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Vitamin D Receptor Regulates Autophagy to Inhibit Apoptosis and Promote Proliferation in Hepatocyte Injury

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Background: Oxidative stress is an important mechanism in liver ischemia/reperfusion (I/R) injury. Hepatocyte apoptosis and proliferation occur in parallel with liver I/R injury, and the degree of apoptosis and proliferation determines the effects on hepatocytes. Vitamin D receptor (VDR) can lessen liver I/R injury, but previous studies focused mostly on inflammation and immunity.

Methods: H_2O_2 was used to induce hepatocyte injury. Before treatment with H_2O_2, Hep-3B cells were pretreated with paricalcitol (PC) and siRNA-VDR. Rapamycin and chloroquine were also applied in the study.

Results: The number of apoptotic cells was measured with an annexin V (AV)-fluorescein isothiocyanate apoptosis detection kit. Expression of proteins was measured by western blotting. As compared with the H_2O_2+Hep-3B group, levels of AV/PI, cleaved caspase-3, and p62 were lower, and expression levels of Bcl-2, proliferating cell nuclear antigen, and VDR were higher, in the PC+H_2O_2+Hep-3B group. When the VDR gene was silenced by siRNA-VDR in the siRNA-VDR+H_2O_2+Hep-3B group, expressions of AV/PI, cleaved caspase-3, and p62 were upregulated, and expressions of Bcl-2, proliferating cell nuclear antigen, and VDR were downregulated, as compared with values for the siRNA-NC+H_2O_2+Hep-3 B group. Treatment with rapamycin or chloroquine partially reversed the effect of PC and siRNA-VDR on apoptosis and proliferation.

Conclusions: VDR mediates hepatocyte apoptosis and proliferation through autophagy.

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Key words: vitamin D receptor, apoptosis, proliferation, autophagy, hepatocyte injury

Introduction

Liver ischemia/reperfusion (I/R) injury has 2 distinct phases: an ischemia injury phase and a reperfusion injury phase. Ischemia is the lack of blood supply and results in oxygen deficiency and disruption of cellular metabolism. Reperfusion—the restoration of blood flow—triggers an inflammatory cascade and worsens organ damage[1,2]. Liver I/R injury is an unavoidable complication in many clinical settings, such as trauma, liver resection, and transplantation, and the outcome of liver surgery depends on the severity of liver I/R injury. Currently, there is no method to prevent liver I/R injury. It is therefore necessary to further study the mechanism and identify new preventive therapies for liver I/R injury.

Although the mechanism of liver I/R injury has been studied for many years, there is no satisfactory technique to prevent it. The evidence indicates that many factors, including exosomes, lncRNA, and ferroptosis, are involved in liver I/R injury[3,4]. Moreover, numerous studies continue to examine the effect of reactive oxygen species (ROS) on liver I/R injury[5,6].

ROS produced during liver I/R are pivotal in initiating

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sterile inflammation. Furthermore, this inflammation and the subsequent cascade response injure hepatocytes[7,8]. Therefore, hepatocyte damage, which promotes and aggravates liver injury during I/R, is a reasonable target for reducing liver injury.

Vitamin D receptor (VDR) is a nuclear receptor that participates in many pathophysiological functions and is activated by natural or synthetic VDR agonists[9]. Ligands of VDR were found to improve liver I/R injury[10,11]. Moreover, VDR expression is lower in hepatic parenchymal cells than in nonparenchymal cells, such as hepatic stellate cells, Kupffer cells, and endothelial cells[12]. Research on VDR in liver injury has focused on hepatic nonparenchymal cells, especially Kupffer cells[10,13]. However, the relationship between VDR and hepatic nonparenchymal cells has received more attention in liver I/R injury.

A PubMed search using the keywords "vitamin D" and "liver ischemia/reperfusion injury" identified 9 articles, 4 of which were reports on the relationship vitamin D and liver I/R injury. Only one report was a study of vitamin D and hepatocytes[14]. Therefore, the effect of VDR on hepatocytes in liver I/R injury is unknown and needs further clarification. We hope the present findings will yield a new strategy for preventing hepatocyte damage in liver I/R injury.

Materials and Methods

Cell Culture

Hep-3B cells (National Collection of Authenticated Cell Cultures, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was a solution containing 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. H_2O_2 was dissolved in phosphate-buffered saline and then diluted in the culture medium to the final concentration. Before co-culturing with H_2O_2 (200 μM) for 24 h, Hep-3B cells were pretreated with paricalcitol (PC, 100 nM, MCE, Shanghai, China), rapamycin (RAP, 100 nM, MCE, Shanghai, China), or chloroquine (CQ, 10 μM, MCE, Shanghai, China) for 24 h.

RNA Interference in Vitro

For the purpose of RNA interference, Hep-3B cells were transfected with control siRNA-NC and siRNA-VDR (Santa Cruz Biotechnology, CA, USA). In accordance with the manufacturer's instructions, Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Shanghai, China) in Opti-MEM (Gibco) was used to start the transfection process.

Annexin V and Propidium Iodide Labeling

The cell samples were stained with an annexin V (AV)-fluorescein isothiocyanate apoptosis detection kit (Sigma-Aldrich, St Louis, USA) according to the manufacturer's instructions. The data were analyzed with Cellquest software (Becton-Dickinson, San Jose, USA) and are presented as the percentage of cells in 4 different population phenotypes: unstained, stained with PI only, stained with AV only, and stained with both markers, relative to the total number of cells analyzed.

Western Blot Analysis

RIPA lysis buffer with Protease and Phosphatase Inhibitor Cocktail (Abcam, Shanghai, China) was used to extract all proteins from cell samples. Protein concentrations were detected with a BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai, China). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and separately transblotted onto polyvinylidene fluoride membranes (Millipore, United States), followed by blocking with 5% nonfat dry milk. These membranes were incubated with primary antibodies against VDR, proliferating cell nuclear antigen (PCNA), and p62 (Abcam, Shanghai, China), as well as with Bcl-2, cleaved caspase-3, and β-actin (Cell Signaling Technology, MA, USA). After incubation with secondary antibodies, the bands were quantified by densitometry using Quantity One software for image analysis.

Statistical Analysis

Data are presented as the mean ± SD from at least 3 independent experiments, and statistical significance was calculated by performing 1-way analysis of variance with SPSS software (Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

VDR Activation Decreased the Number of Apoptotic Cells

After H_2O_2 stimulation, the level of AV/PI was higher in the H_2O_2+Hep-3B group. As compared with values in the H_2O_2+Hep-3B group, PC (a synthetic VDR agonist) activated VDR and decreased the level of AV/PI. However, when siRNA-VDR inhibited the VDR gene in the siRNA-VDR+H_2O_2+Hep-3B group, the level of AV/PI was markedly lower than that of the siRNA-NC+H_2O_2+Hep-3B group (**Fig. 1**).

VDR Mediated Cell Apoptosis and Proliferation

In addition to AV/PI level, we also measured expression of cleaved caspase-3 and Bcl-2. After VDR activation by PC in the PC+H_2O_2+Hep-3B group, expression of

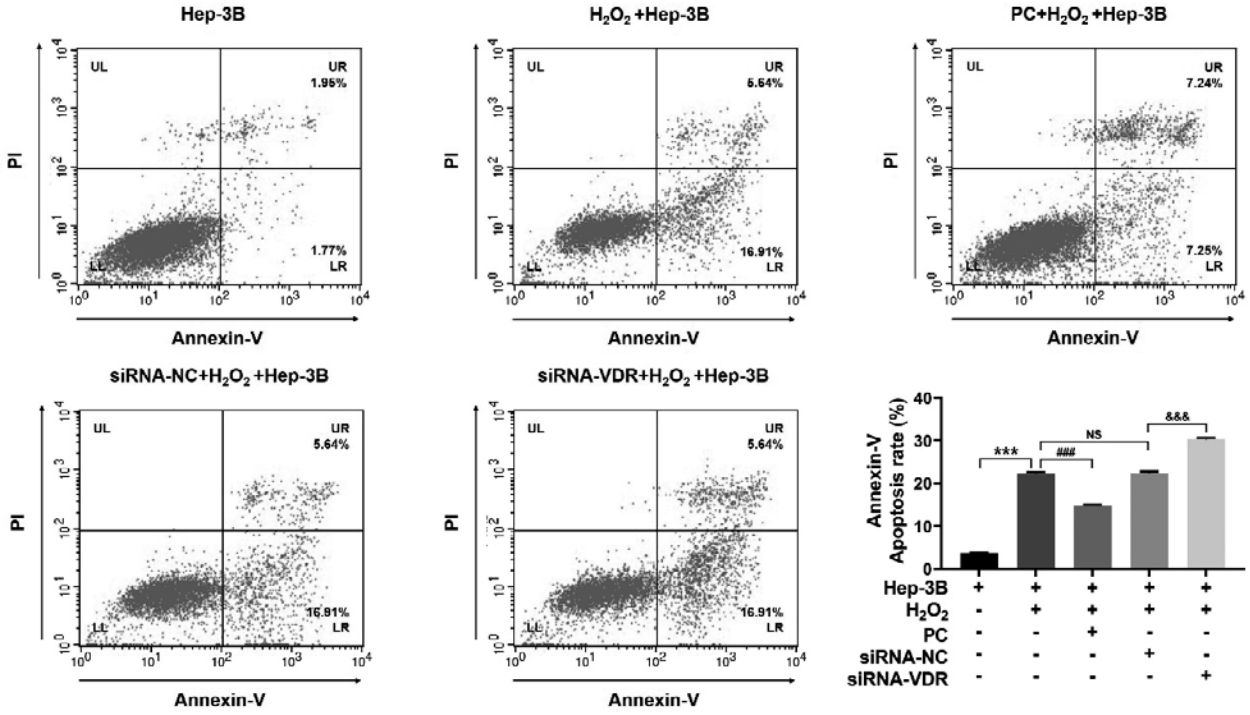

Fig. 1 VDR activation decreased the level of AV/PI. After Hep-3B cells were stimulated with H_2O_2, the level of AV/PI was measured.
***P<0.001; ###P<0.001; &&&P<0.001; NSP>0.05.

cleaved caspase-3 was lower, and the level of Bcl-2 was higher, than the values in the H_2O_2+Hep-3B group. As compared with the siRNA-NC+H_2O_2+Hep-3B group, expression of cleaved caspase-3 was upregulated, and expression of Bcl-2 was downregulated, in the siRNA-VDR +H_2O_2+Hep-3B group (**Fig. 2A**).

Analysis of cell proliferation showed that when PC activated VDR in the PC+H_2O_2+Hep-3B group, PCNA expression was obviously higher than that of the H_2O_2+ Hep-3B group. When the VDR gene was silenced in the siRNA-VDR+H_2O_2+Hep-3B group, PCNA expression was markedly lower than in the siRNA-NC+H_2O_2+Hep-3B group (**Fig. 2B**).

VDR Participated in Cell Apoptosis and Proliferation by Autophagy

Autophagy is critical in cell survival[15], and expression of p62 protein is inversely associated with autophagy. After H_2O_2 stimulation, the level of p62 was higher than that in the Hep-3B group without H_2O_2 stimulation. Expression of p62 was enhanced in the siRNA-VDR+H_2O_2+ Hep-3B group. However, the level of p62 was lower in the PC+H_2O_2+Hep-3B group. The difference in p62 expression between the PC+H_2O_2+Hep-3B group and siRNA-VDR+H_2O_2+Hep-3B group contrasted with the VDR expression profile (**Fig. 3**). Hence, we hypothesize that autophagy is a bridge between VDR and both apoptosis and proliferation in hepatocyte injury.

To demonstrate the relationship between VDR and autophagy, RAP and CQ were used in this study. As compared with levels in the siRNA-VDR+H_2O_2+Hep-3B group, PCNA and Bcl-2 levels were higher, and levels of AV/PI and cleaved caspase-3 were lower, in the RAP+ siRNA-VDR+H_2O_2+Hep-3B group. However, as compared with levels in the PC+H_2O_2+Hep-3B group, levels of AV/PI and cleaved caspase-3 were higher, and levels of PCNA and Bcl-2 were lower, in the CQ+PC+H_2O_2+ Hep-3B group (**Fig. 4, 5**). These findings suggest that autophagy is involved in the association of VDR with cell apoptosis and proliferation.

Discussion

After reperfusion of the ischemic liver, liver I/R injury can be classified as acute and subacute. The acute stage is dominated by the presence of liver-resident macrophages, at 3 to 6 h, while infiltrating neutrophils characterize the subacute stage, at 18 to 24 h[16]. Regardless of stage, ROS are the most important factor in liver I/R injury and are mainly produced during mitochondrial energy metabolism through oxidative phosphorylation in the respiratory chain[17]. Components of hepatocytes damaged by ROS during the ischemic stage activate liver-resident macrophages, initiate an inflammatory cascade,

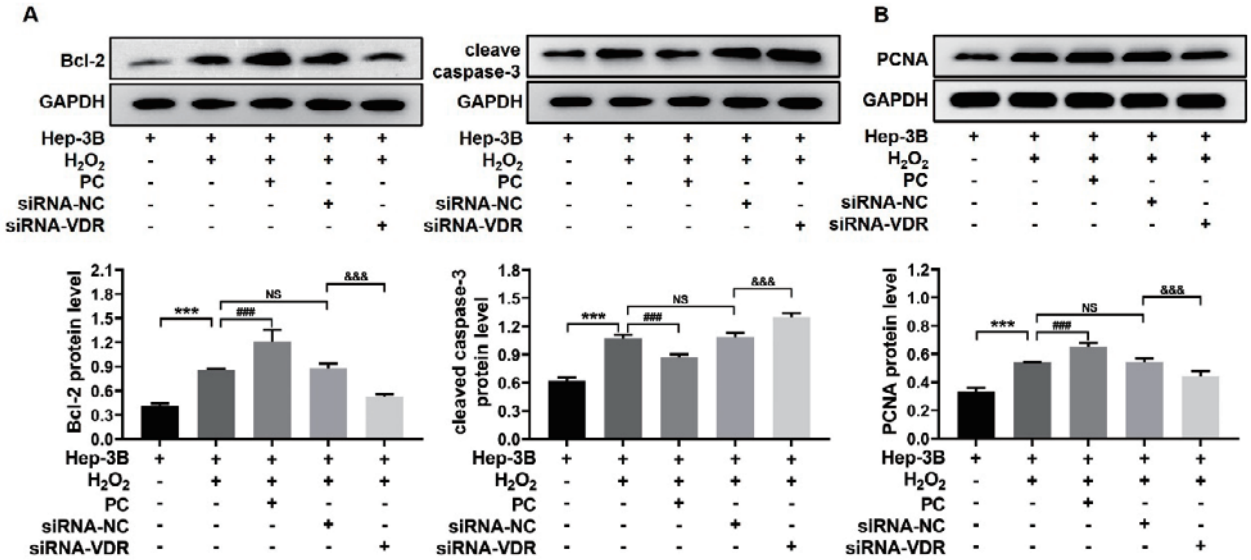

Fig. 2 The effect of VDR on cell apoptosis and proliferation. Expression of Bcl-2 and cleaved caspase-3 was detected by western blotting in (**A**); PCNA expression was tested in (**B**).
***P<0.001; ###P<0.001; &&&P<0.001; NSP>0.05.

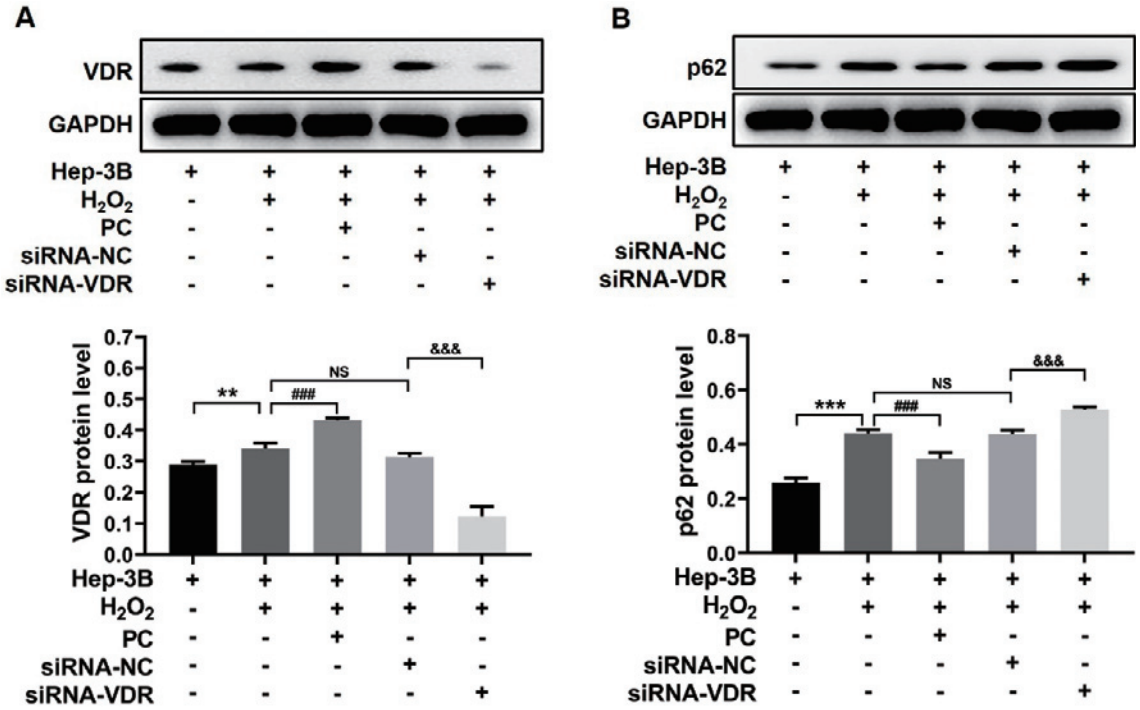

Fig. 3 VDR regulated autophagy in ROS-induced hepatocyte injury. After stimulating Hep-3B cells with H_2O_2, VDR expression was measured in (**A**); p62 expression is shown in (**B**).
**P<0.01 and ***P<0.001; ###P<0.001; &&&P<0.001; NSP>0.05.

and then further aggravate hepatocyte injury[18]. Infiltrating neutrophils from circulation during the subacute stage can also cause hepatocyte injury by generating and releasing ROS[1]. Hence, ROS are closely related with the 2 phases and are a harmful factor in liver injury[1,18]. It was thus reasonable to use ROS to induce hepatocyte injury in our study.

Apoptosis is the highly regulated process of programmed cell death. The products of apoptosis can be cleared and removed by phagocytic cells without inducing an inflammatory response. However, when apoptosis exceeds the handling capacity of an organ or tissue, inflammation and necrosis will result[19]. ROS generated by I/R injury in the liver[20] lead to hepatocyte apoptosis,

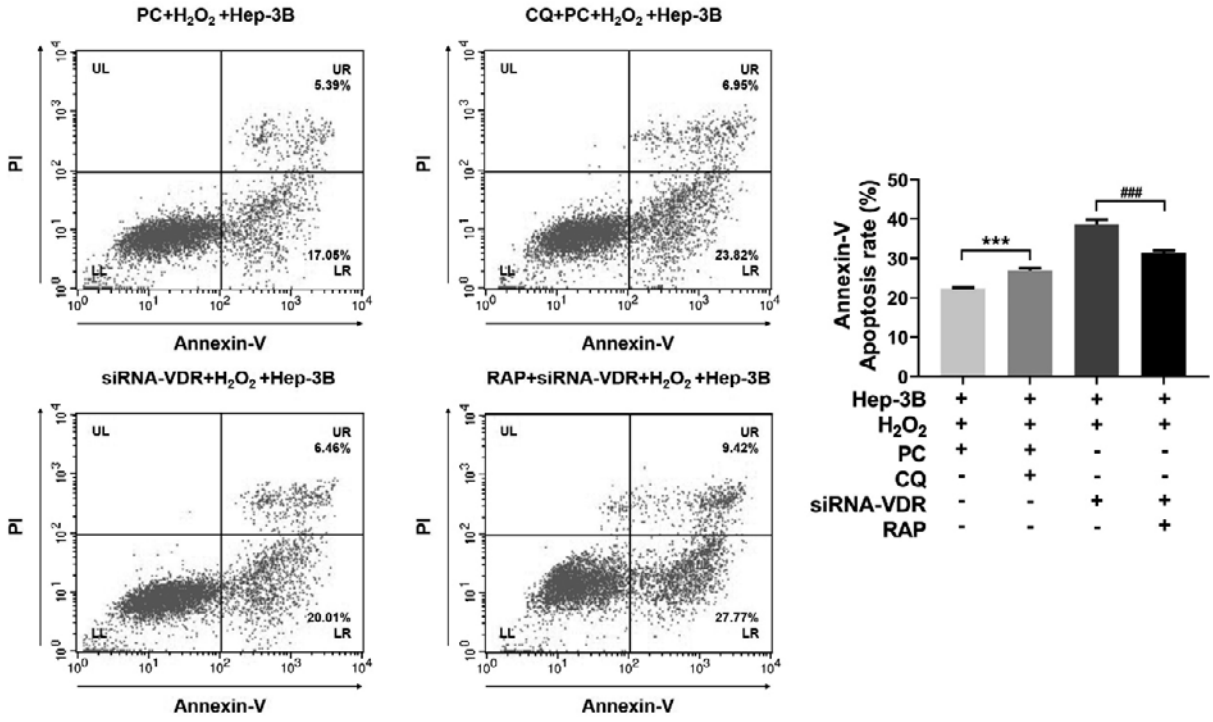

Fig. 4 Autophagy is involved in cell apoptosis. After treatment with RAP and CQ in vitro, the level of AV/PI was detected.
***P<0.001; ###P<0.001.

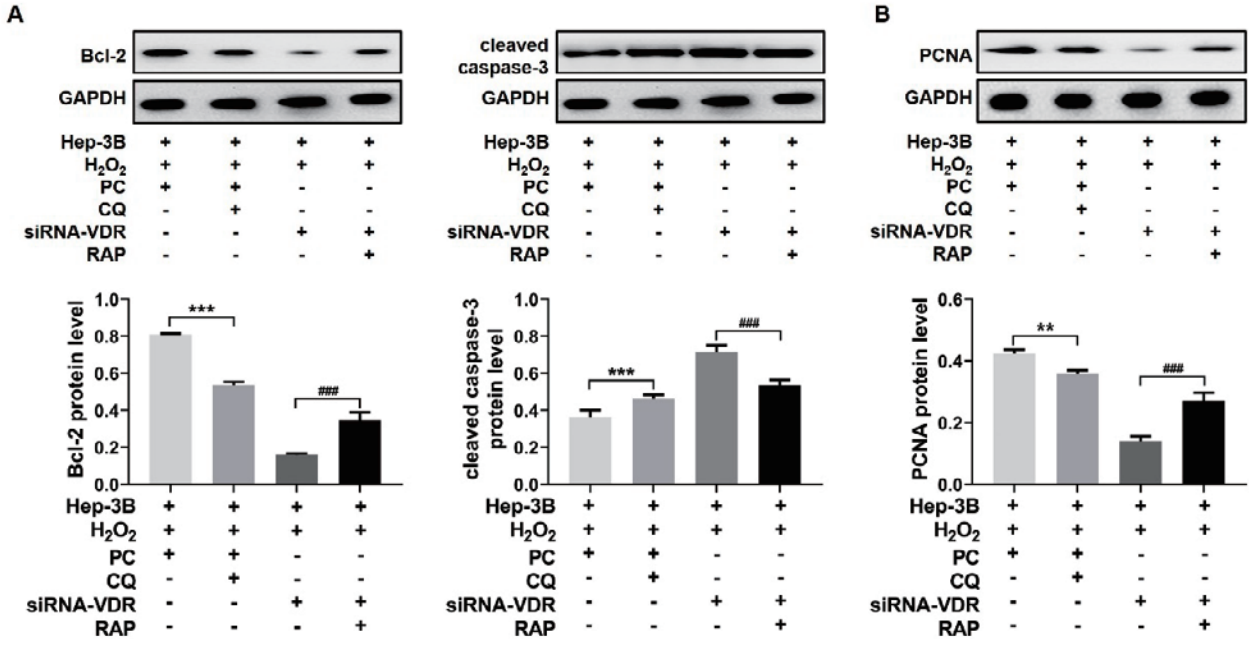

Fig. 5 VDR mediated cell apoptosis and proliferation by autophagy. Expression of Bcl-2 and cleaved caspase-3 was measured in (**A**); PCNA expression was tested in (**B**).
**P<0.01 and ***P<0.001; ###P<0.001.

which occurs in parallel with hepatic dysfunction[21,22]. In our study, apoptotic indicators were upregulated after H_2O_2 stimulation. Therefore, reducing hepatic apoptosis is a therapeutic target for liver I/R injury[23].

Liver regeneration and hepatocyte apoptosis are the hallmarks of liver I/R injury. To restore hepatic function and mass, liver regeneration is initiated during the late phase of reperfusion (24-72 h), which is consistent with hepatocyte proliferation. Hepatocyte proliferation is a vital recovery process for liver I/R injury[1]. PCNA was

measured as an indicator of hepatocyte proliferation in the present research and increased during the study.

Autophagy is indispensable for maintaining a balance in energy metabolism and cellular environmental homeostasis in eukaryotic cells. Autophagy also substantially affects cell apoptosis, and this effect is related to cell type and cellular conditions[24]. Autophagy in liver I/R injury has a dual role in cellular survival and apoptosis, which depend on the degree of I/R injury[25,26]. In our study, differences in p62 level between groups showed that autophagy is involved in ROS-induced hepatocyte injury. Moreover, autophagy protected hepatocytes from ROS damage in this study.

Although VDR ligands were previously found to protect the liver from I/R injury[10,11], the effect of VDR on hepatocytes in liver I/R injury is rarely mentioned. Activated VDR alleviate hepatocyte damage[27] and has an antiproliferative effect on liver cancer[28]. In the present study VDR had a role in cell apoptosis and proliferation, which was not the case in a previous study[28], perhaps because of differences in the microenvironment. In addition, our results showed that p62 expression was inversely correlated with VDR level, which indicates that VDR activation alleviates hepatocyte damage by enhancing autophagy, which is consistent with the findings of other studies[27,29].

In summary, ROS are essential in liver I/R injury, which is characterized by hepatocyte damage. Apoptosis and proliferation are indispensable conditions in liver I/R injury. VDR was involved in mediating hepatocyte apoptosis and proliferation by autophagy. Our study sheds light on the effect of VDR on hepatocytes, and on the mechanisms involved, and may lead to new treatments for liver I/R injury.

Conflict of Interest: None declared.

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