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Cisplatin induces apoptosis of A549 cells by downregulating peroxidase V

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Abstract. – OBJECTIVE: The purpose of the study was to investigate the role of peroxidase V (Prx V) in Cisplatin-induced apoptosis of A549 cells and its underlying mechanism.

MATERIALS AND METHODS: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was conducted to evaluate the regulatory effect of Cisplatin on the survival of A549 cells. ROS (Reactive Oxygen Species) levels in A549 cells induced with 0, 2, 4, and 6 mol/L Cisplatin for 24 h was determined using immunofluorescence. Apoptosis of Cisplatin-induced A549 cells was determined by immunofluorescence and flow cytometry, respectively. Western blot was performed to detect protein levels of Prx V, Bcl-2 (B-cell lymphoma 2), BAD and caspase-3 in Cisplatin-induced A549 cells.

RESULTS: Survival rate of A549 cells gradually decreased with increased dose of Cisplatin. Immunofluorescence results elucidated that cellular ROS level of Cisplatin-induced A549 cells increases in a dose-dependent manner. Both immunofluorescence and flow cytometry results revealed that the apoptotic rate of A549 cells increases with the elevation of Cisplatin dose. Besides, the apoptotic rate and ROS level of A549 cells were reduced by NAC pretreatment. Western blot results showed that the protein level of Prx V remarkably decreased in a dose-dependent manner, whereas Prx II expression did not change. With the treatment prolongation of 6 umol/L Cisplatin in A549 cells, Bcl-2 and caspase-3 were downregulated, while BAD upregulated.

CONCLUSIONS: Cisplatin treatment induces the ROS production, increases the apoptotic rate and downregulates the Prx expression in A549 cells.

Key Words:
Prx V, ROS, Apoptosis, Cisplatin.

Introduction

Non-small cell lung cancer (NSCLC) is a common malignancy with a high mortality rate. Currently, surgical resection is the preferred option for treating NSCLC. Besides, chemotherapy and radiotherapy are performed for NSCLC patients who cannot be operated. Platinum-based combination chemotherapy is the standard treatment of postoperative or advanced lung cancer patients. Cisplatin is a representative drug of platinum and belongs to the cytotoxic cell cycle nonspecific agent[1]. However, the long-term use of Cisplatin may eventually cause drug resistance in a part of tumor patients. A large number of studies have shown that reactive oxygen species (ROS) exerts a crucial role in the progression of various cancers, involving in the regulation of cell proliferation, apoptosis, and aging[2]. Excessive production of intracellular ROS leads to oxidative stress and cell apoptosis[3]. Elevated ROS alters the permeability of mitochondrial membranes, releases cytochrome c, and activates the caspase signaling pathway. Therefore, a medication that increases intracellular ROS is an effective approach for cancer treatment. Scholars[4-9] have shown that some certain drugs can kill cancer cells by increasing intracellular ROS levels.

Peroxiredoxin (Prx) is a class of proteins that could scavenge intracellular ROS and plays a crucial role in peroxidative detoxification[10-12]. Prx V is a member of the Prx family, which is capable of eliminating intracellular ROS and peroxynitrite[13,14]. Previous studies have shown that treatment of sodium nitroprusside dihydrate (SNP) and lipopolysaccharide (LPS) increase protein level of Prx

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V in BV2 neuroglial cells, which is considered to be associated with elevated level of intracellular ROS15. Other studies have shown the protective role of Prx V in P53-induced apoptosis16. Recombinant Prx V protects apoptosis induced by oxidative stress17. However, the mechanism of Prx V in ROS-induced apoptosis of NSCLC cells is still unclear. In this study, we selected A549 cells as the research objects to explore the role of Prx V in Cisplatin-induced cell apoptosis.

Materials and Methods

Cell Lines and Reagents

Cisplatin was obtained from Selleck Chemicals (Houston, TX, USA); A549 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA); Dulbecco's Modified Eagle Medium (DMEM) was provided by Hyclone (South Logan, UT, USA); fetal bovine serum (FBS) was provided by Gibco (Rockville, MD, USA); mouse anti-Bcl2, BAD, caspase-3, Prx V, Prx II, and α-tubulin were obtained from Abcam (Cambridge, MA, USA); Annexin-V-FITC and CMH2DCFDA were provided by Beyotime (Shanghai, China); NAC was obtained from Beyotime (Shanghai, China).

Cell Culture

A549 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. They were maintained in an incubator with 5% CO2 at 37°C.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

A549 cells were seeded in the 96-well plate at a dose of [illegible]/mL and incubated for 22 h. Subsequently, cells were starved for another 2 h. Culture medium was replaced with 20 μL of methyl thiazolyl tetrazolium (MTT) solution (5 g/L) (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 4 h. The supernatant was discarded and 100 μL of DMSO (dimethyl sulfoxide) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well. The absorbance value was recorded at the wavelength of 570 nm with a microplate reader.

Cell Apoptosis Determination

A549 cells were fixed overnight and incubated with 10 μL of Annexin V-fluorescein isothiocyanate (FITC) in the dark for 10 min. 300 μL of binding buffer and 5 μL of Propidium Iodide (PI) were added, followed by apoptosis detection using flow cytometry.

ROS Detection

A549 cells were incubated with the diluted fluoroprobe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Shanghai, China) for 20 min at 37°C with slight shaking for 5 min. After washing with serum-free culture medium, the cells were examined under a fluorescence microscope.

Western Blot

A549 cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Beyotime, Shanghai, China). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membrane (Roche, Basel, Switzerland). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 17.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}$±s). Differences between groups were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

Cisplatin-Induced Apoptosis of A549 Cells

MTT assay was conducted to evaluate the regulatory effect of Cisplatin on the survival of A549 cells. It is found that the survival rate of A549 cells gradually decreases with the increased dose of Cisplatin (Figure 1A). To verify whether Cisplatin treatment could increase ROS level in A549 cells, cells were first induced with 0, 2, 4, and 6 mol/L Cisplatin for 24 h, respectively. Immunofluorescence results elucidated that cellular ROS level increases in a dose-dependent manner (Figure 1B). Subsequently, cell apoptosis was determined by immunofluorescence and flow cytometry. Both experimental results revealed the increased apoptotic rate of A549 cells with the elevation of Cisplatin dose (Figure 1C and 1D). The above

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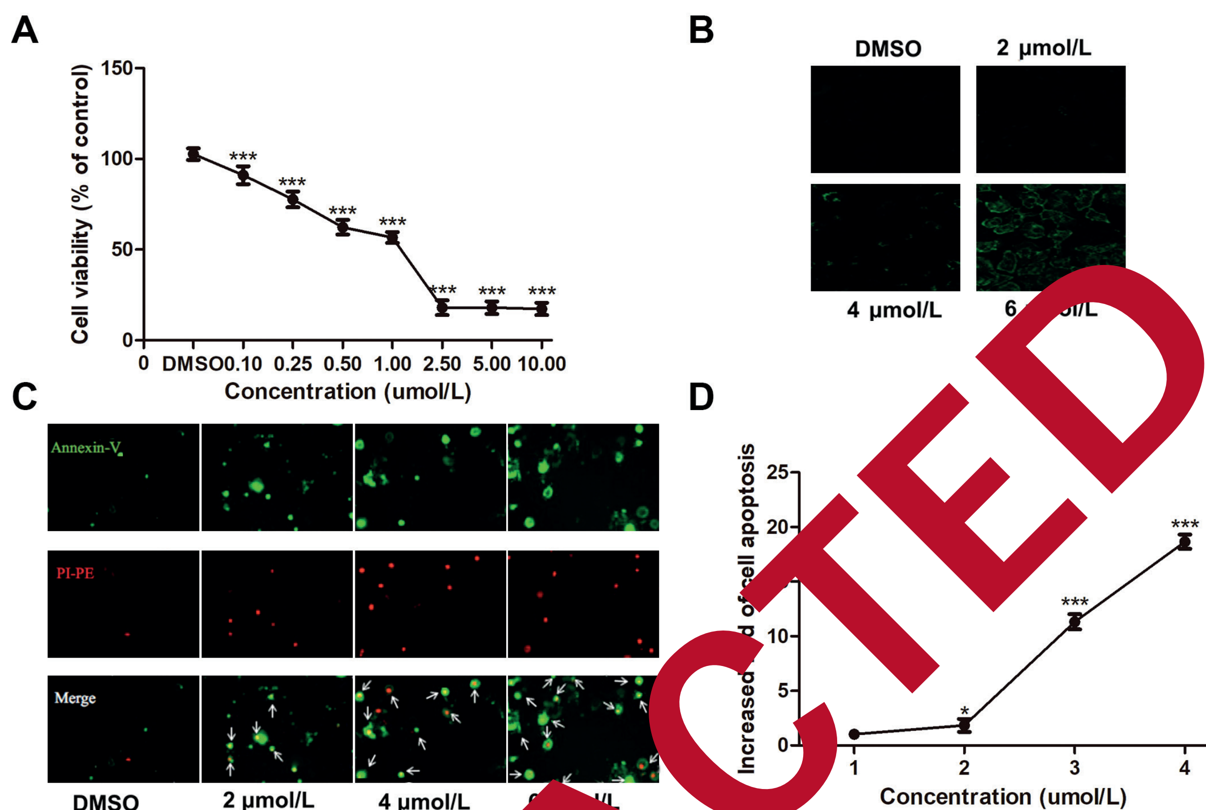

Figure 1. Cisplatin induced apoptosis of A549 cells. [illegible]urviva[illegible] A549 cells was determined using the MTT assay. *B*, ROS level in A549 cells was detected using immunoflu[illegible]en[illegible]*,* A[illegible]ptotic A549 cells induced by Cisplatin treatment. The green light was Annexin-V-FITC staining, red light was [illegible]ing, and merge was the overlap of green and red staining. The light was A549 cells under the white lig[illegible]00 μm[illegible] Quantification of apoptotic A549 cells. Data were expressed as mean±SD, n=3. *p<0.05, ***p<0.001, c[illegible]pared [illegible] DMSO [illegible]p.

data suggested that C[illegible]tin treat[illegible] induces ROS production a[illegible] ap[illegible]is of A5[illegible] cells.

Cisplatin-In[illegible]ced Apopto[illegible]f A549 Cells Wa[illegible]losely Related to ROS Productio[illegible]

We specula[illegible]hat Ci[illegible]atin-induced apoptosis [illegible] cells [illegible] related to the elevation [illegible]ellula[illegible]OS lev[illegible]NAC, as a ROS scavenger, [illegible]utili[illegible] for pretreatment with A549 cells for 30 [illegible] To ve[illegible]y this speculation, A549 cells were t[illegible]ed with DMSO, 4 μmol/L Cisplatin or 4 μmol/L [illegible]platin + 5 mmol/L NAC, respectively. Cell apoptosis and ROS level were then determined. ROS level was found to be remarkably reduced after NAC pretreatment (Figure 2A). Besides, the apoptotic rate of A549 cells was reduced by NAC pretreatment as well (Figure 2B and 2C). These results elucidated that Cisplatin-induced apoptosis of A549 cells is closely related to elevation of ROS level.

Effects of Cisplatin Treatment on Expressions of Apoptosis-Related Genes and Prx V

As a member of the Prx family, Prx V is capable of scavenging ROS in cells. To detect the expression change of Prx V in Cisplatin-induced apoptosis of A549 cells, A549 cells were treated with different doses of Cisplatin (0, 2, 4 μmol/L) for 24 h. Western blot results showed that the protein level of Prx V in A549 cells remarkably decreases in a dose-dependent manner (Figure 3A). We also found that the protein level of Prx V decreases in a time-dependent manner after 4 μmol/L Cisplatin treatment for 0, 3, 6, 9, and 12 h, respectively (Figure 3B). No significant difference was observed in protein level of Prx II. Protein expressions of apoptosis-related genes were detected by Western blot as well. With the treatment prolongation of 4 μmol/L Cisplatin in A549 cells, Bcl-2 and caspase-3 were downregulated, whereas BAD upregulated (Figure 4).

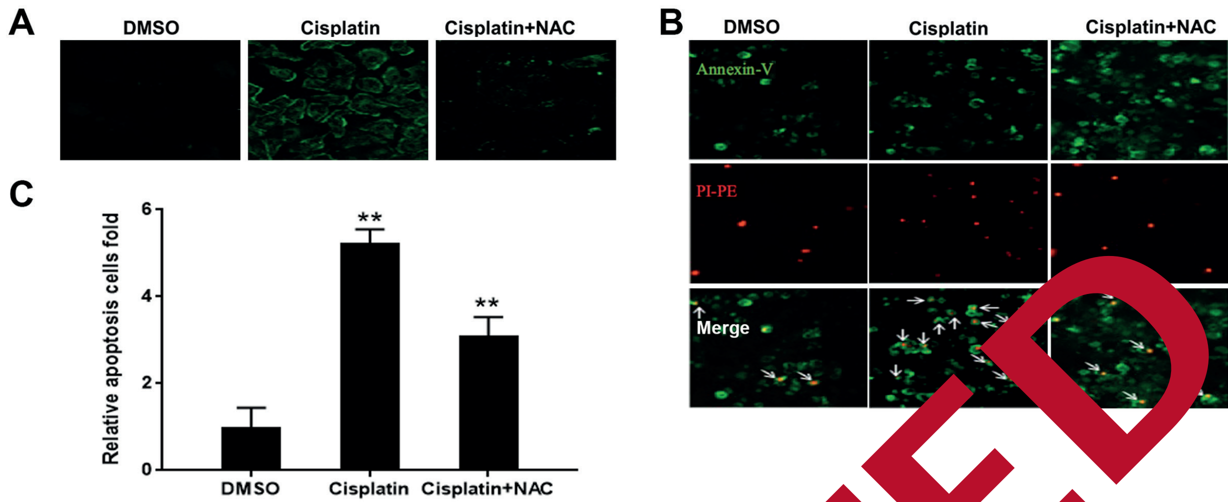

Figure 2. Cisplatin-induced apoptosis of A549 cells was closely related to ROS production. A549 cells were treated with DMSO, 4 μmol/L Cisplatin or 4 μmol/L Cisplatin + 5 mmol/L NAC, respectively. *A,* ROS level in A549 cells was detected using immunofluorescence. ***B,*** Apoptotic A549 cells was detected using immunofluorescence. ***C,*** Apoptotic A549 cells was detected using flow cytometry. Bar = 100 μm.

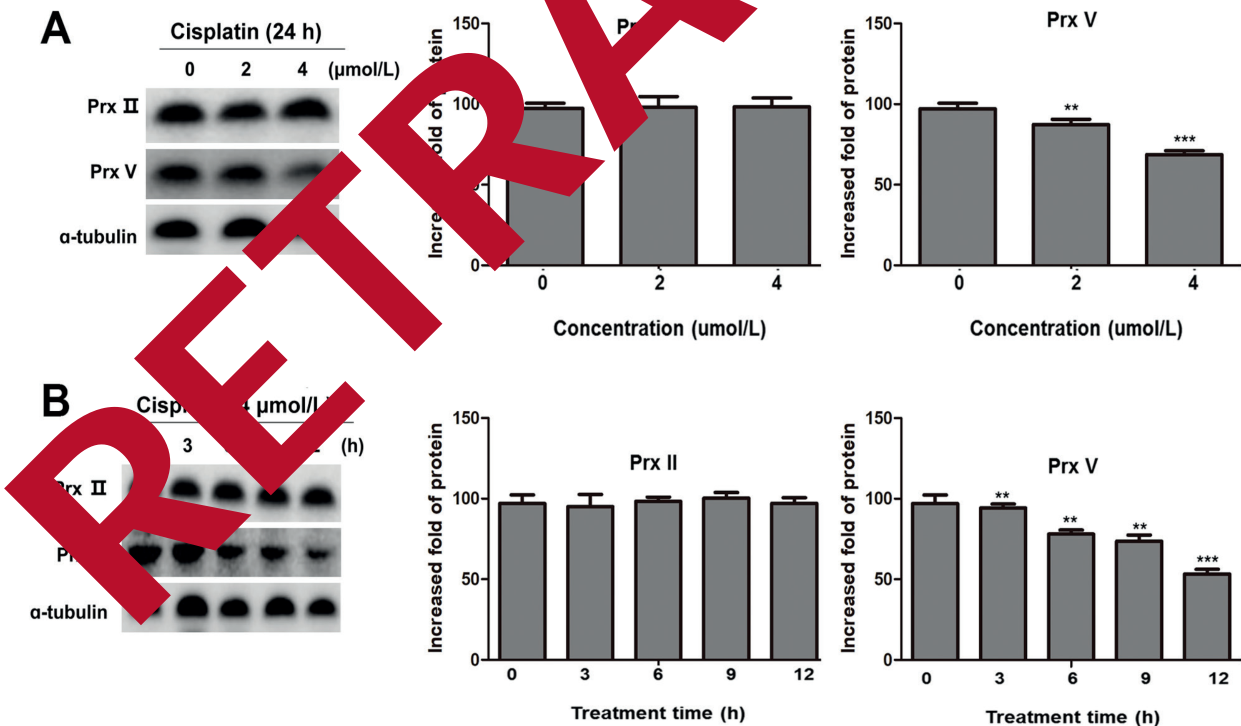

Figure 3. Effect of Cisplatin treatment on protein expressions of Prx V and Prx II. ***A,*** Protein expressions of Prx II, Prx V in A549 cells treated with different doses of Cisplatin (0, 2, 4 μmol/L) for 24 h. ***B,*** Protein expressions of Prx II, Prx V in A549 cells treated with 4 μmol/L Cisplatin treatment for 0, 3, 6, 9, 12 h.

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Discussion

NSCLC has a very high mortality rate, whereas the cure rate after radiotherapy is far away from satisfactory. Chemotherapy in NSCLC patients induces a strong drug resistance and has a certain damage to normal human cells, which severely affects its therapeutic effect. Hence, it is urgent to develop novel therapeutic targets for treating NSCLC. Cisplatin has been widely applied in chemotherapy for tumors and is considered to be one of the most effective treatments for treating solid tumors. However, tumor cells are prone to develop resistance to chemotherapeutic drugs, which has become a major obstacle in cancer treatment. Due to the emergence of drug resistance, the mortality rate of patients remains high. Current studies mainly focus on searching for new targets that could reduce the drug resistance and increase the chemotherapy sensitivity to tumor cells.

This study found that Cisplatin treatment can induce apoptosis in A549 cells and effectively increase intracellular ROS level. It is well known that ROS stimulates the opening of mitochondrial permeability transition pore, cytochrome c release, and caspase-3 activation18. In addition, cell apoptosis leads to the protein processing of pro-apoptotic protein members of the Bcl-2 family, further translo[illegible] to the outer membrane of mitochondri[illegible]n of Bcl-2 exerts an opposite resul[illegible]s promoti[illegible]ell apoptosis19. In the present [illegible]k, our re[illegible]ts showed that ROS scav[illegible]ger [illegible]tively [illegible]duces ROS level, and th[illegible]eby inhibi[illegible]op[illegible]sis of A549 cells. It is ind[illegible]ted th[illegible] Cispla[illegible]nduced apoptosis of A5[illegible]9 c[illegible]rectly related to intracellular RO[illegible] level.

We also [illegible]d activate[illegible]e-3, downregulated Bc[illegible], an[illegible]regulated [illegible]AD in A549 cells after Cisplati[illegible]atment, indicating that the cla[illegible]optotic pa[illegible]ys of caspase-3 and Bcl-

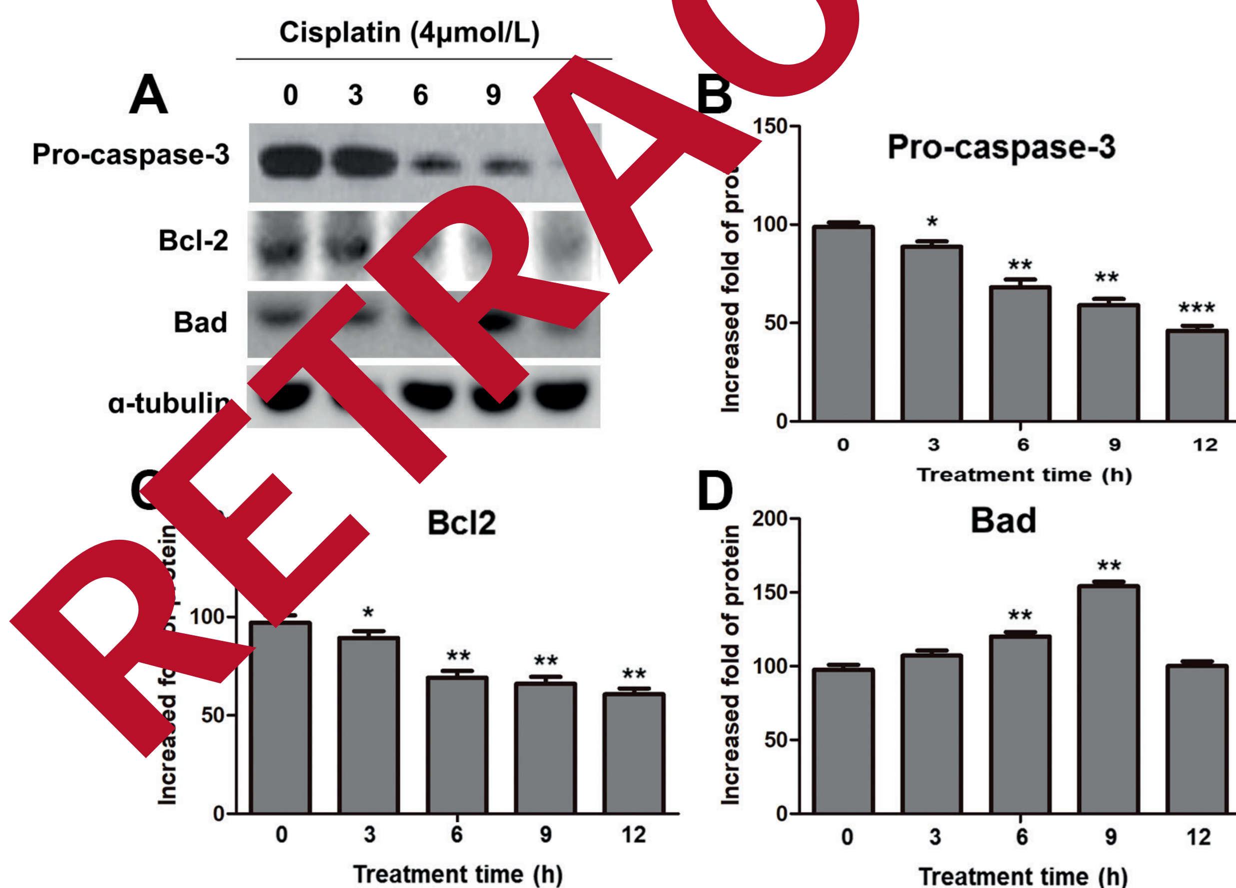

Figure 4. Effect of Cisplatin treatment on protein expressions of Bcl2, BAD, and procaspase-3. Protein expressions of Bcl-2, BAD, and procaspase-3 in A549 cells and the quantification of protein expressions of Bcl-2, BAD, and procaspase-3. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with 0 μmol/L group.

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2 were involved in Cisplatin-induced apoptosis. An increase in intracellular ROS clearly leads to changes in the intracellular level of Prx. As an anti-peroxidase, Prx V regulates ROS level in cells and participates in cell signal transduction, proliferation, and apoptosis. This investigation showed that Cisplatin treatment significantly downregulates protein expression of Prx V, whereas Prx II level did not change, indicating that Cisplatin treatment upregulates ROS level mainly by downregulating the expression of Prx V. However, whether Prx V is the only one in Prx family that is sensitive to Cisplatin remains to be further investigated. We firstly elucidated the effect of Cisplatin on the apoptosis of A549 cells. Our results demonstrated that Cisplatin treatment could downregulate protein level of Prx V and increase cellular ROS level in A549 cells, revealing the regulatory effect of Prx V on Cisplatin-induced ROS production and cell apoptosis in NSCLC. We provided a new theoretical basis for NSCLC treatment targeting on reducing ROS level.

To sum up, Cisplatin treatment remarkably downregulated protein level of Prx V, eleva[illegible] cellular ROS level, and activated apoptoti[illegible] thways in A549 cells. Our study provides [illegible] ideas and therapeutic foundations for clinical [illegible] atment of NSCLC.

Conclusio[illegible]

We found that cispl[illegible] treatmen[illegible]uced the ROS production, in[illegible]ease[illegible] apoptot[illegible]ate, and downregulates t[illegible] Prx expr[illegible]n in A549 cells.

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The Au[illegible] declare that they have no conflict of interest.

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