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Original Article

MicroRNA-181a knockdown protects HepaRG cells from Dichlorvos-induced oxidative stress and apoptosis

Caifeng Gu, Jie Shen, Feng Zhang, Junfeng Chen

The Centre of Emergency and ICU, Jinshan Hospital Affiliated to Fudan University, Shanghai, China

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Abstract: The present study was designed to assess the molecular mechanism of Dichlorvos (DDVP)-induced hepatic cell toxicity in vitro using HepaRG cells. The cytotoxicity was determined by cell viability, apoptosis portion, and reactive oxygenspecies (ROS) generation. The results indicated that DDVP treatment significantly inhibited cell growth, induced cell apoptosis and promoted the production of ROS on HepaRG cells. Microarray analysis showed that miR-181a was significantly upregulated in HepaRG cells treated with DDVP. Furthermore, we found that miR-181a downregulation has a remedy effect on DDVP-induced cell toxicity, while miR-181a overexpression augments the DDVP-induced hepatic cell apoptosis and ROS production. Furthermore studies showed that miR-181a directly targeted Bcl-2, and Bcl-2 downregulation inhibited the remedy effect of miR-181a inhibitor on DDVP induced cell toxicity. It is, therefore, concluded that miR-181a knockdown could protect hepatic cells from DDVP induced oxidative stress and apoptosis by targeting bcl-2.

Keywords: Dichlorvos, HepaRG cell, toxicity, miR-181a, Bcl-2

Introduction

Organophosphate pesticides are one class of the most widely used synthetic chemicals for agricultural and domestic pest control [1]. Among them, Dichlorvos (DDVP) is a commonly used insecticide [2]. It not only results in the environmental pollution due to its use in agriculture, but also poses a great threaten to public health, as DDVP is poisonous when inhaled, swallowed or absorbed through skin and eyes. Recent years, a large number of acute and chronic poisoning events occurred.

Emerging studies suggested DDVP exposure has been related to substantial negative health effects on several organ systems, including the respiratory system [3-5], the nervous system [6, 7] and reproductive system [8, 9]. In addition to that, recently, it has been reported that DDVP also can induce the damage and dysfunction of liver. Binukumar et al. (2010) reported that a mitochondrial energy metabolism impairment and liver dysfunction occurred in rodent model following chronic exposure to DDVP [10]. Zhao et al. (2015) reported a case of DDVP induced autoimmune hepatitis [11]. Bui-Nguyen et al. (2015) demonstrated that DDVP exposure results in large scale disruption of energy metabolism in the liver of the zebrafish [12]. However, the precise molecular mechanism of hepatic response to DDVP exposure remains unclear till now, although its cellular mechanisms have been identified, such as cell apoptosis, ROS production [13].

MicroRNAs (miRNAs) are a class of short (~22 nt), endogenous regulatory RNAs. Changes in microRNA (miRNA) expression occur in many pathological and physiological processes, such as cell viability, apoptosis, and so on [14]. Previous studies have demonstrated that the potential and important role of miRNAs to regulate apoptosis at a variety of levels and in several organisms [15]. However, whether microRNAs involved in DDVP induced hepatic cell toxicity remains unclear.

The present study, therefore, was aimed to detect the miRNA role in DDVP induced hepatic

cell dysfunction. We firstly demonstrated that DDVP could induce HepaRG cells apoptosis and ROS production. Then, we found that miR-181a is up-regulated in DDVP treated HepaRG cells by using microarray analysis and Real-time PCR. Furthermore, we showed that miR-181a involved in the apoptosis process of hepatic cells exposed to DDVP by binding its target gene Bcl-2.

Materials and methods

Chemicals and cell culture

DDVP was obtained from Merck (Germany). HepaRG cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in DMEM (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (HyClone, USA) at 37°C with 5% CO_2.

Caspase activation and western blot

Caspase-3 activity was measured using acommercially assay kit, according to the manufacturer'sinstructions. Total cell protein was isolated from HepaRG cells after treatment. The BCA protein assay kit (Beyotime, Shanghai, China) was used to determine the protein concentration, following manufacturer's instruction. Samples were electrophoresed by using 10% SDS-PAGE. The protein was then transferred onto a PVDF (polyvinylidene fluoride) membrane (Bio-Rad, USA). After blocking in skim milk, the membranes were incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). β-actin was used as internal reference; goat anti-Bcl-2, anti-cleaved-caspase-3 (1:1000) were purchased from Sigma.

Cell viability assay

Cell viability was analyzed by using Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China), followed by manufacturer's instructions. Briefly, the HepaRG cells were seeded into the 96-well plate. 10 μL of Cell Counting Kit-8 solution was then added into each well. The OD450 values were detected by using the Spectra Microplate Reader (BIOTEC). Results were showed as percentage of the vehicle control levels set at 100%. Each treatment was performed in triplicate.

ROS determination

The intracellular amounts of ROS was measured according to the method described by Ben et al. (2016) [16].

Microarray and data analysis

Total RNA was extracted from HepaRG cells treated with DDVP for 24 h, using TRIZOL reagent (Invitrogen) and sent to Biomaker Company form iRNA microarray analysis. Data were analyzed using ANOVA andt tests.

Quantitative real-time PCR

RNA was reverse transcribed to cDNA from 500 ng of total RNA by using a Reverse Tranascription Kit (Tiangen, Beijing, China). Real-time PCR assay were performed with SYBR Green (Tiangen, Beijing, China). All protocols were carried out according to the manufacturer's instructions. The primer sequences in terms of U6, miR-181a were listed as follows: U6: Forward (F): 5'-CTCGCTTCGGCAGCACA-3', Reverse (R): 5'-AACGCTTCACGAATTTGCGT-3'; miR-181a F: 5'-GAACATTCAACGCTGTCGGTG-3', R: 5'-ATCCAGTGCAGGGTCCGAGGTA-3'. Real-time PCR assay was performed on a Real-Time PCR System (Applied Biosystems, USA), and each RT reaction was performed in triplicate, including no-template controls. The reaction was performed in the following conditions: 4 min at 96°C, followed by 39 cycles with denaturing for 17 s at 94°C and annealing for 45 s at 62°C. The relative quantification of miR-181a was normalized to the expression of U6 using the $2^{-\Delta\Delta CT}$ method, respectively.

Luciferase reporter assay

MiR-181a mimics, miR-181a inhibitors, and miRNA normal control (miR-NC) were purchased from GenePharma (Shanghai, China). After placed into 48-well plates, cells were cotransfected with miR-181a, luciferase reporter plasmids (200 ng) containing wild-type (WT) or mutant type (Mut) of Bcl-2 3'UTR. Fourty-eight hours after transfection, luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Each transfectionwas performed in triplicate.

Apoptosis assay

The HepaRG cells transfected with miR-NC, miR-181a mimics, miR-181a inhibitors, pEGFP-

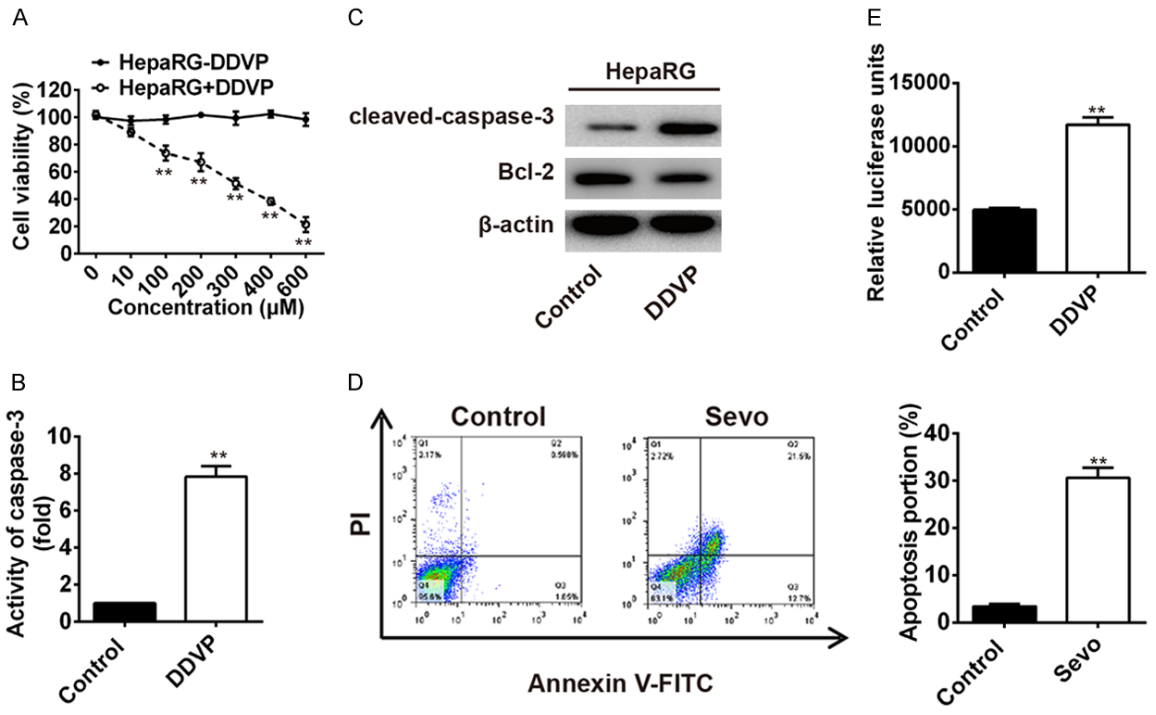

Figure 1. DDVP-induced cell apoptosis and ROS in vitro. A. The inhibit effect of DDVP on HepaRG cells viability. HepaRG cells were treated with DDVP at the indicated concentrations ranging from 10-600 μM for 24 h. MTT assay was used to detect cell viability. The values are expressed as viability percentage. $^{**}P < 0.01$ DDVP treatment group vs. Non-treatment controls. B. DDVP increased caspase-3 activity in HepaRG cells. Cells were treated with DDVP at the concentration of 300 μM for 24 h.$^{**}P < 0.01$ vs. control. C. DDVP increased cleaved caspase-3 expression and decreased bcl-2 expression in HepaRG cells. The protein expression was determined using western blot. D. DDVP induced HepaRG cells apoptosis. The cell apoptosis was detected by flow cytometry.$^{**}P < 0.01$ DDVP treatment group vs. Non-treatment control. E. DDVP promoted ROS production in HepaRG cells.$^{**}P < 0.01$ DDVP treatment group vs. Non-treatment control.

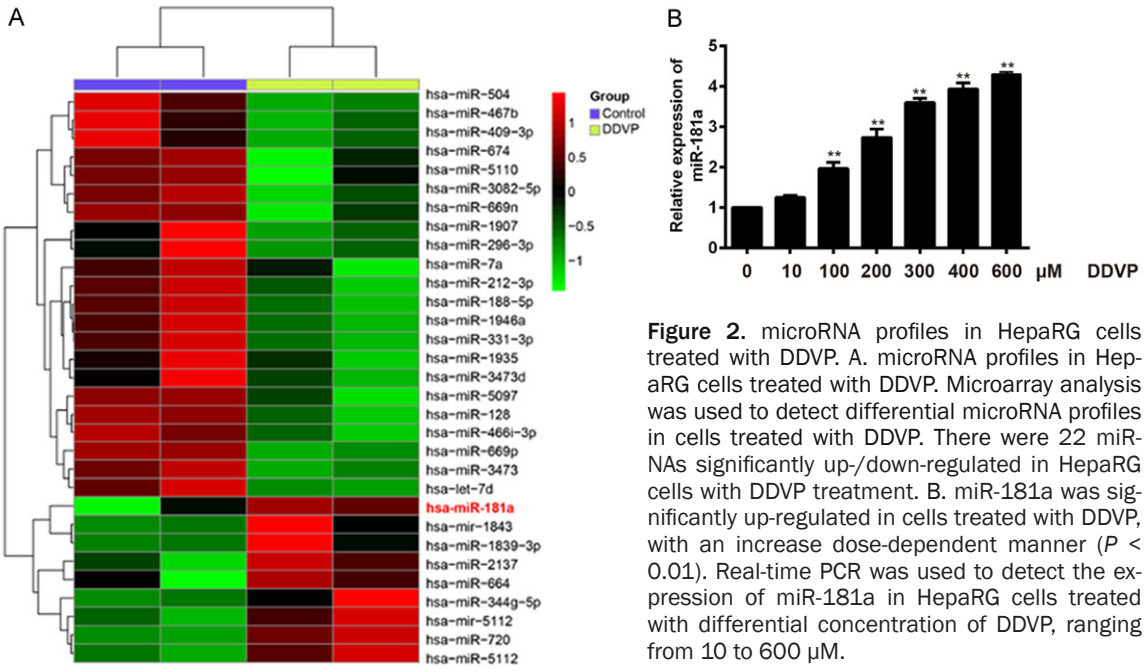

Figure 2. microRNA profiles in HepaRG cells treated with DDVP. A. microRNA profiles in HepaRG cells treated with DDVP. Microarray analysis was used to detect differential microRNA profiles in cells treated with DDVP. There were 22 miRNAs significantly up-/down-regulated in HepaRG cells with DDVP treatment. B. miR-181a was significantly up-regulated in cells treated with DDVP, with an increase dose-dependent manner ($P < 0.01$). Real-time PCR was used to detect the expression of miR-181a in HepaRG cells treated with differential concentration of DDVP, ranging from 10 to 600 μM.

NC, and stably transfected with pEGFP-Bcl-2 were harvested 48 hours after transfection by trypsinization. Apoptotic cells were detected by Annexin V/APC and propidium iodide (PI) apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA), following the manufacturer's recommendations. The cells were analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a Cell Quest software. Cells were distinguished into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and then the relative ratio of early apoptotic cells were compared with control transfection from each experiment. Each sample was run in triplicate.

Statistical analysis

Data were shown as mean ± standard deviation (SD) from atleast three separate experiments. The student's t test and one-way ANOVA were used to conduct the comparison of the different protein, mRNA, luciferase reporter and miRNA expression levels. Statistical analyses were performed by SPSS 16.0 software. A *P*-value less than 0.05 was considered statistically significant difference.

Results

DDVP induced HepaRG cells apoptosis and ROS production

HepaRG cells were treated with differential concentrations of DDVP, ranging from 10 to 600 μM for 24 hours, and cell viability was detected by MTT assay. Results showed that DDVP induced a dose-dependent decrease in cell viability ($P < 0.01$), compared with controls (**Figure 1A**). The DDVP concentration of 300 μM, was therefore, selected as the cell treatment concentration. Caspase-3 activity was significantly increased in DDVP group ($P < 0.01$, **Figure 1B**). Western blot analysis showed that the pro-apoptosis protein cleaved-caspase-3 expression was significantly increased in DDVP group, while the anti-apotosis protein bcl-2 expression was significantly decreased in DDVP group, when compared with controls (**Figure 1C**). DDVP also induced HepaRG cells apoptosis ($P < 0.01$, **Figure 1D**). The levels of ROS production in DDVP group was significantly increased ($P < 0.01$, **Figure 1E**) than that of controls. It is, therefore, indicated that DDVP inhibited cell growth, induced cell apoptosis and promoted the production of ROS on HepaRG cells.

microRNAs profiles in HepaRG cells treated with DDVP

To identify miRNA involved in the mechanism of DDVP treated HepaRG cells, we detected miRNAs expression profiling of HepaRG cells treated with DDVP. By using microarray analysis, we compared miRNA expression profiles in DDVP treated cells to control. miRNAs were considered as differentially expressed when differences in expression levels were significant both in Student's t test ($P < 0.01$) and analysis of microarray test (q value < 5%). Among the individual miRNAs displayed on the microarray, there were 22 miRNAs significantly up-/down-regulated in HepaRG cells with DDVP treatment. The most up-regulated miRNA was miR-181a (**Figure 2A**). Next, we carried out real-time PCR to detect the expression of miR-181a in HepaRG cells treated with differential concentration of DDVP, ranging from 10 to 600 μM. Results showed that miR-181a was significantly up-regulated in cells treated with DDVP, with an increase dose-dependent manner ($P < 0.01$) (**Figure 2B**). Summarizing, these data demonstrated that miR-181a is up-regulated in DDVP treated HepaRG cells, suggesting miR-181a might involve in DDVP induced HepaRG cell toxicity.

The effect of miR-181a on DDVP induced cell apoptosis and ROS production

HepaRG cells were pretreated with miR-181a inhibitor/inhibitor NC for 12 h before DDVP treatment for 24 h (300 μM). Cell viability was significantly inhibited in DDVP + inhibitor NC group ($^{**}P < 0.01$) when compared with the control. However, miR-181a inhibitor significantly reversed DDVP-induced cell viability inhibition ($^{\#\#}P < 0.01$, DDVP + miR-181a inhibitor vs. DDVP + inhibitor NC group, **Figure 3A**). Furthermore, miR-181a significantly inhibited DDVP-induced HepaRG cells apoptosis ($^{\#\#}P < 0.01$, **Figure 3B**), caspase-3 activity increase ($^{\#\#}P < 0.01$, **Figure 3C**), and ROS production ($^{\#\#}P < 0.01$, **Figure 3D**). In contrast, different phenomenon occurred in HepaRG cells treated with miR-181a mimics. miR-181a mimics promoted the DDVP induced cell viability inhibition ($^{\#}P < 0.05$, **Figure 3E**), enhanced DDVP-induced HepaRG cells apoptosis ($^{\#\#}P < 0.01$, **Figure 3F**), upregulated the caspase-3 activity and ROS production ($^{\#}P < 0.05$ for both, **Figure 3G**, **3H**). It is, therefore, suggested that miR-181a down-

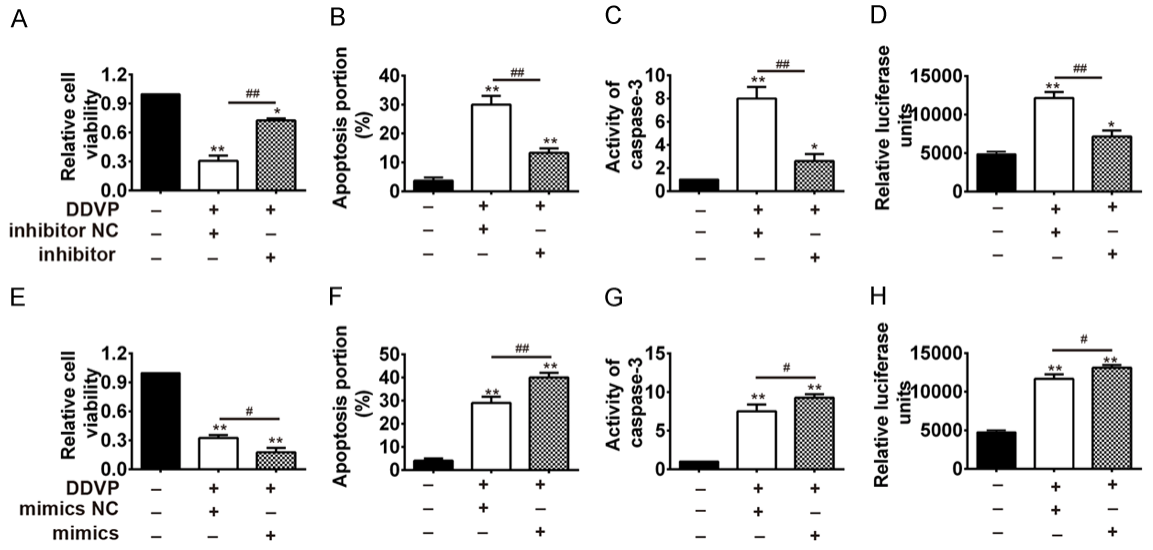

Figure 3. The effect of miR-181a on DDVP induced cell apoptosis and ROS production. HepaRG cells were pretreated with miR-181a inhibitor/inhibitor NC for 12 h before DDVP treatment for 24 h (300 μM). A. The cell viability in different groups was analyzed by using CCK-8 kit. Cell viability was significantly inhibited in DDVP + inhibitor NC group and DDVP + miR-181a inhibitor group when compared with the controls. However, miR-181a inhibitor reversed DDVP-induced cell viability inhibition. B. The cell apoptosis was detected by flow cytometry. miR-181a inhibitor weakened DDVP-induced cell apoptosis. C, D. miR-181a inhibitor decreased DDVP-induced caspase-3 activity and ROS production. E. Cell viability was analyzed by using CCK-8 kit. miR-181a mimics promoted DDVP-induced cell viability inhibition. F. miR-181a mimics upregulated DDVP-induced cell apoptosis. G, H. miR-181a mimics enhanced DDVP-induced caspase-3 activity and ROS production.*$P < 0.05$,**$P < 0.01$, vs. control. #P < 0.05,##P < 0.01, vs. DDVP + inhibitor NC group or DDVP + mimics NC group.

regulationhas a remedy effect on DDVP-induced cell toxicity, while miR-181a overexpression augments the DDVP-induced hepatic cell apoptosis and ROS production.

miR-181a directly targets Bcl-2 mRNA

We used bioinformatic tools (Targetscan and miRBase) to identify the target gene of miR-181a. As shown in **Figure 4A**, Bcl-2 is predicated as the theoretical target gene of miR-181a. Furthermore, to validate whether Bcl-2 is a direct target gene, the 3'-UTR region of Bcl-2 was fused to a luciferase system. Western blot assay showed that the expression of Bcl-2 protein was significantly decreased after miR-181a mimic treatment (**$P < 0.01$, **Figure 4B**), while expression of Bcl-2 protein levels were significantly increased after miR-181a inhibitor treatment, compared to the NC group (##$P < 0.01$, **Figure 4B**). Similar phenomenon was also found at the mRNA levels of Bcl-2 (**$P < 0.01$, ##$P < 0.01$, **Figure 4C**). **Figure 4D** showed that miR-181a obviously suppressed the luciferase activities of the 3'-UTR segment of Bcl-2, whereas miR-181a inhibitor significantly increased the luciferase activities of the 3'-UTR segment of Bcl-2, but not those of the construct containing a mutant binding site (Bcl-2 3'-UTR-mut), compared to the NC group.

Bcl-2 downregulation inhibited the remedy effect of miR-181a inhibitor on DDVP induced cell toxicity

Since Bcl-2 was the target gene of miR-181a, and miR-181a inhibitor has the protection effect of DDVP. Herein, we knock down Bcl-2 to evaluate its protection function of miR-181a on the DDVP-induced cell toxicity, including cell viability, apoptosis, ROS production, and so on. We found that the downregulation of Bcl-2 reversed the miR-181a inhibitor protective effect on the HepaRG cell viability, when compared with non-transfection treatment group (##$P < 0.01$, si-Bcl-2 + miR-181a inhibitor + DDVP vs. miR-181a inhibitor + DDVP group, **Figure 5A**). In the above experiment, we found that miR-181a inhibitor could rescue the increased apoptosis portion, caspase-3 activity and ROS production caused by DDVP treatment. However, when transfected with si-Bcl-2, these effects weak-

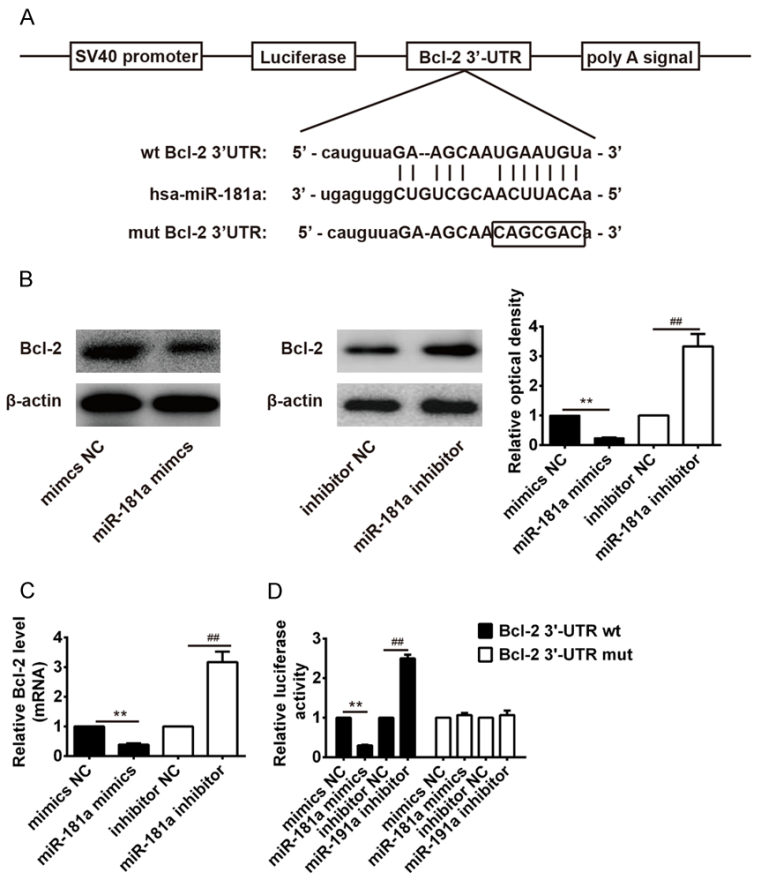

Figure 4. Bcl-2 is the direct target of miR-181a. A. A putative miR-181a-binding site exists in the 3'-UTR of Bcl-2 mRNA and point mutations were generated in the binding site. B. The expression of Bcl-2 protein was analyzed by Western blotting. β-actin was used as control. C. mRNA expression of Bcl-2 was detected by real-time PCR after treatment with miR-181a mimics or miR-181a inhibitor (n=3). $^{**}P < 0.01$ vs. mimics NC group; $^{\#\#}P < 0.01$ vs. inhibitor NC group. D. The luciferase reporter plasmid containing wild-type or mutant Bcl-2 3'-UTR was cotransfected into HepaRG cells with miR-181a mimic/inhibitor or miR-NC. Luciferase activity was determined by the dual luciferase assay and shown as the relative firefly activity normalized to Renilla activity. Each bar represents the mean and s.d. of three independent experiments. $^{**}P < 0.01$ vs. mimics NC group; $^{\#\#}P < 0.01$ vs. inhibitor NC group.

ened, as can be seen from the increased level of apoptosis portion ($^{\#\#}P < 0.01$, si-Bcl-2 + miR-181a inhibitor + DDVP vs. miR-181a inhibitor + DDVP group, **Figure 5B**), caspase-3 activation and expression ($^{\#\#}P < 0.01$, si-Bcl-2 + miR-181a inhibitor + DDVP vs. miR-181a inhibitor + DDVP group, **Figure 5C**, **5E**, **5F**), as well as increased patterns of ROS production ($^{\#\#}P < 0.01$, si-Bcl-2 + miR-181a inhibitor + DDVP vs. miR-181a inhibitor + DDVP group, **Figure 5D**). These results suggested bcl-2 downregulation participated in the protection role of miR-181a involved in DDVP induced hepatic cell apoptosis and ROS production. Summarizing, DDVP induced cell cytoxicity might or at least through miR-181a upregulation by binding its target gene Bcl-2.

Discussion

Since DDVP exposure could pose a serious threaten to human health, and recently studies reported that it could induce the damage and dysfunction of liver [10, 11], however, the precise molecular me-chanism of DDVP induced hepatic cell toxicity is not reported in the literature. In the present study, we firstly found that the HepaRG cells treated with DDVP significantly inhibited cell viability, based which, we propose that hepatic cells might undergo apoptotic cell death when exposed to DDVP for a certain time period. To confirm this, studies of proteins involved in the apoptotic pathway and apoptosis assays were carried out.

Caspases are critical mediators of programmed cell apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins, thereby acting as an important indicator of cell apoptosis [17]. Several studies have been reported that activation of caspase-3 is essential to the apoptosis of hepatic cells [17, 18]. Bcl-2 is known as an anti-apoptosis molecular [19]. In the present study, we observed a significant increase of activation and cleavage of caspase-3, as well as decreased Bcl-2 in DDVP treated HepaRG cells. Our finding is consistent with the reports published by Sunkaria et al. (2012), who found that caspase-3 activation was increased in DDVP treated primary rat microglia [6]. Furthermore, cell apoptosis detected by flow cytometry showed that DDVP induced significantly apoptosis of HepaRG cells. And also, our study found that the DDVP

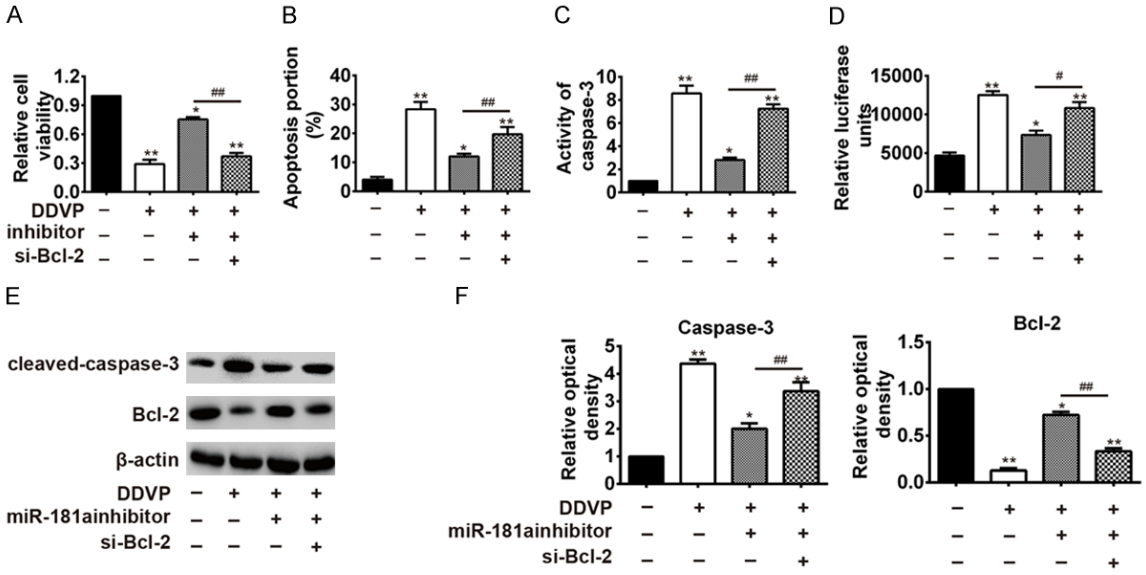

Figure 5. Bcl-2 downregulation inhibited the protective effect of miR-181a inhibitor on DDVP-induced HepaRG cell cytotoxic. Bcl-2 was knocked down by transfected with si-Bcl-2 to evaluate its function in the miR-181a protected DDVP-induced cell toxicity, including cell viability, apoptosis, ROS production. The cells were divided into following groups: control, DDVP group, miR-181a inhibitor + DDVP group, si-Bcl-2 + miR-181a inhibitor + DDVP group. A. The downregulation of Bcl-2 reversed the protective effect of miR-181a inhibitor on the HepaRG cell viability, when compared with non-transfection treatment group. B. miR-181a inhibitor could rescue the increased apoptosis portion caused by DDVP, however, when transfected with si-Bcl-2, these effects diminished, as can be seen from the increased level of apoptosis portion. C. When transfected with si-Bcl-2, theactivity of caspase-3 in HepaRG cell was significantly increased when compared with miR-181a inhibitor + DDVP group. D. The ROS production, valued as relative luciferase units, in HepaRG cells transfected with si-Bcl-2 was significantly increased, when compared with miR-181a inhibitor + DDVP group. E. The expression of caspase-3 and Bcl-2 in HepaRG cells with different treatments was detected by western blot analysis, respectively. F. The caspase-3 and Bcl-2 expression value was determined by relative optical density, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control group. $^{\#\#}P < 0.01$, si-Bcl-2 + miR-181a inhibitor + DDVP vs. miR-181a inhibitor + DDVP group.

induced significantly ROS production in HepaRG cells. Thus, the present study showed that DDVP can inhibit HepaRG cell viability and ultimately apoptotic cell death, and ROS production.

MicroRNAs (miRNAs) are a kind of short length RNA molecules regulating the target gene expression, thereby playing role in biology processes, including proliferation, differentiation, and death [15]. Emerging studies have demonstrated that miR-181a involved in cell apoptosis [20-22]. However, to date, levels of hepatic cell-associated miRNAs in response to DDVP exposure has received limited attention. In the current study, we found that miR-181a was significantly upregulated in HepaRG cells after treatment with DDVP. Furthermore, we treated HepaRG cells with miR-181a inhibitors and mimics, respectively, and found that miR-181a downregulation has a remedy effect on DDVP-induced HepaRG cells apoptosis and ROS production, while miR-181a overexpression augments the DDVP-induced hepatic cell apoptosis and ROS production. It is therefore, suggested miR-181a involved in DDVP induced cell apoptosis.

It is widely acknowledged that microRNAs play biology function by targeting transcription mRNA [23]. MiR-181a has been reported to inhibitcell viability, induce apoptosis and play function in disease, via binding its target gene [20, 24-26]. Bcl-2 is one of the Bcl-2 family membersand specifically considered an important anti-apoptotic protein which plays essential roles in the regulation of apoptosis via the mitochondrial pathway [27, 28]. Previous study demonstrated that downregulation of miR-181a protects mice from LPS-induced acute lung injury by targeting Bcl-2 [25]. Bcl-2 is reported to participate in the apoptosis process of hepatic cells [29]. Accordingly, we speculated that miR-181a target the anti-apoptotic

Bcl-2 in HepaRG cells. In this study, we identified that miR-181a targeted 3'-UTR of Bcl-2. MiR-181a mimic decreased Bcl-2 expression at both transcription and translation levels, while miR-181a inhibitor could promote Bcl-2 expression.

In order to evaluate whether miR-181a participate in DDVP-induced cell apoptosis by binding its target gene Bcl-2, we carried out the following study. Bcl-2 was knockdown and cell apoptosis and ROS production were determined. Results showed that downregulation of Bcl-2 reversed the protective effects of miR-181a inhibitor on DDVP treated HepaRG cells, as the decreased cell viability, the increased cell apoptosis portionand the increased ROS productionwere observed in si-Bcl2 transfected cells, when compared with non-transfection controls. It is, therefore, suggested that DDVP induced cell apoptosis by miR-181a via binding its target gene Bcl-2.

In conclusion, our study revealed that miR-181a inhibitor could protect hepatic cells from DDVP induced oxidative stress and apoptosis by targeting bcl-2. However, our study still have some limitations, further investigations remain to be carried out to verify this point.

Disclosure of conflict of interest

None.

Address correspondence to: Caifeng Gu and Feng Zhang, The Centre of Emergency and ICU, Jinshan Hospital Affiliated to Fudan University, No. 1508, Longhang Road, Shanghai 201508, China. Tel: +86-21-57281687; E-mail: caifenggu@yeah.net (CFG); jgyyzf@126.com (FZ)

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