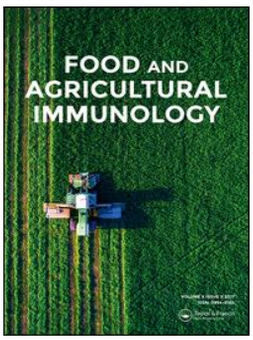

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The cytotoxic effects of spinetoram on human HepG2 cells by inducing DNA damage and mitochondria-associated apoptosis

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ABSTRACT

Spinetoram has been widely used in agriculture due to its target specificity. However, it has not been paid enough attention to the health threats of humans. This study aimed to evaluate the cytotoxic effects of the Spinetoram in human liver cells in vitro. We demonstrated that the viability of HepG2 cells was inhibited by spinetoram in a time- and dose-dependent manner. Intracellular biochemical assays revealed that Spinetoram-induced apoptosis of HepG2 cells was concurrent with a number of activated cell processes, including DNA damage, decrease of mitochondrial membrane potential, release of cytochrome c into the cytosol, marked activation of caspase-9/-3 and cleavage of poly (ADP-ribose) polymerase and up-regulation of Bax/Bcl-2 expression levels. These results indicated that a mitochondrial-mediated pathway contributes to the apoptosis of Spinetoram-induced in HepG2 cells. In summary, this study shows that spinetoram poses a potential risk to human health by inducing DNA damage and mitochondria-associated apoptosis.

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KEYWORDS
Spinetoram; cytotoxic effects; DNA damage; apoptosis; the mitochondrial pathway

1. Introduction

Spinetoram, a macrocyclic lactone compound, was first discovered in soil extracts of the actinomycete *Saccharopolyspora spinose*. spinetoram is now commercialized as a new generation of spinosad pesticide products(Galm & Sparks, 2016) and is composed of two major active ingredients: Spinetoram-J and –L (White et al., 2017). Because of its high insecticidal efficiency, absence of cross resistance, low toxicity toward non-target organisms, and environmentally friendly properties, spinetoram has been widely used in the

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comprehensive management of pests and parasites that affect crops and livestock. It is mainly used to control the insect orders Lepidoptera (*Plutella xylostella*, *Beet armyworm*), Diptera, and Thysanoptera (*Thrips*). The insecticidal efficiency of spinetoram can be attributed to its ability to activate nicotinic acetylcholine receptors (nAChRs) and inhibit gamma-amino butyric acid receptors (GABA) in the insect central nervous system (Khan, Akram, & Shad, 2013; Piner & Uner, 2012). Non-neural cells are not sensitive to the excitatory or inhibitory signalling responses that target nerve cell activities.

Both oral and percutaneous toxicity of spinetoram in rat models have been found to be negligible (LD50 > 5000 mg/kg); moreover, the acute inhalation toxicity is also within safe limits (LC50 > 5.5 mg/L). Therefore, spinetoram has generally been accepted as minimally toxic to human adults. However, several studies have indicated that spinosad can induce DNA damage and apoptosis in human cells. In addition, assessments of the *in vitro* toxicity of spinetoram are limited, and reports suggesting that the compound is toxic to non-target cells highlight the absence of a comprehensive understanding of the mechanism of spinetoram in human cells (Zhang, Luo, et al., 2016). Therefore, further investigating the toxicity of pesticides residuals in human cell are urgently required. The human liver is primarily responsible for metabolizing toxic substances, such as pesticides in food. However, the cytotoxicity of spinetoram on human hepatocytes and the corresponding molecular mechanisms have not yet been completely determined. Here, the cytotoxic effects of spinetoram on human hepatocyte cells were evaluated to assess the safety of these widely used natural product-based pesticides.

DNA damage is a significant parameter in assessing the cytotoxicity and genotoxicity of environmental poisons to human beings (Alavanja, Ross, & Bonner, 2013). Both the toxins themselves as well as their degradation products can adversely affect human health by causing DNA damage (Hongsibsong, Sittitoon, & Sapbamrer, 2017). When cellular DNA is damaged and cannot be repaired, poly(ADP-ribose) polymerase (PARP) plays a crucial role in protecting the cells by repairing the DNA strand breaks either through over-activation of the enzyme or participation in an extracellular signal-regulation pathway (Langelier & Pascal, 2013). In general, there are three main causes of cell death: apoptosis, autophagy, and necrosis. This study verified whether spinetoram reduced human hepatocyte cell viability by inducing apoptosis. Cell death caused by apoptosis occurs via two primary routes known as the intrinsic (mitochondria-mediated) and extrinsic (cell death receptor-mediated) pathways (Guicciardi, Malhi, Mott, & Gores, 2013). The intrinsic pathway is mediated by the mitochondria. Various factors, including the release of a lethal factor cytochrome c into the cytosol, and the up-regulation of caspase and Bcl-2 family proteins, which can be induced by intracellular signals, induce mitochondrial injury (Kale, Osterlund, & Andrews, 2018; Xiong, Mu, Wang, & Jiang, 2014). The decrease in mitochondrial membrane potential (MMP) results in the release of cytochrome c into the cytoplasm. Subsequently, cytochrome c binds Apaf-1 in the presence of dATP following caspase-9 activation, thereby resulting in a caspase cascade. Finally, the activated effector caspase-3 initiates irreversible cell death (Brentnall, Rodriguez-Menocal, De Guevara, Cepero, & Boise, 2013).

Human HepG2 hepatocytes cells, a model cell-line commonly used to study human cellular and molecular biology, were employed in this study. The cytotoxic effects of spinetoram on HepG2 cells were characterized *in vitro* using a series of methods, including cell viability assay, Single Cell Gel Electrophoresis (SCGE), H2AX Foci Staining, fluorescence

microscopy assay, Western blotting, Annexin V-FITC/PI double staining and flow cytometry.

2. Materials and methods

2.1. Chemicals and reagents

Spinetoram (CAS NO: 935545-74-7, 99.5% pure, containing 73.4% XDE-175-J: $C_{42}H_{69}NO_{10}$ pKa 7.86 and 26.6% XDE-175-L: $C_{43}H_{69}NO_{10}$ pKa 7.59) was bought from MOLBASE Shanghai Biotechnology Co., Ltd. (Shanghai, China). Dimethylsulfoxide (DMSO), Rhodamine123(Rh-123),Phenylmethyl sulfonyl fluoride (PMSF), RIPA Lysis buffer, N,N,N′,N′-Tetramethyl ethylenediamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2′,7′-Dichlorodihydrofluorescein diacetate(DCFH-DA), and were all purchased from Sigma-Aldrich (Saint Louis, Missouri, America). Primary antibodies targetingBcl-2, Cytochrome c, Bax, β-actin antibodies, procaspase-9, and PARP were obtained from Sigma-Aldrich Co., LLC. (Saint Louis, Missouri, America). Secondary antibodies that were conjugated with horse radish peroxidase (HRP)were obtained from Sigma-Aldrich Co., LLC. (Saint Louis, Missouri, America). Annexin V-FITC/PI Apoptosis Detection Kit were purchased from Thermo Fisher Scientific (Grand Island, New York, America). The solution of enhanced chemiluminescence was obtained from Tanon (Shanghai, China). Other chemicals and reagents of analytical grade were bought from Shanghai Titan-chem Co. Ltd (Shanghai, China).

2.2. Cell culture

Human HepG2 cells (ATCC, HB-8065, America) were cultured in Dulbecco's modified Eagle's Medium (DMEM, HyClone, Logan, State of Utah, America) supplemented with fetal bovine serum of 10% (FBS, Gibco, Grand Island, New York, America), 1% penicillin-streptomycin solution (HyClone, Logan, State of Utah, America), at 37°C in a humidified atmosphere containing 5%CO_2.

2.3. Drug treatment

The Spinetoram was dissolved in DMSO and the final concentration of this solvent did not exceed 0.1%. The incubation time for Spinetoram (0–20 μM) treatment ranged from 24 to 48 h has indicated in the figures DMSO 0.1% (vehicle) was added to the control group.

2.4. Characterization of cell viability

The cell viability of HepG2 was detected by MTT assay as described in previous literature (Zhang, Liu, et al., 2016). Cell suspensions (1×10^5 cells/mL) were pipetted into 96-well plates for incubation of 24 h, then Spinetoram was added with the final concentration of 5, 7.5, 10, 15,20 μmol/L. Complete DMEM culture medium was used as a blank, fresh medium and cells were used as controls. MTT reagent (20 μL/well, 5 mg/mL) was added to obtain the crystals of formazan, then each plate was incubated at 37°C, 5% CO_2 for 4 h. After 4 h, the above liquid was drawn off and DMSO of 150 μL was added

to dissolve formazan. The absorbance of optical density was measured at 492 and 630 nm respectively by one Synergy H1 microplate reader (Bio-Tech, Inc, Winooski, Vermont, America). The equation for calculating relative inhibition rate was listed as follows: Relative cell viability % = $(OD_{Sample}-OD_{Blank})/(OD_{Control}-OD_{Blank}) \times 100\%$

2.5. Alkaline comet assay

Alkaline comet assay was adopted to detect the DNA damage as described in previous literature (Ateeq, Abul, & Ahmad, 2005; Xu, Yang, Gao, Zhang, & Tao, 2018). The HepG2 cells were collected after being treated by 0, 5, 10, 15 and 20 μM Spinetoram for 12 h, then washed with PBS (pH 7.4) for 3 times to remove Spinetoram. After that, HepG2 cells were mixed with 1% agarose with low melting point at a ratio of 1:5 under 37°C to form monosuspension which was then cast upon the microscopic slide (Cetinkaya, Ercin, Özvatan, & Erel, 2016; Zhang, Luo, et al., 2016). The slide was rinsed by distilled water for 3 times after lysis, immersed in electrophoresis solution of ice alkaline (1 mM EDTA, 300 mM NaOH, with pH of 13.0) at 4°C for 15 min, then the electrophoresis was performed at 20 V (1 V/cm) or 300 mA for 20 min (Ghassemi-Barghi, Varshosaz, Etebari, & Dehkordi, 2016). After 4 h, the slides were washed by neutralized buffer (0.4 mM, pH 7.5) as well as deionized water for three times, before being stained with 35 mL PI solution (20 g/mL). Finally, the cells were photographed and examined with fluorescence microscopy (Lecois, DM3000, GER). DNA damage content was measured by the image analysis system (CASP, www.casp.of.pl).

2.6. Detection of chromatin condensation

Hoechst nuclear staining was adopted to detect the morphological changes of HepG2 cells was after being induced by Spinetoram. In brief, each HepG2 cell was treated with various concentrations of Spinetoram for 24 h. After being rinsed with PBS (pH 7.4) for 3 times, these cells were fixed within the solution of 4% paraformaldehyde at 4°C for 10 min. The fixed cells were then rinsed for 3 times with PBS (pH 7.4) before being stained in 1 mL of Hoechst 33258(5 mg/mL) which was dissolved in 1 mL PBS (pH 7.4) (1:1000). Subsequently, these cells were all incubated at 37°C for 20 min. After being rinsed with PBS (pH 7.4) for 3 times, the morphology of the Spinetoram-treatments HepG2 cells was photographed and examined with the fluorescence microscopy (Lecois, DM3000, GER).

2.7. Assay of apoptosis

Annexin V- FITC/PI apoptosis kit (Invitrogen™) was utilized for estimating apoptosis which was induced by Spinetoram. In brief, HepG2 cells cultured within culture dish of 6 cm were treated with different concentrations of Spinetoram for 24 h incubation. After that, cells were collected (1×10^5 cells per dish), then, they were rinsed for 2 times with PBS (pH 7.4). HepG2 cells were labelled with 10 μL of PI and 5 μL of Annexin V-FITC after centrifugation at 2000rpm for 15 min. Lastly, apoptosis was tested by flow cytometer (BD, *FACSCalibur*), and the software Flow Jo was adopted to analyze the data.

2.8. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential (MMP,) was considered as one of the early signals for cell apoptosis. Spinetoram's effect on mitochondrial membrane potential (MMP) was detected with Rhodamine 123 staining. Cells were seeded in 12-well plates with a density of 1×10^4 cells per well and incubated for 24 h. After being treated with different concentrations of Spinetoram for 6 h, the cells were rinsed for 3 times with PBS (pH 7.4) to remove Spinetoram and incubated with Rh123 (5 mg/mL) for 20 min at 37°C. After that, the cells were rinsed for another three times with PBS (pH 7.4) to remove extracellular fluorescent dyes. Fluorescence microscopy was used to examine HepG2 cells' fluorescence intensity after being treated with Spinetoram. Meanwhile, the treated cells were collected by centrifugation after being washed and tinted with Rh 123 for flowcytometry assay, and software ImageJ was adopted to analyze the data.

2.9. Western blotting

To explore the underlying mechanisms of Spinetoram-induced HepG2 cell death, western blot was used to conduct specific proteins analysis. In brief, with Spinetoram in 6 cm cell culture for 6 h and being centrifuged at 1000 g for 5 min, cells were gathered after incubation. After that, cells were rinsed for 3 times with PBS (pH 7.4) and harvested, the RIPA lysis buffer with 1 mM PMSF was used to lyse cell protein for 30 min. After centrifugation at 12, for 15 min, BCA protein assay was utilized to measure the concentrations of proteins, which could be inactivated by being mixed with 2× sample buffer and heated at 100°C for 5 min. Equal volume of proteins was exposed to 8–15% of SDS-PAGE, then they were transferred by the electrophoresis on the membranes of polyvinylidene fluoride (PVDF) (Millipore Corporation, America). Membranes were all blocked with 5% of skim milk powder within Tris-buffered saline–Tween (TBST; 150 mM NaCl, 10 mM Tris-HCl, 0.1% of Tween-20, pH 7.5) for 2 h and then incubated at4 °C along with the following primary antibodies: PARP, caspase-3, Bax, Bcl-2, β-actin, and cytochrome c (1:1000 of dilution ratio) overnight. After washing for 3 times with 1×Tris-buffered saline as well as tween 20 (TBST), Peroxidase labelled goat anti-rabbit secondary antibody (1:5000; St. Croix) was added and then each PVDF membrane was incubated at room temperature for 2 h. The solution of enhanced chemiluminescence (ECL) (Thermo, Cleveland, Ohio, America) was used to examine the bound antibody, followed by developing with electro-generated chemiluminescence.

2.10. Activity detection of caspase-3

Caspase-3activities play important roles in the process of cell apoptosis. To verify the participation of the two caspases, the cells of HepG2 were treated with 0, 5, 10,15 and 20 μM Spinetoram for 6 h, then collected and washed three times. Lysate was centrifuged at 12,000 g, at 4°C for 5 min after cells being lysed by wards lysate buffer. Supernate was collected and BCA kit was used to determine the concentration of protein. Then the cells extracted were incubated with 20 ng of Ac-DEVD-*p*NA (activity of caspase-3) in one 96-well plate at 37°C for 4 h, and one Synergy H1 microplate reader was used to measure the UV-Vis absorbance at 405 nm.

2.11. Statistical analysis

At least 3 independent experiments were performed for every experiment, it was found that findings of all independents were closely related with each other. One-way variance analysis was conducted for data, followed by one t-test, *p*-values (less than 0.05). SPSS 22.0 was used to perform each statistical analysis (SPSS Inc., Chicago, Illinois, America).

3. Results

3.1. Spinetoram inhibited HepG2 cell viability.

Spinetoram's cytotoxic effect on HepG2 cell viability was detected by MTT assay. Inhibition rates for the HepG2 cells were 17.12 ± 2.54%, 33.07 ± 1.99%, 42.19 ± 3.52%, 77.15 ± 1.78% and 90.50 ± 3.15% respectively after 24 h of treatment with Spinetoram, which finally reached to 22.07 ± 2.59%, 40.81 ± 2.09%, 56.17 ± 3.14%, 85.53 ± 3.09% and 96.69 ± 2.37% respectively after 48 h treatment. The values of LC_{50} were shown in Table 1. Results demonstrated that HepG2 cell viability was inhibited by Spinetoram in time- and dose-dependent manner (Figure 1).

3.2. Spinetoram induced DNA damage in HepG2 cells

To have an understanding of whether Spinetoram causes DNA damage, assay of alkaline comet was applied as migrated DNA fragments could form one comet-like image. In the Spinetoram treatments, the control group (Figure 2(A)), in which the heads of cornet centred on the high-density DNA along with smooth margin and intact nuclei. With an increase of Spinetoram concentration, the comet tails increased while head size decreased in comparison (Figure 2(B)). While Table 2 shows that the severity of impairment triggered by Spinetoram induces the generation of typical DNA fragmentation within the cells of HepG2 is dose-dependent.

3.3. Apoptosis of HepG2 cells induced by Spinetoram

After being treated with Spinetoram of 0, 5, 10, 15 and 20 μM for 24 h, an increasing number of HepG2 cells began to undergo chromatin condensation and fragmentation (Figure 3(C)). In order to certify the cell apoptosis, flow cytometer was used to perform the annexin V-FITC/PI staining (Figure 3(A)). When cells undergo early apoptosis, phosphatidyl serine binds to Annexin-V and then binds to FITC to visualize green fluorescence. On the other hand, when in the late stage of apoptosis or death, the structural

Table 1. The half maximal inhibitory concentration (IC50) of HepG2 cells exposed to Spinetoram in 24 and 48 h.

					95%(confidence limits)	
Cell lines	Teatment time(h)	a(intercept)	b(slope)	IC_{50}(μM)	Lower	Upper
HepG2	24	1.42	3.62	10.73 a	9.89	11.50
	48	2.17	3.08	8.27 b	7.39	9.25

Note: Small alphabet indicated statistically significant differences between any two groups in the same column ($P \leq 0.05$).

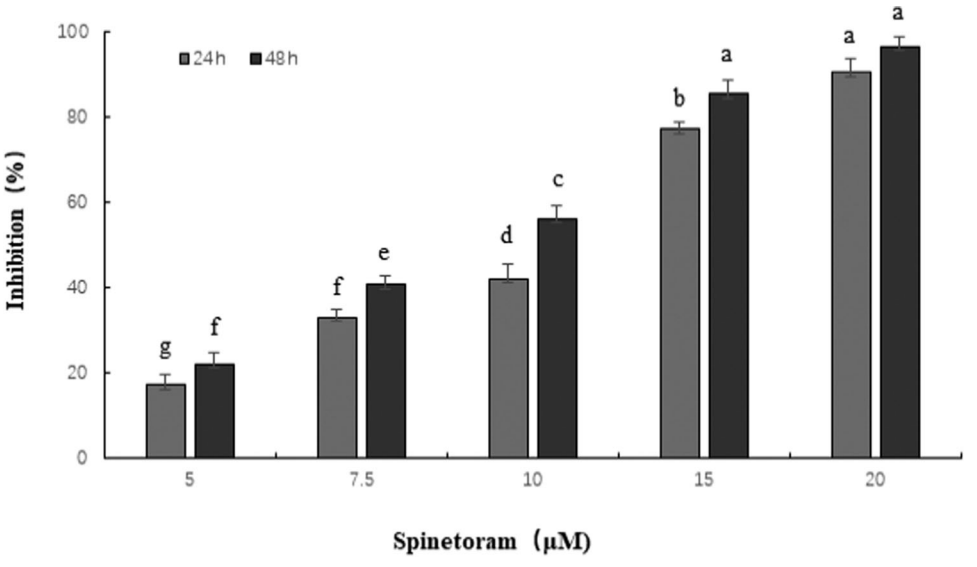

Figure 1. Effect of Spinetoram on the viability of HpeG2 cells. HepG2 cells viability varied in response to various concentrations of Spinetoram for 24 and 48 h. Small alphabets indicate significant differences ($P \leq 0.05$) between any two groups. Data are expressed as mean ± SD of three separate sets of independent experiments.

integrity of the cell membrane is destroyed, and the PI dye enters the cell to bind to the DNA to exhibit red fluorescence. The results showed that the ratio of apoptotic cells at the early stage raised from 1.50 ± 1.03% for cells without Spinetoram treatment to 3.52 ± 1.41%, 4.58 ± 1.29%, 13.60 ± 3.66%, 35.60 ± 2.79% respectively and at the late stage, the apoptotic rate raised from 1.19 ± 1.09% for the cells without Spinetoram treatment to 8.86 ± 1.57%, 16.50 ± 2.76%, 34.60 ± 3.05% for cells treated with different concentrations of Spinetoram, respectively. The quantitative analysis showed that the significant increase of HepG2 apoptosis is dose-dependent (Figure 3(B)).

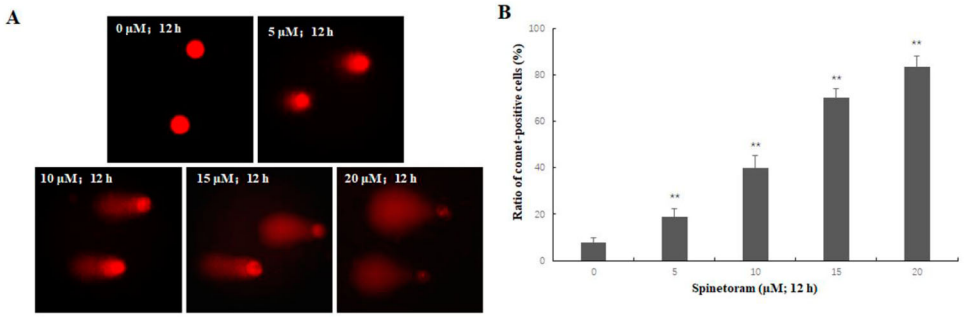

Figure 2. Spinetoram induced DNA damage in HepG2 cells. (A) DNA fragments are shown as comet images in alkaline gel electrophoresis (200×). The percentage of comet-positive cells in the treatment of Spinetoram for 12 h analysis results (B). Data are represented as means ± SD from three independent experiments. (*) $P \leq 0.05$; (**) $P < 0.01$.

Table 2. DNA damage (mean %Tail DNA; Tail Length; Tail Moment) in HepG2 cells detected by alkaline comet assay after exposed to the different concentrations of Spinetoram for 12 h.

Concentrations (μM)	Comet assay parameters		
	Tail DNA (%)	Tail Length (μm)	Tail Moment
0	0.18 ± 0.06 d	3.33 ± 0.58 c	0.13 ± 0.01 c
5	17.42 ± 2.34 c	27.00 ± 3.01 b	7.40 ± 6.14 c
10	42.29 ± 4.14 b	55.00 ± 7.57 a	28.33 ± 3.72 b
15	51.18 ± 6.27 b	66.33 ± 7.02 a	43.86 ± 5.23 b
20	62.18 ± 4.50 a	76.00 ± 6.24 a	59.74 ± 6.20 a

Note: Data represent mean values (±SD) of three independent experiments. Different small letters in the same column indicate significant differences ($P \leq 0.05$) between any two groups.

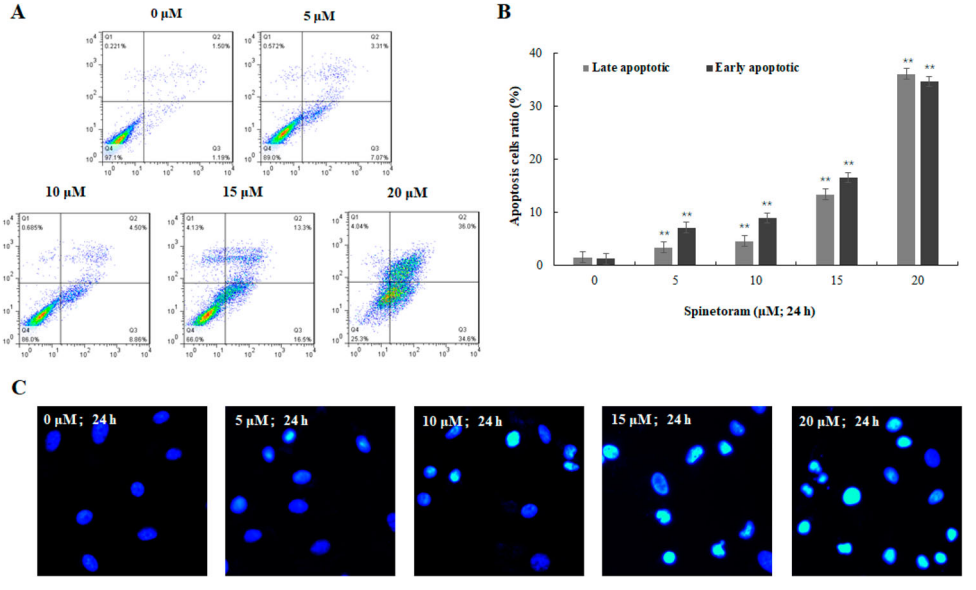

Figure 3. Spinetoram induced apoptosis in HepG2 cells. (A) The lower left panel shows the normal cells, the lower right panel shows the early apoptotic cells, and the upper right panel shows the late apoptotic cells or undergoing necrotic cells (24 h). (B) Apoptosis cell ratio. Data are represented as the means ± SD from three independent experiments. (**) $P \leq 0.01$. (C) Cell nuclei were observed by fluorescence microscopy (200×). Typical apoptosis morphological changes are shown in treated cells including chromatin condensation and DNA fragmentation.

3.4. Mitochondrial dysfunction in HepG2 cells induced by Spinetoram

MMP change shows that mitochondrial ultrastructure with damaged iconic changes is an early characteristic of the mitochondrial apoptosis pathway(Wang et al., 2014). Flow cytometry and Fluorescent microscopy were utilized to examine MMP quantification inHepG2 cells on the treatment of Spinetoram. In our study, it was discovered that HepG2 cells exposure to different concentrations (0, 5, 10, 15 and 20μM) of Spinetoram for 6 h led to gradual dose-dependent decline in fluorescence intensity (Figure 4). The results demonstrated that Spinetoram could induce mitochondrial dysfunction in HepG2 cells.

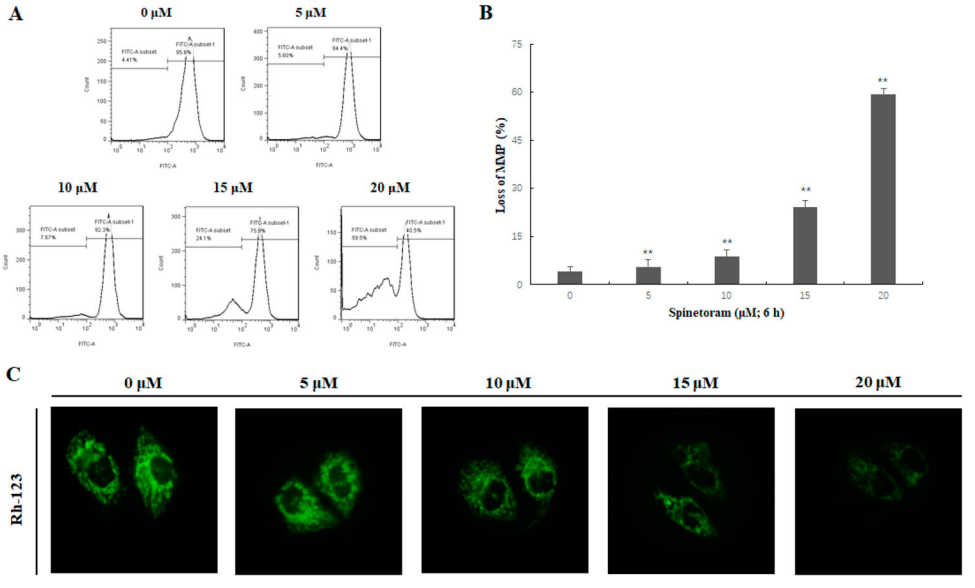

Figure 4. Spinetoram induced ΔΨm collapse on the HepG2 cells. (A) Cells were treated with Spinetoram at various concentrations (0, 5, 10, 15, and 20μM) for 6 h. (B) Changes in ΔΨm were evaluated by the flow cytometry method of Rh-123. Data are the means ± SD of three independent experiments. (∗) $P \leq 0.05$ and (∗∗) $P \leq 0.01$. (C) Intracellular Rh-123 level was observed by confocal laser scanning microscope.

3.5. Influence of Spinetoram on the apoptosis-related protein level in HepG2 cells

To elaborate the reaction mechanism of Spinetoram-triggered apoptosis, extracted apoptosis-associated proteins of HepG2 cells treated with different concentrations of Spinetoram (0, 5, 10, 15 and 20 μM) were measured by western blot. The release of cytochrome c increased dose-dependent for the Spinetoram-induced HepG2 cells (Figure 5(A)). Moreover, the expression level of Bax/Bcl-2 was upregulated dose-dependently after Spinetoram treatment (Figure 5(B,C)).

3.6 Effect of Spinetoram on activation of caspase-3/-9 and PARP cleavage in HepG2 cells

Caspase plays a significant role in regulating apoptosis. In proapoptotic stage, Spinetoram-treated HepG2 cells were examined for PARP cleavage andcaspase9/-3 activation through Western blot or colorimetric enzymatic assay. PARP cleavage and caspase-3 and caspase-9 activation increased with the concentration of Spinetoram (Figure 5(D)). The results showed that the Spinetoram-induced apoptosis could be mediated by caspase-9/-3 activation.

4. Discussion

Over recent years, the natural product pesticides have gradually been challenged (Yang et al., 2018; Zhang, Luo, et al., 2016). Increasing evidence demonstrates that anti-insect antibiotics can exert toxicological effects on non-target organisms (Zhang et al., 2017,

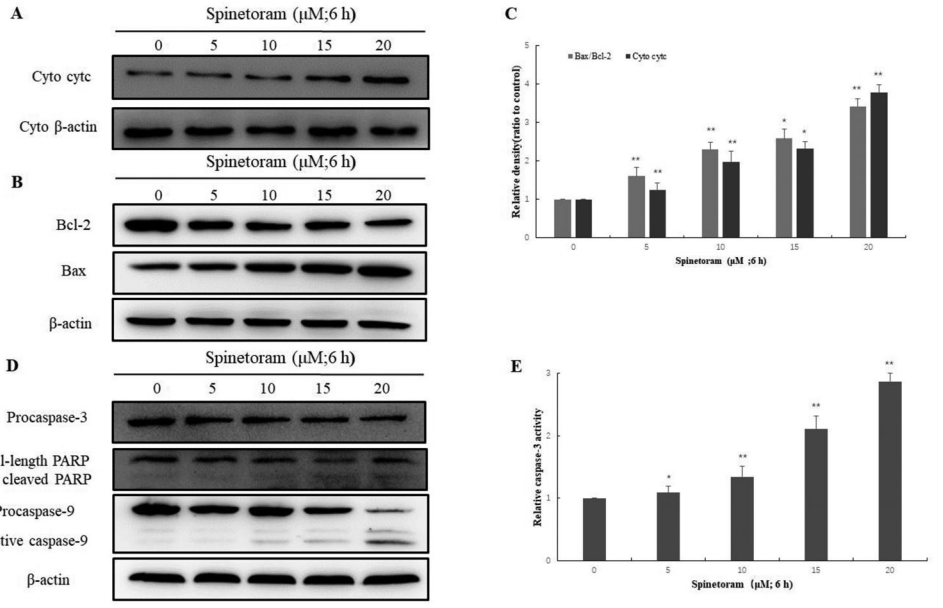

Figure 5. Effect of Spinetoram on the expression of apoptosis-related proteins in HepG2 cells. Whole cell extracts were prepared for cells treated with Spinetoram for 6 h the expression levels of Cyto-cyt c, Bax, Bcl-2, PARP, caspase-3 and caspase-9 were determined by western blotting assay (A, B and D). Cyto denote cytosolic fractions. b-actin was used as internal control. Expression of Cyto-cytc and Bax/Bcl-2, the ratio was adjust-regulated by Spinetoram-treatment in HepG2 cells (C). Caspase-3 activity increased vitality (E). Data are expressed as mean ± SD of three separate sets of independent experiments. (**) $P \leq 0.01$.

2018). Therefore, further investigation into the toxicity of pesticides residuals in human cells is urgently required. Spinetoram was awarded the 2008 Presidential Green Chemistry Challenge Prize because of its environment friendly characteristics, broad-spectrum insecticidal properties, and low toxicity to non-target organisms (Park et al., 2012). It also offers a higher level of prolonged activity than other spinosad products. Nevertheless, current research has indicated the adverse effect of spinosad on the non-target cells, but there are few reports about the toxicity of Spinetoram in non-target cells and absence of a comprehensive understanding of its mechanism in the human hepatocyte cells (Yang et al., 2016; Zhang et al., 2019). Therefore, in this study, evaluation demonstrated the presence of underlying genotoxicity and apparent cytotoxicity of Spinetoram in human HepG2 cells, and whether or not its action mechanism depends upon the pathway of mitochondrial apoptosis was also explored.

Previous studies showed that both oral and percutaneous toxicity of the original drug of Spinetoram in rat models are negligible and the acute inhalation toxicity are also within safe level. Therefore, Spinetoram's biological safety on human HepG2 cells were assessed by MTT assays to determine the cytotoxicity of different Spinetoram concentrations on HepG2 cells viability. The results showed that Spinetoram is much more toxic than spinosad and it had significant *in vitro* cytotoxic effects on human HepG2 cells.

Alkaline single-cell gel electrophoresis was used to detect DNA strand breaks induced by spinetoram in the HepG2 cells. Figure 2 shows the damage caused by spinetoram in the

test group compared with the control group. The resulting data illustrates a decrease in the cell head size with a concomitant rise in the dimensions of comet tails in a dose-dependent manner associated with an increase in the concentration of spinetoram. This experimental data demonstrated DNA damage induced by spinetoram in HepG2 cells. In addition, an Annexin V-FITC/PI apoptosis kit was used to stain the HepG2 cells, and flow cytometry was adopted to analyze cell apoptosis. The proportion of apoptotic cells verified the hypothesis that spinetoram could induce apoptosis in a dose-dependent way. Furthermore, microscopic observations of divided and condensed nuclei indicated chromosomal DNA fragmentation. These results offered an initial demonstration that spinetoram can induce the death of HepG2 cells via apoptotic pathways.

The two most significant characteristics of the intrinsic mitochondria-mediated apoptotic pathway are: (i) the release of cytochrome c and (ii) the collapse of the MMP (Dou et al., 2018). A decrease in MMP is primarily caused by the opening of a permeability transition pore (PTP), which results in the loss of potential difference on both sides of the inner mitochondrial membrane (Sileikytė et al., 2011). The MMP collapse induced by spinetoram was demonstrated using fluorescent microscopy (Figure 4). Cytochrome c is considered a marker for the mitochondrial apoptotic signal transduction pathway (Garrido et al., 2006). Upon activation of the apoptotic signalling pathway, cytochrome c is affected by the opening of the mitochondrial PTP and then released from the mitochondria into the cytoplasm, thereby leading to an elevated cytochrome c level (Figure 5). Apoptotic bodies are formed between cytochrome c and both apoptotic protease-activating factor (Apaf-1) and procaspase-9 in the presence of dATP, thereby causing caspase-9 activation (Figure 5(D)) and resulting in a caspase cascade. Finally, the activated effector caspase-3 (Figure 5(E)) causes irreversible cell death. Taken together, these findings indicate that this mitochondrial-mediated pathway potentially contributes to Spinetoram-induced apoptosis in HepG2 cells.

Apoptosis is regulated by various factors, among which the Bcl-2 family of proteins is known to be one of the most important (Imran et al., 2017). Here, the two most involved proteins of the Bcl-2 family were apoptosis-inhibiting Bcl-2 and apoptosis-promoting-Bax. The expression and balance of Bcl-2 and Bax and the release of cytochrome c are closely related to the maintenance of MMP. These processes regulate the occurrence and intensity of apoptosis, and the content ratio of Bcl-2, Bax, and cytochrome c is an important "regulatory switch" for apoptosis. By regulating the mitochondrial PTP, the life and death of a cell is determined. Our results indicated that the Bax/Bcl-2 ratio was positively correlated with the concentration of spinetoram. The increase in the Bax/Bcl-2 ratio resulted in the release of cytochrome c from mitochondria, the collapse of the MMP, and the activation of caspase related protease and PARP cleavage. The final results indicated that these steps contributed to spinetoram-induced apoptosis of the HepG2 cells.

Furthermore, the present study evaluated the presence of the apparent cytotoxicity and underlying genotoxicity of spinetoram in human HepG2 cells and determined whether its mechanism of action involves the mitochondrial apoptosis pathway. The biological safety of spinetoram in the human HepG2 cells was assessed using MTT assays to determine the cytotoxicity of various concentrations of spinetoram on cell viability. The results indicated that spinetoram is much more toxic than spinosad and exerts considerable *in vitro* cytotoxic effects on human HepG2 cells. Spinetoram induces the accumulation of

mitochondrial dysfunction in HepG2 cells. Spinetoram treatment induced a concentration-dependent increase in the Bcl-2/Bax ratio, which could trigger the release of cytochrome c and initiate the activation of caspase-9/-3 and the cleavage of PARP.

In summary, these results indicate that spinetoram exerts cytotoxic effects on human HepG2 cells by inducing DNA damage and mitochondria-associated apoptosis. These results not only provide guidance toward understanding the safety of spinetoram but also offer a theoretical basis for understanding its toxicity mechanism. Further research is warranted to identify whether there is a correlation between the apoptosis described and the potential genotoxicity of spinetoram.

Disclosure statement

No potential conflict of interest was reported by the authors.

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