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ROS-Dependent Cell Death Induced by Parthenolide in Human Hepatoma Cell HepG2

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Abstract

Objective: The incidence and mortality of malignant tumors are increasing year by year around the world. However, the pathogenesis is still unclear. The anti-tumor activity of parthenolide (PN) and its cytotoxicity and acting mechanism on hepatoma cell line HepG2 were mainly studied in this paper. **Methods:** CCK-8 method was used to detect the effect of PN on the toxicity of HepG2 at different time points and concentrations; flow cytometry (FCM) was adopted to detect the effect of PN on mitochondrial membrane potential (MMP), intracellular calcium ions, cell cycle and apoptosis; Western blot was used to detect the effect on HepG2 cell death (apoptosis and autophagy)-related protein and cycle-related protein; FCM was adopted to detect the effect of PN on the ROS in HepG2 cell; the effect of PN combined with ROS scavenger N-acetyl-L-cysteine (NAC) on the above-mentioned indicators of HepG2. **Results:** With the increase of the action time and dosing concentration, the proliferation of HepG2 cell was inhibited and its vitality gradually decreased. FCM showed that, with the increase of PN action time, the MMP gradually decreased; calcium ions flowed inwards; the cell cycle was arrested in phase G1; the cell apoptosis rate, especially late apoptosis and necrotic cells, increased. The shear expression of apoptosis-related proteins caspase3 and caspase9 was up-regulated; the shear expression of AIF, MIF and PARP1 proteins associated with Caspase-independent apoptosis, *i.e.* Parthanatos apoptosis, showed time-dependent up-regulation; the long and short expressions of anti-apoptosis protein FLIP showed different degrees of decrease; the expression of autophagy-related proteins LC3A/B and becin-1 was up-regulated; the expression of P62 protein was down-regulated; the expression of cycle-related proteins P53, P27 and P21 increased significantly; the expression of CyclinD1 and CyclinE1 decreased. FCM was used to detect the increase of ROS with the action time of PN; and its generation level showed an increasing trend; after the combination with ROS scavenger NAC, there was no

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significant difference in cell viability with the control group. There was no significant difference in the expression level of relevant cell death protein with DMSO control group. There was no difference in intracellular ROS generation level with the control group. **Conclusion:** PN induces the ROS generation in HepG2 cell, blocks its cycle and causes apoptosis and autophagy to play an anti-tumor effect.

Subject Areas

Integrated Chinese and Western Medicine

Keywords

Parthenolide, ROS, Cell Death

1. Introduction

The causes of liver cancer are complicated. Rapid population growth and the aging trend are the main reasons, some of which are related to economic and social development [1]. According to epidemiological statistics [2] [3], among all malignant tumors, liver cancer is the sixth largest cancer in the world. About 50% of liver cancer cases occur in China; its mortality is the second highest in the world, accounting for more than half of all liver cancer deaths worldwide. Liver cancer has low cure rate and high mortality rate, thus the main focus is prevention. At present, the risk factors for hepatocellular carcinoma (HCC) include the infection with HBV, HBC or chronic liver diseases caused by drinking and exposure to aflatoxin, etc. Chronic liver diseases further develop into HCC [3]. Many natural compounds are found in the long-term life practice of human society to have good anti-tumor activity and have no obvious toxic effect on normal cells. Therefore, it is of great significance to explore the anti-tumor activity of natural products and study their targets and mechanisms for revealing the pathogenesis of malignant tumors and how to prevent and treat them. PN is a natural compound of sesquiterpene lactone extracted from tansy. As recorded in early literature [4], people in Europe and America have used it as an antipyretic analgesic to treat fever, psoriasis, toothache, insect bites, rheumatism, asthma, stomachache, menstrual problems and other diseases since ancient times. In follow-up studies, it was also found to be effective against migraines and arthritis. Relevant pharmacological studies have shown that it has multiple important pharmacological activities. Some studies have shown that [5] it acts as an anti-herpes simplex virus by reducing cell activity. Some other studies have shown that [6] it plays an anti-atherosclerosis role by inhibiting the nuclear transcription factor kappaB to reduce the arterial inflammatory response and plaque formation in apoE mice. In 1973, it was first proved to have anti-tumor activity [7]. Relevant studies have shown that the mechanism of its anti-tumor activity is correlated to its strong inhibition of NF-κB [8]. Subsequent studies have also

focused on its anti-tumor related molecular biological mechanisms. The main anti-tumor mechanisms found mainly include: 1) the study of Gopal, Y N *et al.* [9] proved that it can specifically consume histone deacetylase I protein to inhibit the proliferation of tumor cells and promote the apoptosis of tumor cells. 2) The experimental study of Liu *et al.* [10] found that PN inhibits DNA methyltransferase 1 (DNMT1) by alkylation to activate HIN-1 gene, thus inhibiting the proliferation of tumor cells and promoting apoptosis; 3) The study of Wen *et al.* [11] found that PN can induce intracellular ROS accumulation by mediating oxidative stress, and promote cell apoptosis by breaking the oxidation-reduction balance; 4) The study of Li *et al.* [12] found that PN regulates the expression levels of downstream apoptotic protein and cyclin, thus inhibiting the proliferation and promoting apoptosis of gastric cancer cells SGC-7901. In conclusion, as a natural product, PN has numerous anti-tumor targets; and its detailed mechanism has not been fully elucidated. In this paper, the main anti-tumor targets and mechanism of PN in breaking the oxidation-reduction system of liver cancer HepG2 cell were mainly discussed.

2. Main Experimental Materials and Methods

2.1. Sources of Main Experimental Materials

Human hepatoma cell line: identified and provided by American ATCC cell bank.

PN: purchased from the official website of MCE, with dimethyl sulfoxide (DMSO) used as a solvent, 200 mmol/L storage solution prepared, separated and stored in −80˚C refrigerator for future use.

Relevant reagents for cell culture: DMEM high glucose (Hyclone), containing L-Glutamine, without Sodium Pyruvate; PBS (Hyclone), without calcium ions or magnesium ions; pancreatin (Gibco), containing 0.25% EDTA.

Fetal bovine serum (FBS): Israel BI.

N-acetyl-L-cysteine (NAC): purchased from the official website of MCE and stored at a concentration of 1 mol/L.

Experimental antibodies: all experimental primary antibodies were purchased from American CST and secondary antibodies from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.

2.2. Methods

2.2.1. Cell Culture

After conventional recovery, HepG2 hepatoma carcinoma cells were cultured in a culture dish containing DMEM high sugar medium containing 10% FBS at 37.0˚C and in cell incubator containing 5% CO_2. The culture solution was exchanged every other day, passed or inoculated into culture plate when the cell density grew to 75% - 90% for subsequent experiments.

2.2.2. CCK-8 Cytotoxicity Experiment

HepG2 cells were digested, mixed, resuspended and evenly inoculated into 96-hole plate. After 12 h of conventional culture, the cells adhered well to the

wall and were in the logarithmic phase. The cell density in each hole was controlled to be about 75%. The test was divided into 2 groups: DMSO group and PN group. 100 μL culture solution containing six concentration gradients (15 μM, 17.5 μM, 20 μM, 22.5 μM, 25 μM and 27.5 μM) was added to the cells in the PN group in the form of culture solution exchange. The DMSO group was the control group of the PN group. 100 μL culture solution containing 1/1000 DMSO was added in the form of culture solution exchange. Three parallel holes were set in each group. After incubation in the incubator for 24 h, 48 h and 72 h, respectively, 100 μL culture solution containing 10 μL CCK-8 solution was added to each hole in the form of culture solution exchange. Background holes were set. After incubation in cell incubator for about 50 min, microplate reader was used to determine the absorbance (A) at 450 nm. Cell inhibition rate was calculated: cell inhibition rate = [(Ac control hole − As experiment hole)/(Ac control hole − Ab blank hole)] × 100%. Tumor cytotoxicity map was drawn. SPSS24.0 was used to calculate the IC50 value of PN acting on HepG2. Appropriate concentrations were taken for subsequent experiments.

2.2.3. Effect of PN on MMP in HepG2 Cells Detected by FCM

After digestion and resuspension, HepG2 cells were evenly incubated into 6 cm plates, which were divided into a DMSO group and a PN group. The DMSO group was the control group. Three time points, 6 h, 12 h and 24 h, were set in the PN group. When cells were in the logarithmic phase and the cell density was around 75%, a culture solution containing 25 μM PN was added at different time points. After incubation in the incubator, cells in each group were collected, washed with PBS for two times, centrifuged at 150 g to discard the supernatant. 0.5 Ml culture solution and 0.5 ml·JC^{-1} staining solution were added according to the proportion of 100,000 - 600,000. The above-mentioned cell precipitate was slowly and evenly resuspended. After incubation in the incubator for 20 min, the precipitate was centrifuged at 150 g for 5 min to discard the supernatant. After adding 1 ml·JC^{-1} staining buffer (1×), the cells were resuspended and centrifuged for 2 times to discard the supernatant. 200 μL·JC^{-1} staining buffer was added. The mixture was resuspended. Upflow cytometer was used for detection. The experiment was repeated for three times.

2.2.4. Effect of PN on Calcium Ions in HepG2 Cells Detected by FCM

After digestion and resuspension, HepG2 cells were evenly incubated into 6 cm plates, which were divided into a DMSO group and a PN group. The DMSO group was the control group. Three time points, 6 h, 12 h and 24 h, were set in the PN group. When cells were in the logarithmic phase and the cell density was around 75%, a culture solution containing 25 μM PN was added at different time points. After incubation in the incubator, cells in each group were collected, washed with PBS for two times, The cells were centrifuged at 1000 RPM for 5 min to discard the supernatant. The PBS with a Flow-3 calcium probe concentration of 5 μM was used to slowly blow the suspended cell precipitate. It was incubated in incubator for 35 min. After centrifugation, PBS was used to resus-

pend cells twice. Upflow cytometer was used for detection. The experiment was repeated for three times.

2.2.5. Effect of PN on Cycle of HepG2 Cells Detected by FCM

After digestion and resuspension, HepG2 cells were evenly incubated into 6 cm plates, which were divided into a DMSO group and a PN group. The DMSO group was the control group. Three time points, 6 h, 12 h and 24 h, were set in the PN group. When cells were in the logarithmic phase and the cell density was around 75%, The culture solution containing 25 μM PN was added at the same time point in the PN group. The mixture was incubated in the incubator. After 6 h, 12 h and 24 h of incubation, the mixture was washed with pre-cooled PBS and centrifuged at 300 g for 5 min to discard the supernatant. 1 mL 75% pre-cooled ethyl alcohol. After resuspension, the mixture was placed at −20˚C for fixation overnight. Propidium iodide staining solution was prepared according to reagent specification: 0.5 mL stain buffer, propidium iodide staining solution (20X) 25 μL and RnaseA (50×) 10 μL was used for each sample. The staining solution prepared in advance was stored away from light at 4˚C. When cells were fixed overnight, the cells were centrifuged at 300 g for 5 min to discard the supernatant, resuspended with pre-cooled PBS, and centrifuged and precipitated. 0.5 mL propidium iodide staining solution was added to each tube of sample. The cell precipitate was slowly and fully resuspended and incubated away from light at room temperature for 30 min. Upflow cytometer was used for detection. The experiment was repeated for three times. FlowJo streaming software was used to analyze DNA content in cells.

2.2.6. Effect of PN on Apoptosis of HepG2 Cells Detected by FCM

After digestion and resuspension, HepG2 cells were evenly incubated into 6 cm plates, which were divided into a DMSO group and a PN group. The DMSO group was the control group. Three time points, 6 h, 12 h and 24 h, were set in the PN group. When cells were in the logarithmic phase and the cell density was around 75%, a culture solution containing 25 μM PN was added at different time points. After incubation in the incubator, cells in each group were collected, 4˚C pre-cooled PBS was used to wash cells twice, 300 g each time. The cells were centrifuged at 4˚C for 5 min. 1 × Binding Buff was used to resuspend cells. In each group, 100 μL cell suspension was taken from each group and placed in a light-free flow tube. 5 μL Annexin V-Alexa Fluor 488 and 10 μL PI staining solution were added to gently mix. Meanwhile, the single staining group of Annexin V-Alexa Fluor 488 and PI was established to adjust the fluorescence compensation. After reaction in dark place and at room temperature for 15 min, 200 μL PBS was added to mix. Upflow detection was directly carried out. The experiment was repeated for three times.

2.2.7. Effect of Combination of PN with NAC on ROS in HepG2 Cells Detected by FCM

After digestion and resuspension, HepG2 cells were evenly incubated into 6 cm

plates, which were divided into four groups: That is DMSO group, PN group, NAC group and PN+NAC group. When cells were in the logarithmic phase and the cell density was around 75%, The culture solution containing 1/1000 DMSO was used in the DMSO group. 25 μM PN was added in the form of culture solution exchange in the PN group. Culture solution containing 1 mM NAC were added in the form of culture solution exchange in the NAC group. In the PN + NAC group, 1mM NAC was added in advance for incubation for 1 h; then 25 μM PN was added for co-culture. The cells of each group were placed in an incubator containing 5% CO_2 for incubation at 37˚C for 24 h. Next, cells were collected from each group, washed with PBS, centrifuged at 1000 RPM for 5 min. The above-mentioned cells were resuspended with 300 μL ROS probe diluted in PBS (10 μM) and incubated in an incubator containing 5% CO_2 at 37˚C for 30 min. After centrifugation, 200 μL PBS was used for resuspension, after which upflow cytometer was used for detection. The experiment was repeated for three times.

2.2.8. Effect of PN on Expression Levels of Apoptosis, Autophagy and Cycle-Related Proteins in HepG2 Cells Detected by Western Blot

Cells in logarithmic phase were treated with culture solution containing 25 μM PN for 6 h, 12 h and 24 h. The DMSO was a control group without PN. The cells in each group were lysed with appropriate cell lysates; BCA protein concentration was determined; the protein concentration in each group was adjusted to be consistent. Protein loading buffer containing SDS and bromophenol blue was added to fully mix. The mixture was boiled at 100˚C for 10 min to fully denature the protein, which was placed in ice bath for future use. Excessive protein was separated and stored at −80˚C. SDS-PAGE electrophoresis gel was configured (different concentrations of underlying gel were configured according to the molecular weight of the target protein). After gel configuration, it was placed in the electrophoresis tank. Sufficient electrophoresis solution and sample were added for electrophoresis. 80V was applied first. The voltage was increased to 100 V when the Marker was separated and the protein entered the underlying gel. The electrophoresis continued for about 90 min. When the bromophenol blue indicator went to the lower edge of the gel, the gel position of target protein was cut according to Marker; the gel was placed on a transfer sandwich clip soaked in a transfer film buffer; a PVDF membrane of gel size was cut and activated in methanol. The clip was sealed in the order of membrane positive and gel negative and placed in membrane transfer tank, which was filled with pre-cooled electrophoretic solution. The membrane was transferred at 300 mA constant current in ice bath for about 1.5 h. Then PVDF membrane was taken from the clip and placed in TBST shaker containing 5% skim milk powder and sealed at room temperature for about 1.5 h. PVDF membrane was taken out and slightly washed with TBST and then placed in the antibody diluent containing primary antibody diluted at a ratio of 1:1000 and in a shaker at 4˚C overnight for about 14 h. It was rewarmed in a shaker for 1 h and washed with TBST for three times, 5 - 6 min each time. Later, the PVDF membrane was placed in the secondary antibody with marker HRP diluted at a ratio of 1:1000, incubated in a

shaker at room temperature for about 2 h and washed with TBST for three times, 5 - 6 min each time. The membrane was finally placed in a chemiluminescence developer, and exposed and developed in a chemiluminescence instrument after full reaction. The experiment was repeated for three times.

2.3. Statistical Method

SPSS24.0 was used for statistical analysis of all experimental data. Generally, normal analysis and test of homogeneity of variance were carried out on the data. The results were expressed as $X \pm S$. T test was adopted for comparison between the two groups and one-way analysis of variance for comparison among groups. The difference was statistically significant for $P < 0.05$.

3. Results

3.1. Effect of PN on Cytotoxicity

The results of CCK-8 cytotoxicity experiment showed that HepG2 cells died with the increase of PN concentration and prolongation of time (**Figure 1**).

3.2. Effect of PN on MMP Detected by FCM

The decrease of MMP is a landmark event in the early stage of apoptosis [13]. JC^{-1} FCM showed that the MMP decreased with the extension of PN action time. After 24 h of PN action, HepG2 MMP almost completely lost (**Figure 2**).

3.3. Effect of PN on Intracellular Calcium Ions Detected by FCM

After incubation, each group was detected by upflow cytometry with calcium ion probe Flow-3. The detection showed that intracellular calcium levels gradually increased with the extension of PN action time. Compared with the DMSO group, the inward flow of calcium ions in HepG2 cells peaked when PN acted on such cells for 24 h. Since extracellular calcium ion flow caused intracellular calcium ion level to increase and overload, mitochondrial permeability increased; cytochrome C was released, which triggered the relevant apoptotic signal [14]. The combination of this experiment and the above-mentioned JC-1 MMP indicated that a certain concentration of PN can influence the mitochondrial pathway and trigger endogenous apoptosis (**Figure 3**).

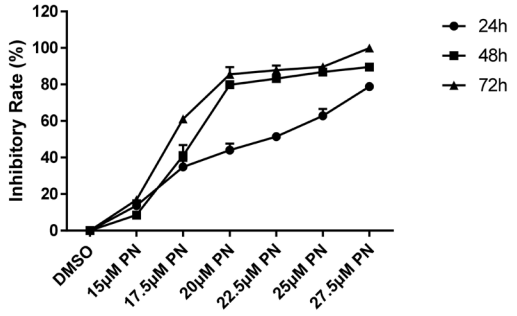

Figure 1. Effect of PN on Toxicity of Hepatoma Carcinoma Cell HepG2 at Different Concentrations and Time Points Detected by CCK-8.

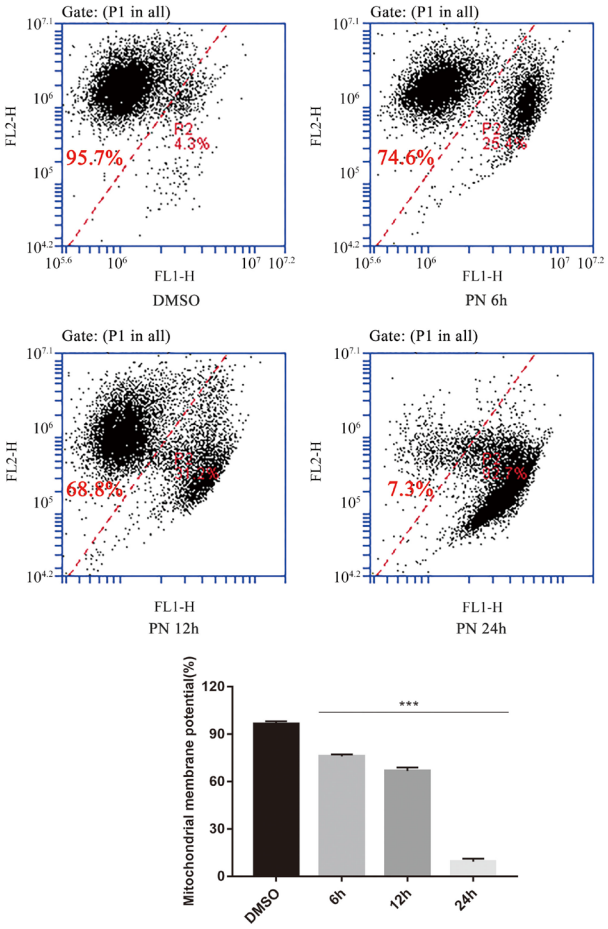

Figure 2. Effect of PN on Hepatoma Carcinoma Cell HepG2 MMP Detected by JC-1 FCM. Note: *** represents $P < 0.001$, compared with the DMSO group.

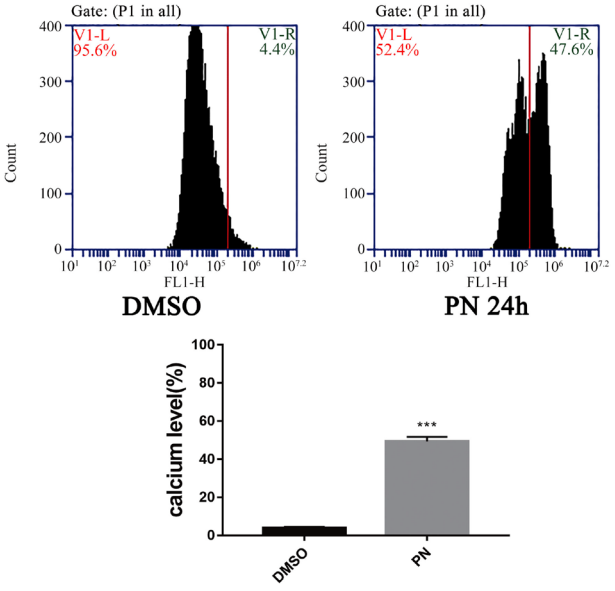

Figure 3. Effect of PN on Hepatoma Carcinoma Cell HepG2 Calcium Ion Detected by Calcium Ion Probe Flow-3JC-1 FCM. Note: *** represents $P < 0.001$, compared with the DMSO group.

3.4. Effect of PN on HepG2 Cell Cycle Detected by FCM

Uncontrolled cell proliferation caused by cell cycle regulation disorder is a sign of cancer [15]. According to PI staining combined with FCM, compared with the DMSO group, the number of phase 1 cells in the group where HepG2 cells were treated with PN for 24 h increased significantly (**Figure 4(a)**). In order to further verify the role of PN in cell cycle arrest, the detection of relevant cyclin by WB showed that PN can significantly up-regulate the cycle-related proteins P53, P27 and P21 in HepG2 cells with the extension of the action time, while cyclinD1 and cyclinE1 proteins decreased. (**Figure 4(b)**) This indicated that PN inhibited DNA replication and HepG2 cell proliferation by arresting HepG2 cells in G1 phase. Abnormalities were found at cellular immunity checkpoints. HepG2 was induced to irreversible death through related signal transduction mechanism.

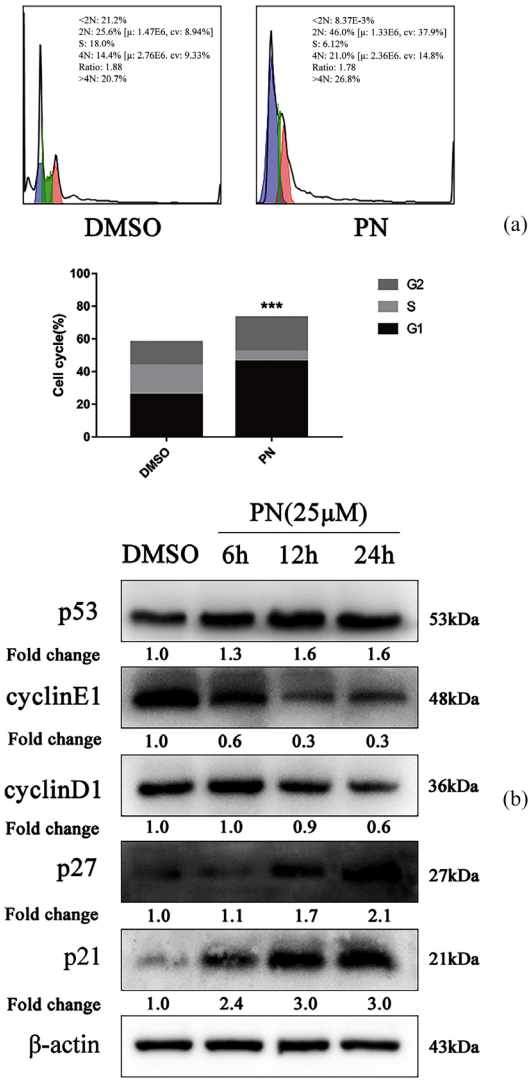

Figure 4. (a) Effect of PN on Hepatoma Carcinoma Cell Cycle Detected by PI Single Staining. Note: *** represents $P < 0.001$, compared with the DMSO group; (b) Effect of PN on Hepatoma Carcinoma Cell HepG2 Cycle Detected by WB.

3.5. Effect of PN on HepG2 Cell Apoptosis Detected by FCM

Cell apoptosis is a programmed cell death without inflammatory reaction and is natural death of cells [16]. However, if normal cell proliferation and differentiation are out of control, cell apoptosis cannot occur normally, tumorigenesis may be induced. The typical morphological characteristics of apoptotic cells were observed during HepG2 death induced by PN. Annexin V-Alexa Fluor 488 + PI double staining combined with FCM were hereby used and apoptotic proteins detected to further verify the mode of PN-induced cell death and the occurrence of early and middle and late apoptosis. Apoptotic FCM **Figure 5(a)** showed that the incidence of HepG2 cell apoptosis increased gradually with the extension of PN action time and mainly was middle and late apoptosis. There was significant difference between the 24 h group and the DMSO group. Apoptosis-related proteins are as shown in **Figure 5(b)**. The shear expression of HepG2 apoptosis-related proteins Caspase-9 and Caspase-3 was activated. The protein level increased with the extension of treatment time. The results showed that PN induced Caspase cascade reaction in cells to have and caused cell apoptosis. While the expression of anti-apoptosis proteins c-FLIPL (long) and c-FLIPs (short) decreased with the extension of PN action time. The results showed that PN promoted HepG2 cell apoptosis by down-regulating c-FLIP.

3.6. Effect of PN on Expression Levels of HepG2 Autophagy-Related Proteins Detected by WB

Since PN is a natural product, cytotoxicity may be caused in many ways and targets. As another form of programmed cell death, autophagy regulates cell homeostasis by degrading long-lived proteins, protein polymers, and damaged organelles. It can also inhibit tumor formation by limiting inflammation, removing toxic unfolded proteins and removing damaged mitochondria that generate reactive oxygen species (may damage DNA) [17]. Losing those protective measures will promote cancer. Thus the effect of PN in autophagy was verified. We found that with the extension of PN action time, the expression of proteins LC3B and beclin-1 increased and that of protein p62 decreased, indicating that PN can promote HepG2 autophagy (**Figure 6**).

3.7. Effect of PN Combined with NAC on ROS Generation in HepG2 Cells Detected by FCM

The experimental results showed that with the extension of PN action time, the amount of ROS generated in HepG2 cells increased significantly. The difference in the 24 h group and the DMSO group was significant. After the combination of PN with NAC, there was no significant difference in the amount of ROS generated with the DMSO group, but there was significant difference with the PN group. This experiment indicated that NAC can almost completely block the ROS generation in HepG2 cells, so as to block the generation of ROS-dependent cell death (**Figure 7**).

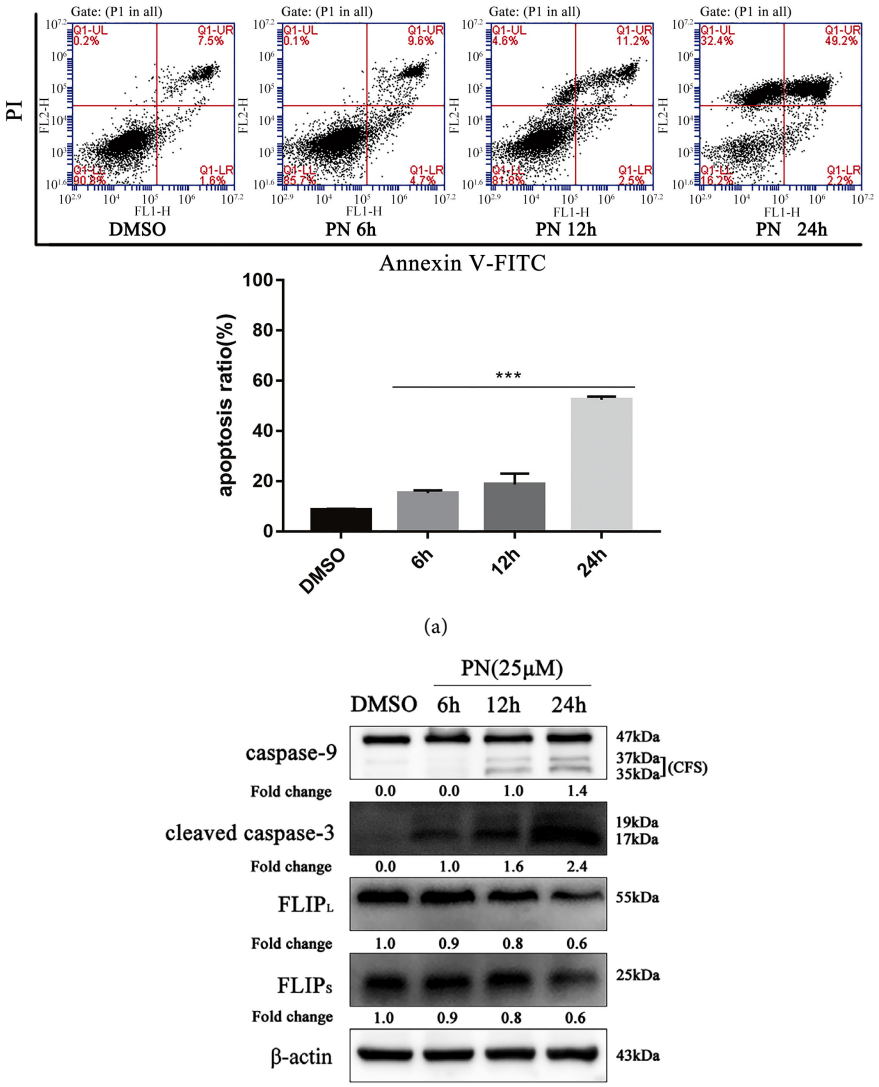

Figure 5. (a) Promotion of Hepatoma Carcinoma Cell HepG2 Apoptosis by PN Detected by Annexin V + PI Double Staining. Note: *** represents $P < 0.001$, compared with the DMSO group; (b) Effect of PN on Hepatoma Carcinoma Cell HepG2 Apoptosis-related Protein Detected by WB.

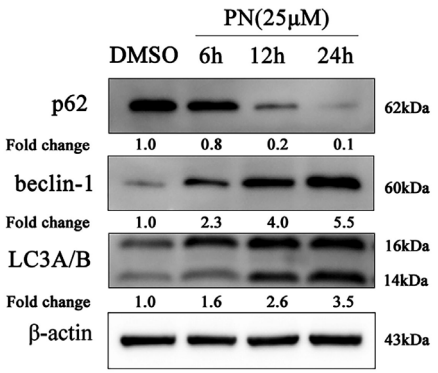

Figure 6. Effect of PN on Hepatoma Carcinoma Cell HepG2 Autophagy-related Proteins Detected by WB.

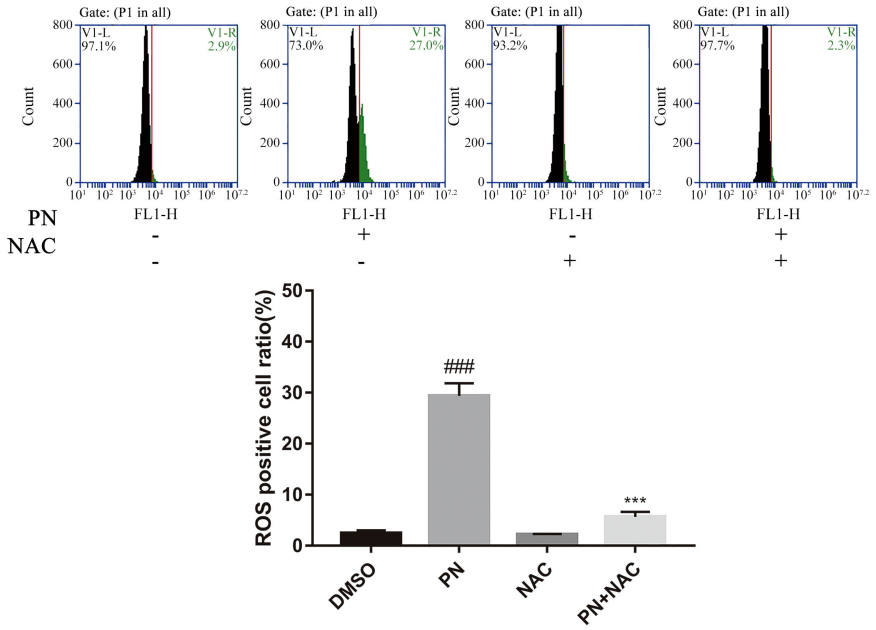

Figure 7. Changes in ROS Generation Level in Hepatoma Carcinoma Cell HepG2 after the Application of PN and the Combination of PN and NAC. Note: ### represents $P < 0.001$, compared with the DMSO group; *** represents $P < 0.001$, compared with the PN group.

3.8. Effect of PN Combined with NAC on HepG2 Cell Viability and the Expression Level of Relevant Cell Death Proteins

After the combination of PN with NAC (as shown in **Figure 8(a)** and **Figure 8(b)**), there was no significant difference in cell viability and the expression of relevant cell death proteins with the DMSO group (**Figure 8**).

4. Discussion

PN is a natural sesquiterpene lactone extracted from feverfew and has excellent anticancer and anti-inflammatory activities [18]. A great number of previous studies have proved that PN can inhibit many types of tumor cells; and some targets and acting mechanisms have been identified. For instance, PN can promote cell apoptosis by inhibiting NF-κB signaling pathway，cause oxidative damage to tumor cells by inducing ROS generation, cause mitochondrial dysfunction, activate the relevant signaling pathway and cause cell death. It can also inhibit the phosphorylation of Tyr705 by combining with signal transducers and transcriptional activators and cause dephosphorylation of STATs, thus dimer cannot be formed and cannot enter the nucleus to play a biological role, causing cell death. Besides, PN plays an anti-tumor role by interfering with microtubule formation, maintaining JNK activity, regulating DNA methylation, and promoting the ubiquitination of MDM2 without side effects on normal cells [19].

In this study, CCK-8 cytotoxicity test showed that PN can inhibit HepG2 cell viability and cause cell death; and this effect is time and concentration-dependent. Further study showed that in HepG2 cells treated with PN, the MMP

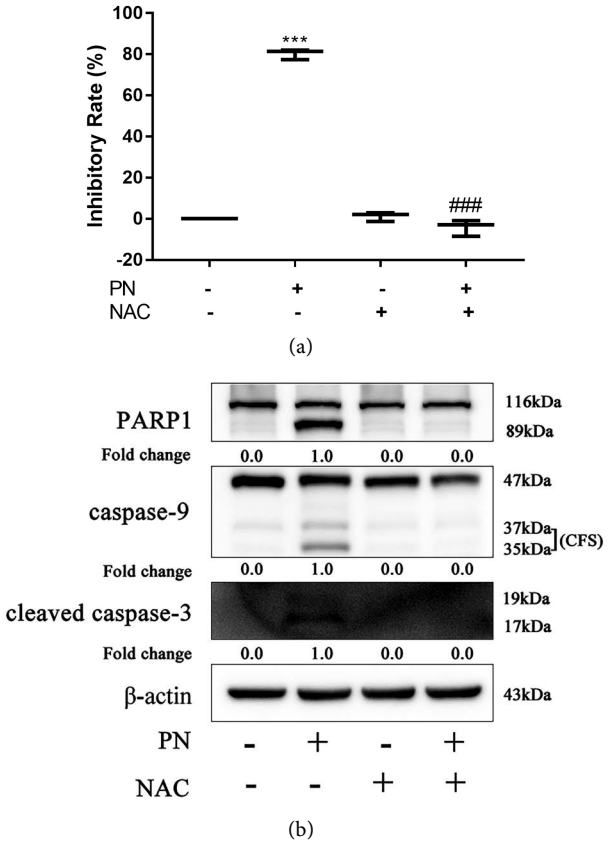

Figure 8. (a) Effect of PN Combined with NAC on Hepatocellular Carcinoma Cytotoxicity. Note: *** represents $P < 0.001$, compared with the DMSO group; ### represents $P < 0.001$, compared with the PN group; (b) Effect of PN Combined with NAC on Apoptosis-related Proteins in Hepatocellular Carcinoma Cells.

decreased and calcium ions flowed inwards. Both of the two are one of the events of early cell apoptosis. This indirectly indicated that PN caused mitochondrial dysfunction, causing activation of cell-related signaling pathways and apoptosis.

In order to further verify the effect of PN in HepG2 apoptosis, AnnexinV + PI double staining FCM showed that with the extension of PN action time, HepG2 developed from early apoptosis to late apoptosis and necrosis. Among the factors that induce apoptosis, cell cycle arrest played a key role. The action principle of many chemotherapeutics used clinically today is to arrest the tumor cell cycle to inhibit the proliferation of tumor cells and cause the death of tumor cells. PI single staining combined with FCM showed that PN arrested HepG2 cells in phase G1. The number of phase S cells in DNA replication decreased significantly. In order to further figure out the mechanism of PN acting on HepG2 cells and inducing their cycle arrest and apoptosis, WB and cell cycle-related proteins showed that the expression of proteins P53, P27 and P21 in HepG2 cells was up-regulated, while the expression of proteins cyclinD1 and cyclinE1 decreased. Both of the two changes were time-dependent. Previous studies [20] have shown that in cell cycle, the regulatory function of tumor suppressor gene

P53 is mainly reflected in the monitoring of calibration points in G1 and G2/M phases and is closely correlated to the transcriptional activation. Upon cell apoptosis, the cyclin-dependent kinase inhibitor P21 is induced by p53 in dependent and non-dependent manners after oxidative stress. The expression of p21 causes cell cycle arrest. On the one hand, P21 can combine with a series of cyclin-cdk complexes and inhibit the corresponding protein kinase activity. Rb cannot be phosphorylated by Cyclin-cdk. The non-phosphorylated Rb remains bound to the E2F. Thus, the transcription regulator E2F cannot be activated, causing arrest in phase G1. On the other hand, the downstream gene Cyclin B1 of P53 participates in arrest in G2/M phases [21]. Like protein P21, protein P27 inhibits the phosphorylation of Rb by cyclic protein, preventing E2F from being separated from Rb and thus completely blocking the transcription of the gene needed for G1/S conversion. This includes cyclins, such as cyclins A, D and E [22]. According to WB and apoptosis-related proteins, we found that PN caused the classical Caspase cascade mediated apoptosis and generated AIF-mediated Caspase-independent apoptosis, *i.e.* Parthanatos, to participate in the reaction. When DNA damage is caused by oxidative stress on cells, DNA damage may cause overactivation of PARP1, causing (ADP-ribose) (PAR) polymer synthesis and accumulation and consumption of NAD + and ATP. However, this does not seem to be the main cause of cell death. PAR polymer sends a signal to the mitochondria and directly combines with the PAR polymer binding site on the apoptosis-inducing factor (AIF), inducing mitochondrial release and transfer to the nucleus. Once mitochondrion enters the nucleus, it causes large-scale DNA fragmentation (≈50 kb) and chromatin concentration through the unidentified nuclease (PAAN) associated with hay fever AIF, finally leading to cell apoptosis [23]. The experimental results showed that when hepatoma carcinoma cell HepG2 was treated with PN, the shear expression level of protein PARP1 increased. Nucleoprotein AIF and MIF were activated, proving that PN can induce Caspase-independent Parthanatos apoptosis in HepG2.

As a basic cellular protective mechanism, autophagy acts as a "housekeeper" in the normal physiological process. Cells degrade their contents by devouring their own cytoplasmic protein or organelle, forcing their envelopes into the vesicle and fusing with lysosomes to form autophagy lysosomes, including the removal of long-lived, clumped and misfolded proteins and the removal of damaged organelles to achieve the normal metabolism of the cell itself [24]. The experiment showed that PN can induce autophagy in HepG2 and that the expression of autophagy proteins LC3B and beclin-1 showed time-dependent increase.

Mitochondrial function in cells decreased. The energy failure is a key factor that causes cell aging and death [25]. In the above experiment, we have proved that PN can cause mitochondrial dysfunction, thus initiating the relevant signaling pathway to induce cell death. As a source of energy in cells, mitochondria inevitably have aerobic respiration to produce ATP. Aerobic respiration inevitably generates ROS. The generation of ROS can cause mitochondria themselves to be attacked. however, there is a complete set of oxidation-reduction system in

normal cells. This system can remove excess free radicals in time to protect the naked mitochondria from damage [26]. In our study, the ROS level in the hepatoma carcinoma cell treated with PN showed time-dependent increase, while in HepG2 cells pre-treated with NAC, PN-induced cell death can be almost completely blocked. There was no difference in the ROS level with the control group.

5. Conclusion

In conclusion, as a natural sesquiterpene lactone, PN can induce cell cycle arrest, apoptosis and autophagy in HepG2 cells through the ROS-dependent pathway. This action may be correlated to the direct action on GSH mercaptan and Michael addition reaction [27]. This mechanism and target provide a new strategy for clinical prevention and treatment of malignant tumors.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A. and Jemal, A. (2018) Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA*: *A Cancer Journal for Clinicians*, **68**, 394-424. https://doi.org/10.3322/caac.21492

[2] Mcglynn, K.A., Petrick, J.L. and London, W.T. (2015) Global Epidemiology of Hepatocellular Carcinoma. *Clinics in Liver Disease*, **19**, 223-238. https://doi.org/10.1016/j.cld.2015.01.001

[3] Lafaro, K.J., Demirjian, A.N. and Pawlik, T.M. (2015) Epidemiology of Hepatocellular Carcinoma. *Surgical Oncology Clinics of North America*, **24**, 1-17. https://doi.org/10.1016/j.soc.2014.09.001

[4] Knight, D.W. (1995) Feverfew: Chemistry and Biological Activity. *Natural Product Reports*, **12**, 271. https://doi.org/10.1039/np9951200271

[5] Benassi-Zanqueta, É., Marques, C.F., Nocchi, S.R., Dias Filho, B.P., Nakamura, C.V. and Ueda-Nakamura, T. (2018) Parthenolide Influences Herpes Simplex Virus 1 Replication *in Vitro*. *Intervirology*, **61**, 14-22. https://doi.org/10.1159/000490055

[6] López-Franco, O., Hernández-Vargas, P., Ortiz-Muñoz, G., Sanjuán, G., Suzuki, Y., Ortega, L., *et al.* (2006) Parthenolide Modulates the NF-κB-Mediated Inflammatory Responses in Experimental Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **26**, 1864-1870. https://doi.org/10.1161/01.ATV.0000229659.94020.53

[7] Suzuki, T., Saitoh, Y., Isozaki, S. and Ishida, R. (1973) Simple Method for Portal Vein Infusion in the Rat. *Journal of Pharmaceutical Sciences*, **62**, 345-347. https://doi.org/10.1002/jps.2600620245

[8] Zhang, S., Lin, Z.N., Yang, C.F., Shi, X., Ong, C.N. and Shen, H.M. (2004) Suppressed NF-kappaB and Sustained JNK Activation Contribute to the Sensitization Effect of Parthenolide to TNF-Alpha-Induced Apoptosis in Human Cancer Cells. *Carcinogenesis*, **25**, 2191-2199. https://doi.org/10.1093/carcin/bgh234

[9] Gopal, Y.N., Arora, T.S. and Van Dyke, M.W. (2007) Parthenolide Specifically Depletes Histone Deacetylase 1 Protein and Induces Cell Death through Ataxia Te-

langiectasia Mutated. *Chemistry & Biology*, **14**, 813-823. https://doi.org/10.1016/j.chembiol.2007.06.007

[10] Liu, Z., Liu, S., Xie, Z., Pavlovicz, R.E., Wu, J., Chen, P., *et al.* (2009) Modulation of DNA Methylation by a Sesquiterpene Lactone Parthenolide. *Journal of Pharmacology and Experimental Therapeutics*, **329**, 505-514. https://doi.org/10.1124/jpet.108.147934

[11] Wen, J., You, K.R., Lee, S.Y., Song, C.H. and Kim, D.G. (2002) Oxidative Stress-Mediated Apoptosis. The Anticancer Effect of the Sesquiterpene Lactone Parthenolide. *The Journal of Biological Chemistry*, **277**, 38954-38964. https://doi.org/10.1074/jbc.M203842200

[12] Li, H., Lu, H., Lv, M., Wang, Q. and Sun, Y. (2018) Parthenolide Facilitates Apoptosis and Reverses Drug-Resistance of Human Gastric Carcinoma Cells by Inhibiting the STAT3 Signaling Pathway. *Oncology Letters*, **15**, 3572-3579. https://doi.org/10.3892/ol.2018.7739

[13] Kroemer, G., Dallaporta, B. and Resche-Rigon, M. (1998) The Mitochondrial Death/Life Regulator in Apoptosis and Necrosis. *Annual Review of Physiology*, **60**, 619-642. https://doi.org/10.1146/annurev.physiol.60.1.619

[14] Ermak, G. and Davies, K.J.A. (2002) Calcium and Oxidative Stress: From Cell Signaling to Cell Death. *Molecular Immunology*, **38**, 713-721. https://doi.org/10.1016/S0161-5890(01)00108-0

[15] Sherr, C.J. (1996) Cancer Cell Cycles. *Science*, **274**, 1672-1677. https://doi.org/10.1126/science.274.5293.1672

[16] Hengartner, M.O. (2000) The Biochemistry of Apoptosis. *Nature*, **407**, 770-776. https://doi.org/10.1038/35037710

[17] Yun, C. and Lee, S. (2018) The Roles of Autophagy in Cancer. *International Journal of Molecular Sciences*, **19**, 3466. https://doi.org/10.3390/ijms19113466

[18] Mathema, V.B., Koh, Y., Thakuri, B.C. and Sillanpää, M. (2012) Parthenolide, a Sesquiterpene Lactone, Expresses Multiple Anti-Cancer and Anti-Inflammatory Activities. *Inflammation*, **35**, 560-565. https://doi.org/10.1007/s10753-011-9346-0

[19] Tollini, L.A., Jin, A., Park, J. and Zhang, Y. (2014) Regulation of p53 by Mdm2 E3 Ligase Function Is Dispensable in Embryogenesis and Development, But Essential in Response to DNA Damage. *Cancer Cell*, **26**, 235-247. https://doi.org/10.1016/j.ccr.2014.06.006

[20] Gartel, A.L. and Tyner, A.L. (2002) The Role of the Cyclin-Dependent Kinase Inhibitor p21 in Apoptosis. *Molecular Cancer Therapeutics*, **1**, 639-649.

[21] Abbastabar, M., Kheyrollah, M., Azizian, K., Bagherlou, N., Tehrani, S.S., Maniati, M., *et al.* (2018) Multiple Functions of p27 in Cell Cycle, Apoptosis, Epigenetic Modification and Transcriptional Regulation for the Control of Cell Growth: A Double-Edged Sword Protein. *DNA Repair*, **69**, 63-72. https://doi.org/10.1016/j.dnarep.2018.07.008

[22] Fatokun, A.A., Dawson, V.L. and Dawson, T.M. (2014) Parthanatos: Mitochondrial-Linked Mechanisms and Therapeutic Opportunities. *British Journal of Pharmacology*, **171**, 2000-2016. https://doi.org/10.1111/bph.12416

[23] Ravanan, P., Srikumar, I.F. and Talwar, P. (2017) Autophagy: The Spotlight for Cellular Stress Responses. *Life Sciences*, **188**, 53-67. https://doi.org/10.1016/j.lfs.2017.08.029

[24] Vakifahmetoglu-Norberg, H., Ouchida, A.T. and Norberg, E. (2017) The Role of Mitochondria in Metabolism and Cell Death. *Biochemical and Biophysical Research*

Communications, **482**, 426-431. https://doi.org/10.1016/j.bbrc.2016.11.088

[25] Mailloux, R.J. (2018) Mitochondrial Antioxidants and the Maintenance of Cellular Hydrogen Peroxide Levels. *Oxidative Medicine and Cellular Longevity*, **2018**, Article ID: 7857251. https://doi.org/10.1155/2018/7857251

[26] Ghantous, A., Sinjab, A., Herceg, Z. and Darwiche, N. (2013) Parthenolide: From Plant Shoots to Cancer Roots. *Drug Discovery Today*, **18**, 894-905. https://doi.org/10.1016/j.drudis.2013.05.005

[27] Duan, D., Zhang, J., Yao, J., Liu, Y. and Fang, J. (2016) Targeting Thioredoxin Reductase by Parthenolide Contributes to Inducing Apoptosis of HeLa Cells. *Journal of Biological Chemistry*, **291**, 10021-10031. https://doi.org/10.1074/jbc.M115.700591