that inhibition of proliferation of HOS cells was mostly related to cell apoptosis, as evidenced by Hoechst staining and flow cytometry analysis, suggesting that the anticancer effect of UV-Tianjin on HOS cells was mediated through the induction of apoptosis.

The intrinsic, mitochondrial pathway is activated by intracellular signals generated in response to cellular stress. It is regulated by Bcl-2 family proteins, including the anti-apoptotic factor (Bcl-2, Bcl-xL, Mcl-1) and the pro-apoptotic factor (Bax, Bak) (D'Orsi et al. 2017). An increase of Bax/Bcl-2 ratio may induce a loss in $\Delta\Psi_m$, release of Cyt c into the cytoplasm, and subsequent activation of caspase, which eventually leads to occurrence of cell apoptosis (Matsuyama and Reed 2000). In our results, UV-Tianjin decreased the ratio of red/green fluorescence intensity, indicating a decreased $\Delta\Psi_m$ and depolarization of the mitochondrial membrane. In addition, our results also demonstrated that UV-Tianjin decreased the levels of Bcl-2, Bcl-xL, Mcl-1, procaspase-9 and procaspase-3 and increased the levels of Bax, Bak, and Cyt c in a dose-dependent manner. Moreover, the caspase-9 specific inhibitor z-LEHD-fmk significantly attenuated UV-Tianjin-induced apoptosis. All these evidences suggest that the mitochondrial pathway is involved in UV-Tianjin-induced apoptosis in HOS cells.

Many chemotherapeutic agents exert their anticancer effects by inducing the generation of ROS (Park et al. 2015; Wang et al. 2016). In the present study, the production of intracellular ROS increased remarkably in HOS cells treated with UV-Tianjin, while inhibition of ROS production by the antioxidant NAC attenuated UV-Tianjin-induced cell viability loss and apoptosis, suggesting that UV-Tianjin-induced apoptosis in HOS cells was closely associated with the production of ROS, which may act as upstream signaling molecules to initiate mitochondria-mediated cell apoptosis (Wang 2001).

As one of the most important signal transduction systems of organism, MAPK pathways participate in many cellular physiological functions, including cellular proliferation and apoptosis (Sun et al. 2015). Our results demonstrated that UV-Tianjin increased the levels of phosphorylated JNK, p38 and ERK in a dose-dependent manner in HOS cells. Moreover, UV-Tianjin-induced apoptosis was remarkably attenuated by JNK inhibitor SP600125, ERK inhibitor PD98059 and p38 inhibitor SB203580, suggesting that MAPK pathways were involved in UV-Tianjin-induced apoptosis in HOS cells.

In summary, these results demonstrated that UV-Tianjin induced apoptotic cell death in HOS cells through the intrinsic mitochondrial pathways *via* triggering ROS generation and activating MAPK pathways, suggesting that UV-Tianjin might be a potential candidate for the treatment of human osteosarcoma. This study provides an insight into the molecular mechanisms of SeV-induced apoptosis in human osteosarcoma cells.

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Conflict of interest. The authors have no conflicts of interest to declare.

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