Mechanism of Hydrogen Gas Promoted Apoptosis of Lung Adenocarcinoma A549 cells through XIAP and BIRC3

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Research

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Abstract

Background/AIM

Lung cancer is the most common reason of cancer-related death in worldwide. Hydrogen gas has been found to have effects on a variety of diseases. At present, it is not reported that the effect of hydrogen gas on lung cancer domestic and overseas. Therefore, we designed this experiment to test the differences in the expression of XIAP, BIRC3 and BAX In vivo and in vitro.

Materials and methods

A549 cells in logarithmic phase were treated by 20%, 40%, 60% hydrogen gas respectively. Then the apoptosis of different groups were detected by Flow cytometry. We identify the differential expressed genes(DEGs) by transcriptional. The protein expression of XIAP, BIRC3 and BAX were detected by western blot and immunohistochemistry.

Result

The results demonstrated that hydrogen gas can significantly induce apoptosis compared with the control group. The expression of XIAP and BIRC3 were downregulated in hydrogen group.

Conclusion

Hydrogen gas may promote the apoptosis of lung cancer A549 cells by reducing the expression of XIAP and BIRC3 protein.

1. Introduction

Lung cancer has the highest incidence and mortality rate in the world. It brings about a great threat to people's health. In China, cancer has become the most fateful disease since 2010. Among men, lung cancer is the first cancer(1). The incidence of lung cancer in women is second, behind only to breast cancer. In China, the 5 year survival rate of all cancers is 36.9%, while the 5 year survival rate of lung cancer is only 16.8%, which is obviously lower than the overall level of cancer. Since 1970, with the progress of science and technology, the survival period of most cancers, such as cervical cancer, blood system tumor and lymphatic cancer, has been improved, but the increase of lung cancer is very small. In China, from 2000 to 2011 the large data showed that the incidence of pancreatic cancer and colorectal cancer had declined, but the incidence and mortality of lung cancer remain high, which was always the first in the mortality rate. With the development of the economy, people's health awareness is gradually improved, and people have higher requirements for the quality of life and the cure rate of the disease. It is the urgent desire of all medical staffs and the broad masses of the people to find an economical and effective treatment for lung cancer.

Hydrogen, as a new medical gas, has been found to be effective for many diseases. Researchers found that hydrogen has selective antioxidant, anti-inflammatory and anti apoptotic effects on many diseases. Hydrogen has the characteristics of small molecular weight and rapid dispersion. After entering the human body, it can quickly spread in tissue, reach every part of the tissue, and selectively remove toxic hydroxyl radical and nitrite anion, but it can not affect the biological activity of normal free radicals. Recent studies have shown that under normal atmospheric pressure, hydrogen can reduce inflammation induced by concanavalin and dextran sulfate, lipopolysaccharide and Yeast Polysaccharides in animal models. Drinking hydrogen can reduce the oxidative stress caused by inflammatory factors, regulate the expression of nuclear factor- kappa B and tumor necrosis factor(TNF), and ameliorate the symptoms of rheumatoid arthritis. Studies have shown that hydrogen can act as a signal molecule in the cell, and its action is similar to nitric oxide, carbon monoxide and hydrogen sulfide. Many studies have shown that hydrogen molecules can exert their biological activities by regulating the activity and expression of related proteins and affecting the activity of signaling pathways. The study of the effect of hydrogen is mainly focused on ischemia reperfusion injury, acute lung injury and so on. Hydrogen has not been reported in the treatment of lung cancer. The purpose of this study is to investigate the effect of hydrogen on apoptosis of lung adenocarcinoma A549 cells. Combined with transcriptome sequencing, we explored the molecular mechanism of hydrogen affecting the apoptosis of lung adenocarcinoma A549 cells. We desired our research would be of some value for the future therapy of lung cancer in clinic.

High throughput technology has been more and more applied to clinical research. RNA sequence and microarray dataset has played a great role in exploring molecular markers, assessing therapeutic effects and revealing possible molecular mechanisms. In our previous studies(2), transcriptome sequencing of hydrogen treated lung adenocarcinoma A549 cells was carried out, and 823 differentially expressed genes were identified. There were 168 up-regulated genes and 655 down regulated genes. The purpose of this study was to determine the possible mechanism of hydrogen effect on the apoptosis of lung cancer A549 cells by using the results of transcriptional sequencing, and the results were verified by Western blot and immunohistochemistry.

2. Materials And Methods

2.1 Cell culture and hydrogen treatment

A549 cells were resuscitated according to the routine. Cell culture was carried out using RPMI-1640 medium containing 10% fetal bovine serum. The cells were passed 1 times every 1-2 days, according to cell growth. After reaching the expected number of cells, the cells were intervened in hydrogen incubator for 2 hours with different concentrations of hydrogen. The control group was placed in the common cell culture box. After 3 days of intervention, the cells were collected and the total protein was extracted to carry out the experiment.

2.2 Detection of apoptosis by flow cytometry

Trypsin was used to digest and collect cells, then centrifugation and discarding supernatant. The cells were added with ice PBS 250 μl and then 750 μl ice ethanol was added by drop. The final ethanol concentration is up to 75%. The suspension is placed at -20 C for at least 2 hours in the refrigerator. Take the fixed cells from the fridge, centrifuge for 3 minutes with 2000 turns, discard the supernatant, and add PI/RNase Staining Buffer.

2.3 Subcutaneous tumor formation in nude mice

Acquisition of 5-6 week male nude mice from card Vince experimental Animals Inc, Changzhou, Jiangsu. The nude mice were anaesthetized with ether. First, the skin was sterilized with alcohol, and the A549 cell suspension was inoculated into the axilla of the forelimb of nude mice. The cell concentration was $250x10^6$ /ml, each was inoculated with 0.1 ml. After subcutaneous tumor formation, the control group was still fed normally, and the hydrogen group was given 60% concentration of hydrogen inhalation once a day for four hours each time. Cisplatin group was given intraperitoneal injection of cisplatin 4 mg/kg for one times a week. After 3 weeks, the nude mice were sacrificed, then the tumor tissue was removed and fixed in 4% neutral formaldehyde.

2.4 Immunohistochemistry

The fresh tumor tissue was immobilized in 4% neutral formaldehyde for 48-72 hours. The tissue was first dehydrated in gradient alcohol, then transparent in turpentine, and finally immersed in a wax cylinder and embedded into wax blocks. The wax blocks were cut into pathological sections with a thickness of 4 μm. The tissue section was followed by roasting, dewaxing, hydration, antigenic heat repair, 3% hydrogen peroxide blocking endogenous peroxidase activity, 5% goat serum blocked for 60 minutes at room temperature. The sections were subsequently incubated overnight at 4˚C with primary antibody. The negative control group was incubated with PBS instead of the primary antibody. The slides were incubated with a biotinylated goat anti-mouse horseradish peroxidase conjugated secondary antibody (proteintech, Wuhan, China) for 30minutes at 37℃ centigrade bath. The slices were stained with DAB reagent and distilled water to stop dyeing. After DAB staining, first use of hematoxylin to stain the slices, followed by 1% hydrochloric acid alcohol for color separation, and then use ammonia water to return blue, using anhydrous ethanol soaked for 5 minutes. After the tissue slices were dried, the neutral gum was used to seal them.

2.5 Transcript Analysis

After cultured A549 cells reached the experimental number, the control group was routinely cultured, and the experimental group was treated with 60% hydrogen. Two groups of specimens were sent to Source of NOA in Beijing to extract total RNA by TRIzol method. Samples were tested by agarose gel electrophoresis, NanoPhotometer® spectrophotometer Qubit® 2.0 FluorometerAgilent 2100 bioanalyzer and so on. After the establishment of the library, the quality of the library was checked to ensure the quality of the library, followed by Illumina HiSeq/MiSeq sequencing. In comparison between the two groups, the differential genes were screened from two levels of significant and multiple differences (*P*value(pval)<0.005, $|\log2FoldChange|>1$). KEGG Pathway enrichment was used to analyze the major signaling pathways involved in differential genes.

2.6 Western blot

First, SDS-PAGE gel was prepared, then samples and marker were added to the lane. A constant voltage 110 V was used to separate the different molecular weight proteins. We used 200mA steady current to conduct transmembrane at 4 ℃. 5% skimmed milk was used to block the Polyvinylidene Fluoride(PVDF) membrane for 1 hours at room temperature. PVDF membrane was incubated overnight at 4℃ with the following primary antibodies: XIAP(1:1000 proteintech, Wuhan, China), BIRC3(1:750 proteintech, Wuhan, China), BAX(1:1000 Cell Signaling Technology, Beverly, MA, USA) or β-actin(1:1000 proteintech, Wuhan China). PVDF membrane immersed in secondary antibody (1:8000 proteintech, Wuhan, China) solution and incubated at room temperature for one and a half hours. Use the ECL developer to develop. All the antibody reagents were brand Proteintech.

2.7 Statistical Analysis

SPSS21.0 statistical software was used to analyze the data and the experimental data were expressed in x±s. The average number between the two groups was compared with the t test of two independent samples. The multiple groups of data were normal distribution and Fang Chaqi test, and a single factor analysis of variance was used for statistical test. The test level was set at α=0.05, and all results were statistically significant as compared with $P < 0.05$.

3. Results

3.1 Detection of apoptosis by flow cytometry

A549 cells in each group were treated with hydrogen at concentrations of 20%, 40% and 60% respectively, then the cells were collected and detected by flow cytometry. The results showed that the apoptosis rate of the control group was 1.27%±0.096%, and the apoptotic cells rate in 20% hydrogen group, 40% hydrogen group and 60% hydrogen group was 2.25%±0.076%, 3.08%±0.040% and 7.76%±0.050%, respectively. The rates of apoptotic cells in the hydrogen group were significantly higher than that in the control group, $P<0.001$. It is suggested that hydrogen at 20%, 40% and 60% levels can promote the apoptosis of lung cancer A549 cells (Fig. 1).

3.2 Differential gene screening combined with transcriptome sequencing results

The transcriptome sequencing results showed that there were 823 differentially expressed genes in A549 cells of the hydrogen group compared with the untreated group. There were 168 up-regulated genes and 655 down regulated genes. Using KEGG database to enrich Pathway, six genes related to apoptosis were found. They were XIAP, BIRC2, BIRC3, BAX, PIK3CD and ATM. The log2Fold_change were -1.0014, -1.579, -2.0717, 1.0262, 1.2161, -3.4458, respectively. In this experiment, we selected XIAP, BIRC3 and BAX to test at protein level (Fig.2).

3.3 Western blot was used to detect the expression of XIAP, BIRC3 and BAX in lung adenocarcinoma A549 cell line

The results showed that the ratio of gray value of XIAP to β-actin in the control group, 20% hydrogen group, 40% hydrogen group and 60% hydrogen group were 0.355±0.012、0.300±0.016、0.303±0.019、0.231±0.022, respectively. There were statistical differences among the groups. The ratio of gray value of BIRC3 to β-actin in the control group, 20% hydrogen group, 40% hydrogen group and 60% hydrogen group were 0.702±0.178、0.536±0.122、0.388±0.052、0.415±0.092、respectively. There were statistical differences between the groups. The expression of XIAP and BIRC3 decreased in 20% hydrogen group, 40% hydrogen group and 60% hydrogen group, and there were a significant difference compared with the control group (P < 0.05). There was no difference in the expression of BAX between the 20% hydrogen group, 40% hydrogen group, 60% hydrogen group and the control group, and there was no significant difference between the two groups (P>0.05) (Fig.3A, Fig.3B).

3.4 The effect of hydrogen on the tumor formation of A549 cells in nude mice

After subcutaneous inoculation of nude mice, cisplatin and hydrogen were treated. Compared with the control group, the volume of tumor in cisplatin group was significantly lower than that of the control group, 0.0834+0.0474 cm vs 0.3043+0.0884 cm, there was statistical difference between groups (P <0.001). The tumor volume in the hydrogen group and control group were 0.3205±0.1652 cm^3 vs 0.3043±0.0884 cm^3, and there was no significant difference between the two groups (P>0.05).

3.5 The results of immunohistochemistry

The XIAP score was (+ + +) in the control group, and (+ +) in the hydrogen group and the cisplatin group. The scores of BIRC3 in control group, hydrogen group and cisplatin group were (+ + +). The scores of BAX in control group, hydrogen group and cisplatin group were (+ + +). Image protein Plus6.0 software was used to analyze the average optical density of immunohistochemical pictures. XIAP was mainly expressed in the cytoplasm. The average optical density values of the control group, hydrogen group and cisplatin group were 0.090±0.033、0.041±0.012 and 0.024±0.001 respectively. There were statistical differences between the hydrogen group and cisplatin group compared with the control group (P<0.05). There was no statistical difference between the hydrogen group and cisplatin group (P>0.05). BIRC3 was mainly expressed in the cytoplasm. The average optical density of the control group, hydrogen group and cisplatin group was 0.322±0.028、0.258±0.035 and 0.299±0.031 respectively. There were statistical differences between the hydrogen group and cisplatin group compared with the control group (P<0.05), and there was no statistical difference between the hydrogen group and cisplatin group (P>0.05). BAX was mainly expressed in the cytoplasm. The average optical density values of the control group, hydrogen group and cisplatin group were 0.264±0.023、0.254±0.024 and 0.276±0.037, respectively. There was no statistical difference between each group (P>0.05)(Fig.4A, Fig.4B).

4. Discussion

In China, lung cancer has become the highest incidence of disease, which poses a great threat to the health of the people. The disease also increases the cost of medical expenses. According to our previous experimental results(2), the migration ability of A549 cell was reduced in hydrogen group, all differences were statistical significance, when compared with the control group. The invasion results demonstrated that, 40% and 60% H_2 could suppress A549 cell invasion significantly, especially in 60% H_2 group. This study found that hydrogen, a new medical gas in recent years, can promote the apoptosis of lung adenocarcinoma cell line A549, and the expression of XIAP and BIRC3 protein in A549 cells after hydrogen treatment is reduced. The A549 cell suspension was injected subcutaneously into the nude mice. After successful modeling, 60% hydrogen inhalation or cisplatin was injected into nude mice. The results showed that the expression of XIAP and BIRC3 in the H2 group was lower than that in the control group. The expression of XIAP and BIRC3 in the tumor tissues of the cisplatin group was also lower than that of the control group. The above results suggest that hydrogen intervention in A549 cells is related to the expression of XIAP and BIRC3. XIAP and BIRC3 belong to the inhibitor of apoptosis protein, which may be related to hydrogen induced apoptosis of A549 cells. In 1975, Dole and so on confirmed the therapeutic effect of high pressure hydrogen on cancer in squamous cell skin cancer models. This is the first report on the treatment of cancer by hydrogen. Since then, the flammable and explosive properties of hydrogen have limited its research in medical subjects. Most of the research on hydrogen has focused on the efficacy of hydrogen-rich water. Some researchers compared the effects of peritoneal injection of hydrogen or hydrogen water on hydrogen metabolism in rats. It is found that peritoneal injection of hydrogen or hydrogen water can rapidly increase the concentration of hydrogen in the exhaled gas of rats, and there is a quantitative relationship. However, intraperitoneal injection of hydrogen can maintain high hydrogen concentration in exhaled breath for a long time. It can be seen that direct intake of hydrogen molecule gas model, than hydrogen water can maintain the concentration of hydrogen in the body for a longer time, thus maintaining the therapeutic effect of hydrogen. The researchers also compared the different ways of intraperitoneal injection of hydrogen water, intravenous injection of hydrogen and intraperitoneal injection of hydrogen, and also found that intraperitoneal injection of hydrogen can make the rabbits longer maintain the hydrogen concentration in exhaled gas longer. It can be seen that the intake of hydrogen by gas has certain advantages over hydrogen rich water, and it can maintain the efficacy of hydrogen for a longer time. In recent years, many studies have found that hydrogen has a good therapeutic effect on brain injury, hemorrhagic shock, acute cerebral infarction and other diseases (3-5). However, the hydrogen concentration used in the above studies is between 1.3%-3%, and there is no research on high concentration hydrogen (>4%). In this experiment, the AMS-H-01 hydrogen atomizer (Asclepius, Shanghai, China) is used to produce hydrogen with the highest concentration of 66%. It is convenient for us to explore the mechanism of high concentration hydrogen to promote the apoptosis of lung cancer A549 cells. Previous studies have proved that hydrogen has anti-inflammatory, antioxidation, anti apoptosis and other effects on ischemia reperfusion injury, sepsis, acute necrotizing pancreatitis, diabetes and other diseases to reduce cell apoptosis and protect the normal physiological function of cells. The protein expressions of Caspase-3, Caspase-8, caspase-9, BAX and Bcl-2 were different. In our study, we found that hydrogen can promote the apoptosis of lung cancer A549 cells, thereby further inhibiting the growth and expansion of tumor. There are two reasons. Firstly, the tumor cells have different physiological metabolism from the normal cells, the tumor cells grow exuberant, the demand for energy metabolism is high, and the tumor cells are well tolerated to the anoxic environment. So the factors inducing apoptosis may be different between normal cells and tumor cells. Hydrogen can promote apoptosis of tumor cells. Secondly, a lot of previous studies(6-8) have shown that hydrogen has a selective reduction effect and reduced ROS, which is the main mechanism for its therapeutic effect. However, as a medical gas, it is similar to hydrogen sulfide, nitric oxide and carbon monoxide. Hydrogen is very likely to be a independent molecule involved in multiple signaling pathways such as proliferation, migration, cycle, immunity and inflammation. XIAP and BIRC3 belong to the family of apoptosis suppressor (IAPs). In 1993, the IAPs family was

first discovered in the baculovirus genome screening, and it was believed could inhibit apoptosis. It is an endogenous inhibitor of cysteine hydrolytic enzyme (caspase). Later studies found that IAPs is involved in multiple signaling pathways such as cell death, immunity, inflammation, cell cycle and cell migration. IAP family members are highly expressed in most human cancers and have unique abilities to modulate cell death and survival. They are associated with chemoresistance, disease progression and poor prognosis of cancer(9,10). Our experimental results show that high concentration of hydrogen, especially 60% of hydrogen, can reduce the expression of XIAP in lung cancer A549 cells. Many studies(11-13) have shown that XIAP regulates mitochondrial membrane permeability. Changes in mitochondrial membrane permeability can stimulate cytochrome C release into the cytoplasm, thereby activating the activity of caspase protease. Hydrogen may interfere with the information transduction of the above signaling pathways, and promote the apoptosis of tumor cells. In recent years(14), researchers have discovered two new characteristics of cancer: energy metabolism recombination and immune escape. The uncontrolled proliferation of tumor cells must require an energy supply system adapted to its rapid proliferation, and mitochondria are the key organelles of the energy supply of tumor cells. It is very likely that hydrogen participates in the energy metabolism of tumor cells, breaking the energy balance of tumor cells, thus inducing apoptosis of tumor cells.

The results showed that high concentration of hydrogen was associated with low expression of BIRC3 in lung cancer A549 cells. Xiaojie Jiang et al(15) found that cIAP2/BIRC3 is highly expressed in human gallbladder carcinoma and is associated with poor prognosis of gallbladder cancer. M Nagata etc(16) found that BIRC3 is highly expressed in oral squamous cell carcinoma and is a potential target for treatment. BIRC3 is related to the survival rate of patients. Decreasing the expression of BIRC3 can significantly increase the apoptosis of cancer cells. It has been found that BIRC3 is highly expressed in renal cell carcinoma than normal renal tissue(17). All the above studies showed that the expression of BIRC3 was higher in cancer tissues than in normal tissues. Reducing BIRC3 expression is beneficial to cancer treatment and improving prognosis. Hydrogen can reduce the expression of BIRC3 and further improve the prognosis of patients with lung cancer.

The development of lung cancer is a multistage and multistep process. The imbalance of apoptosis and inhibition of apoptosis in lung cancer cells is the basis for the rapid growth of the tumor. The study on the effect of hydrogen on the apoptosis of cancer is in the initial stage, and the deeper mechanism needs further study to reveal. In the past, due to the characteristics of flammability and explosion, it is difficult to obtain high concentration of hydrogen under atmospheric pressure, which limits the development of hydrogen medicine. In recent years, with the development of hydrogen manufacturing equipment, it is no longer difficult to obtain high concentration hydrogen at atmospheric pressure. The hydrogen and oxygen atomizer is used at any time, making hydrogen at any time and without storage, which greatly improves the safety of hydrogen. The study of hydrogen will enter a high speed development stage.

Declarations

Ethics approval and consent to participate

The study has been approved by the ethics committee of the Third Hospital of Hebei Medical University.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

CG is the designer of the project and provides the fund. ZY performed the WB and immunohistochemistry, and was a major contributor in writing the manuscript. YZ's contribution is to conduct animal experiments. WLF and WDC analyzed the experimental data. All authors read and approved the final manuscript.

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Figures

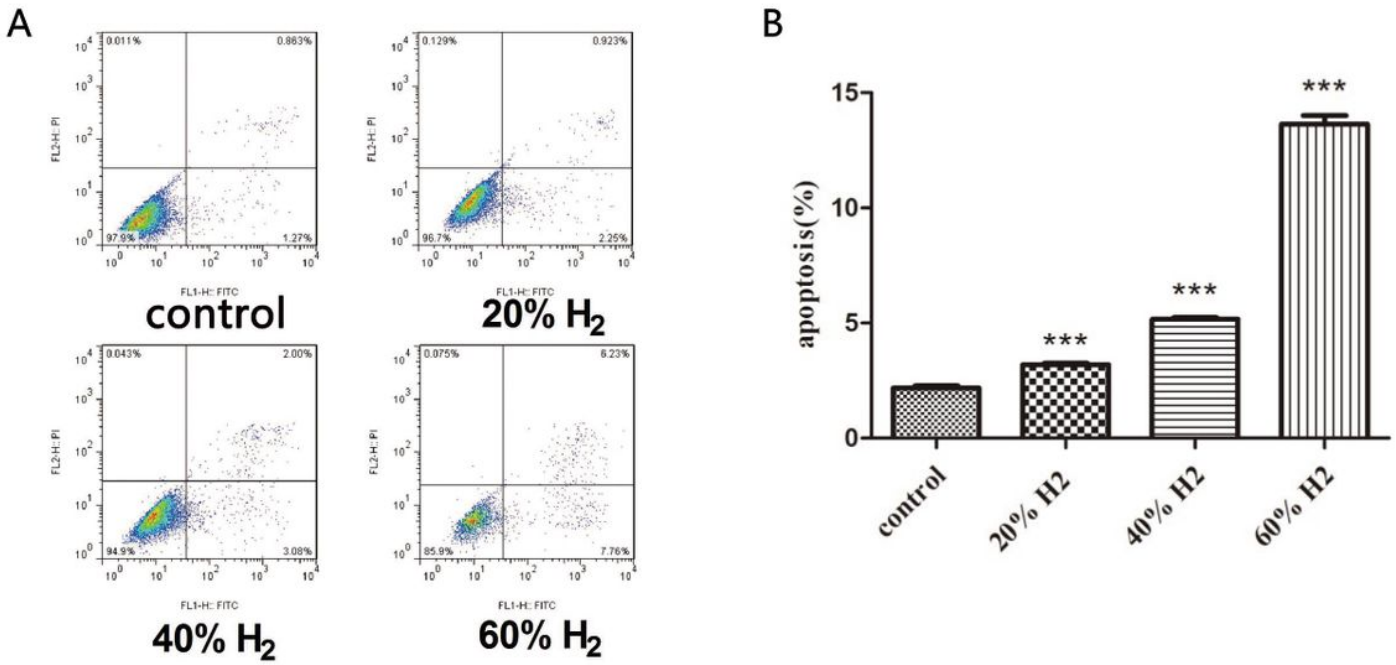
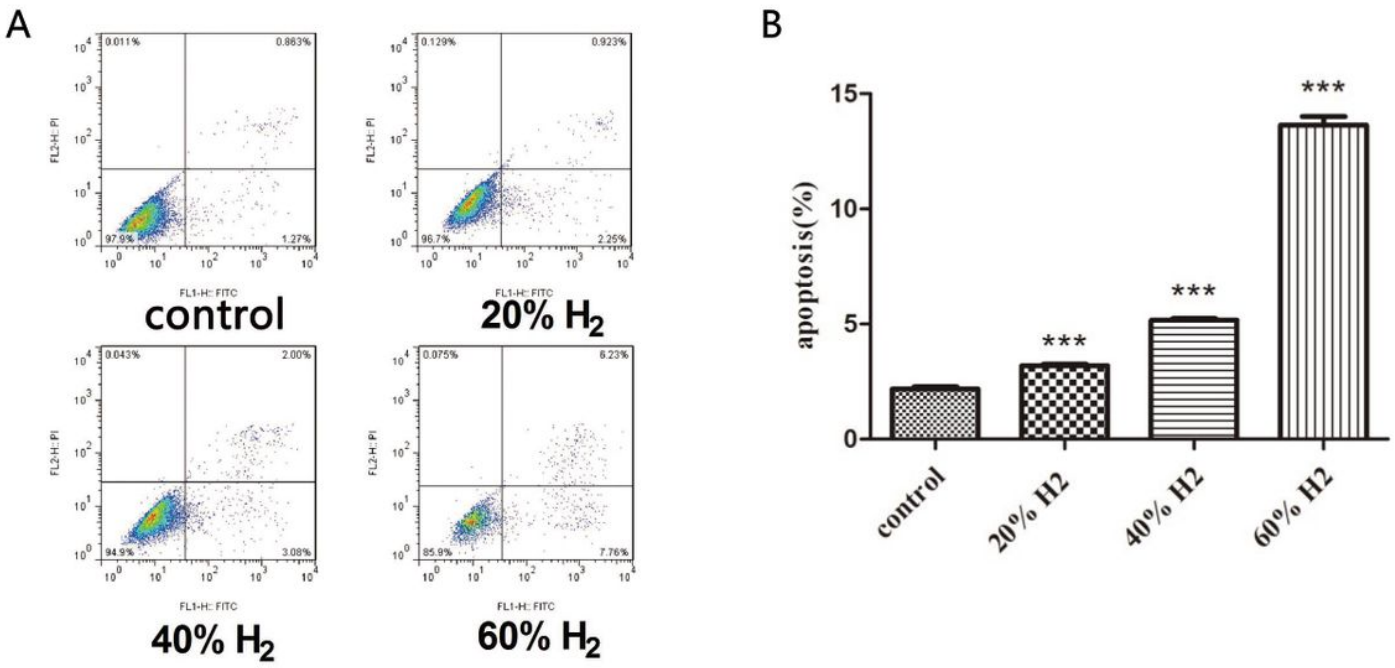


Figure 1

A549 cells were treated with multiple concentrations of hydrogen and detected by flow cytometry. A：Flow cytometry was used to detect cell apoptosis treated by different concentrations of hydrogen in A549 cell. B：The bar graphs were on behalf of the number of apoptosis cells in different group. The experiments were repeated at least three times. Each bar represents the mean of three independent experiments. The error bar in the column chart is SEM. (n = 3, * P < 0.05, **P < 0.01, ***P < 0.001).



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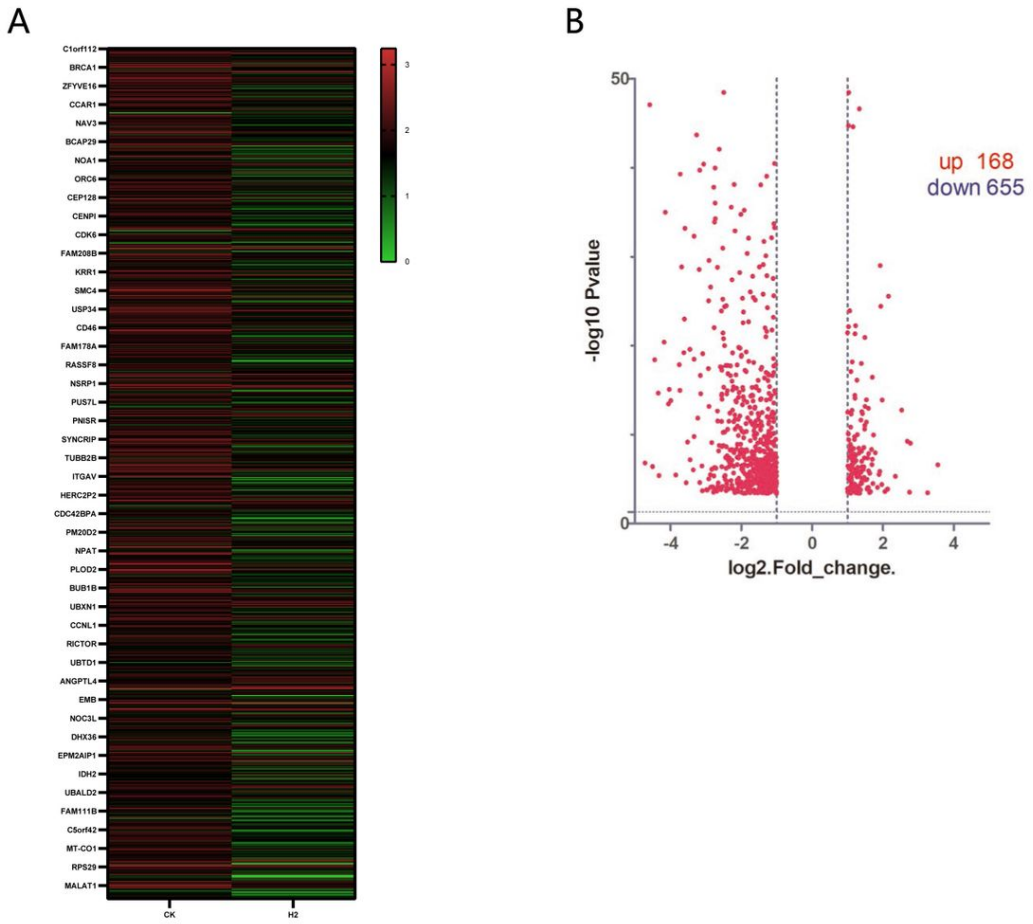
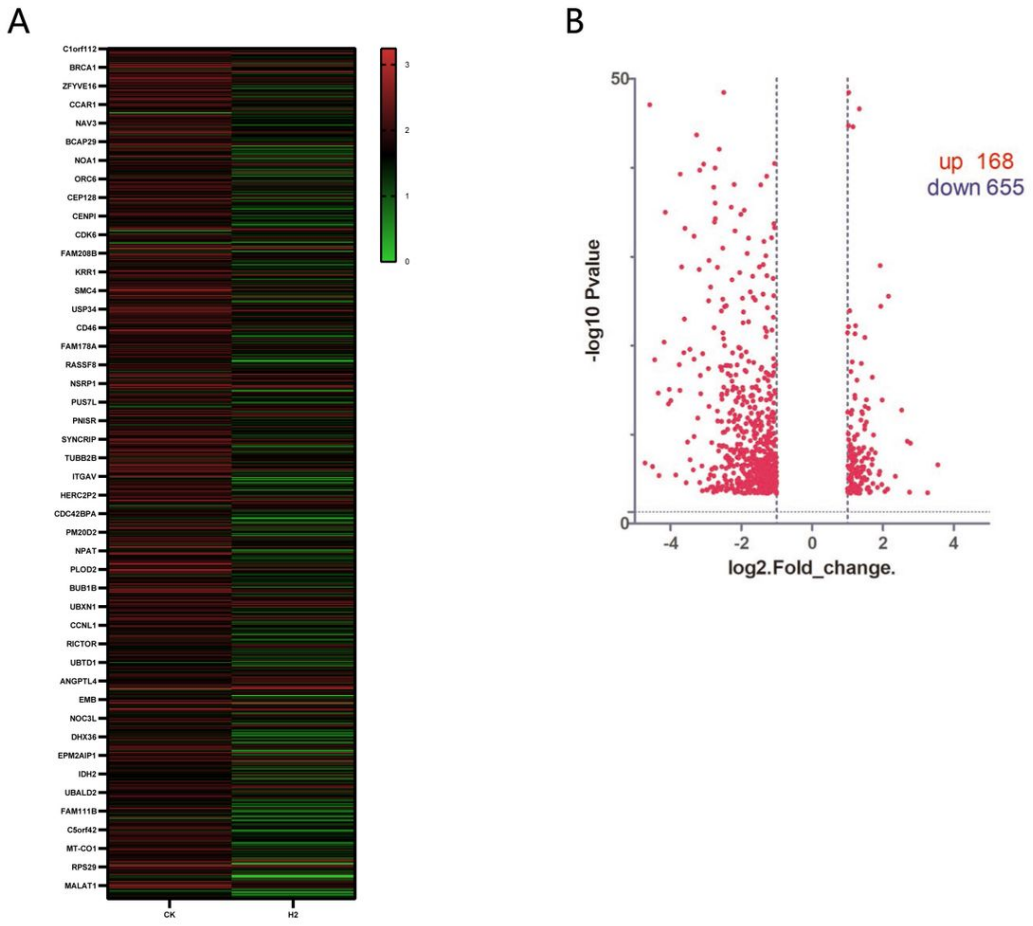


Figure 2

The DEGs between H2 and control groups were decided by transcriptome sequencing. A: The heat map of DEGs in A549 cells of H2 and control groups. B The volcano map of DEGs in A549 cells treated with H2 or not. (log2.Fold_change>1 or log2.Fold_change<-1)



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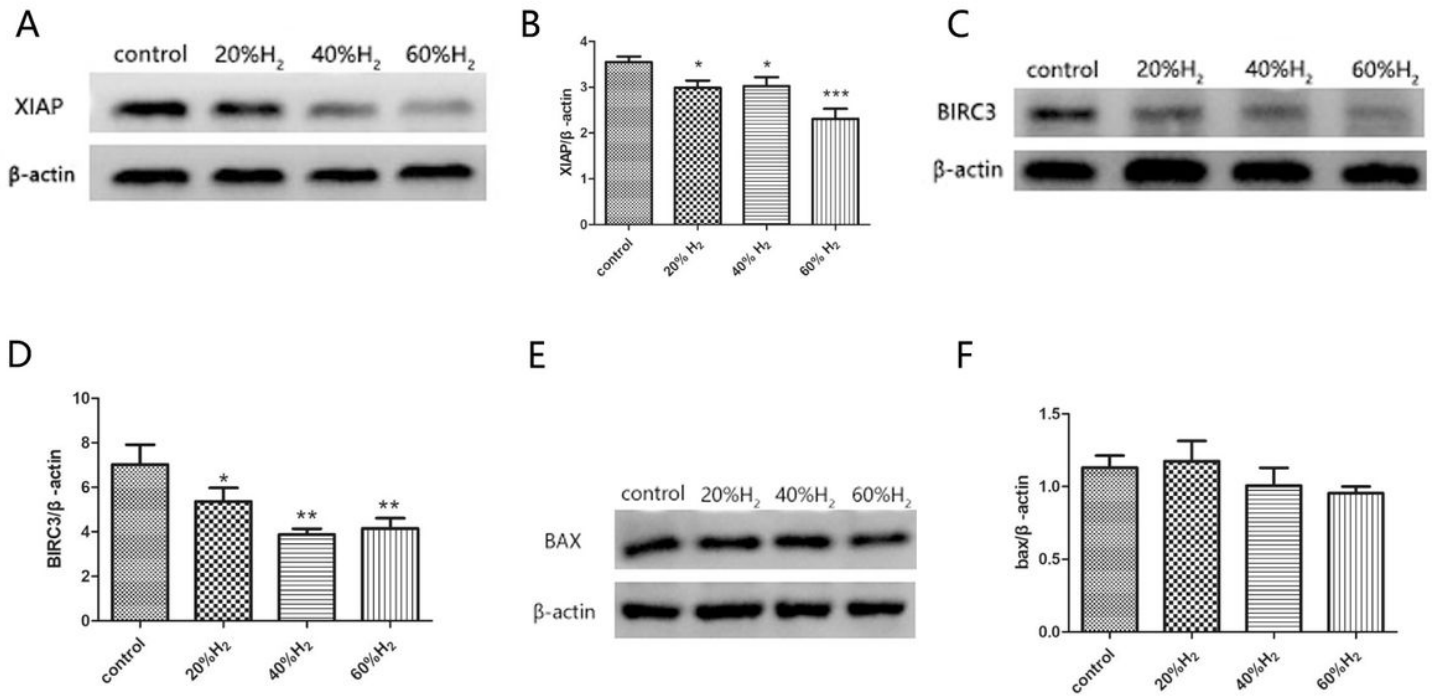

Figure 3

The protein levels of XIAP, BIRC3,and bax in A549 cells. A: Western blotting analysis of the effect of H2 on the expression levels of XIAP in A549 cells. B The bar graphs were represented
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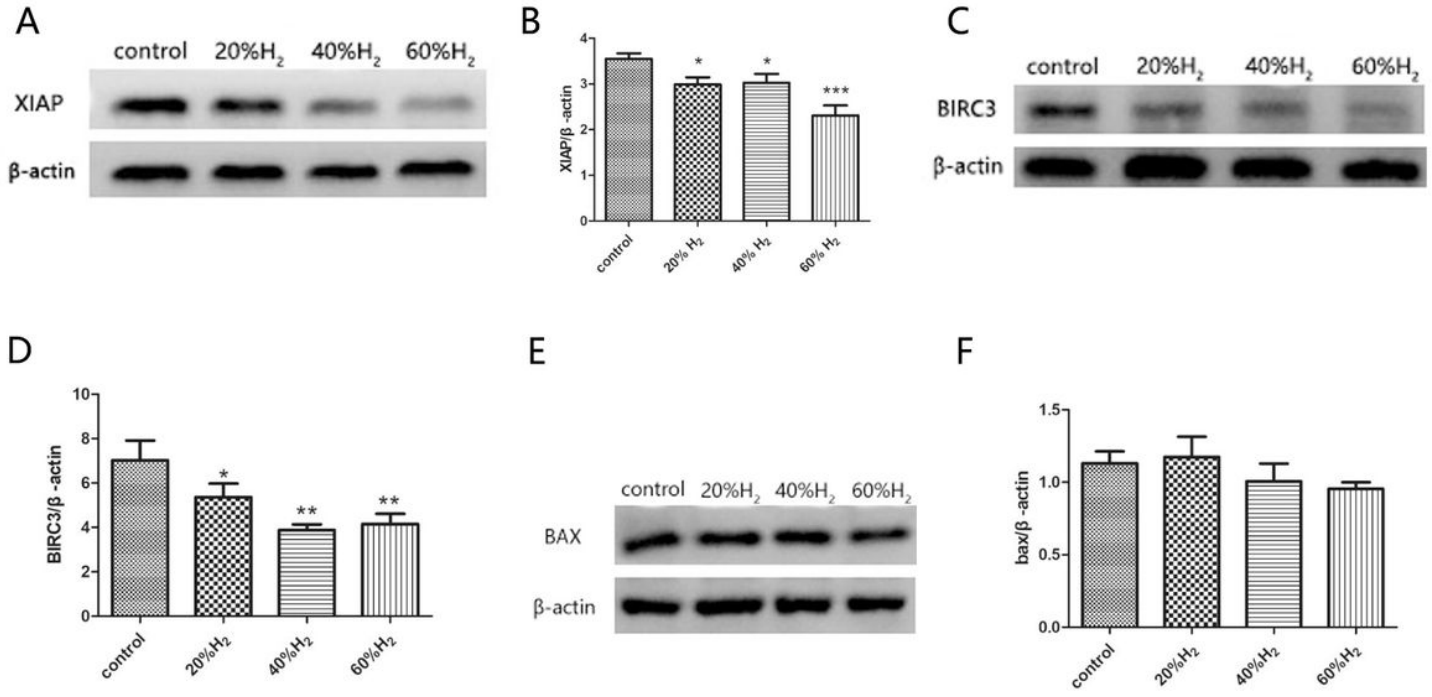

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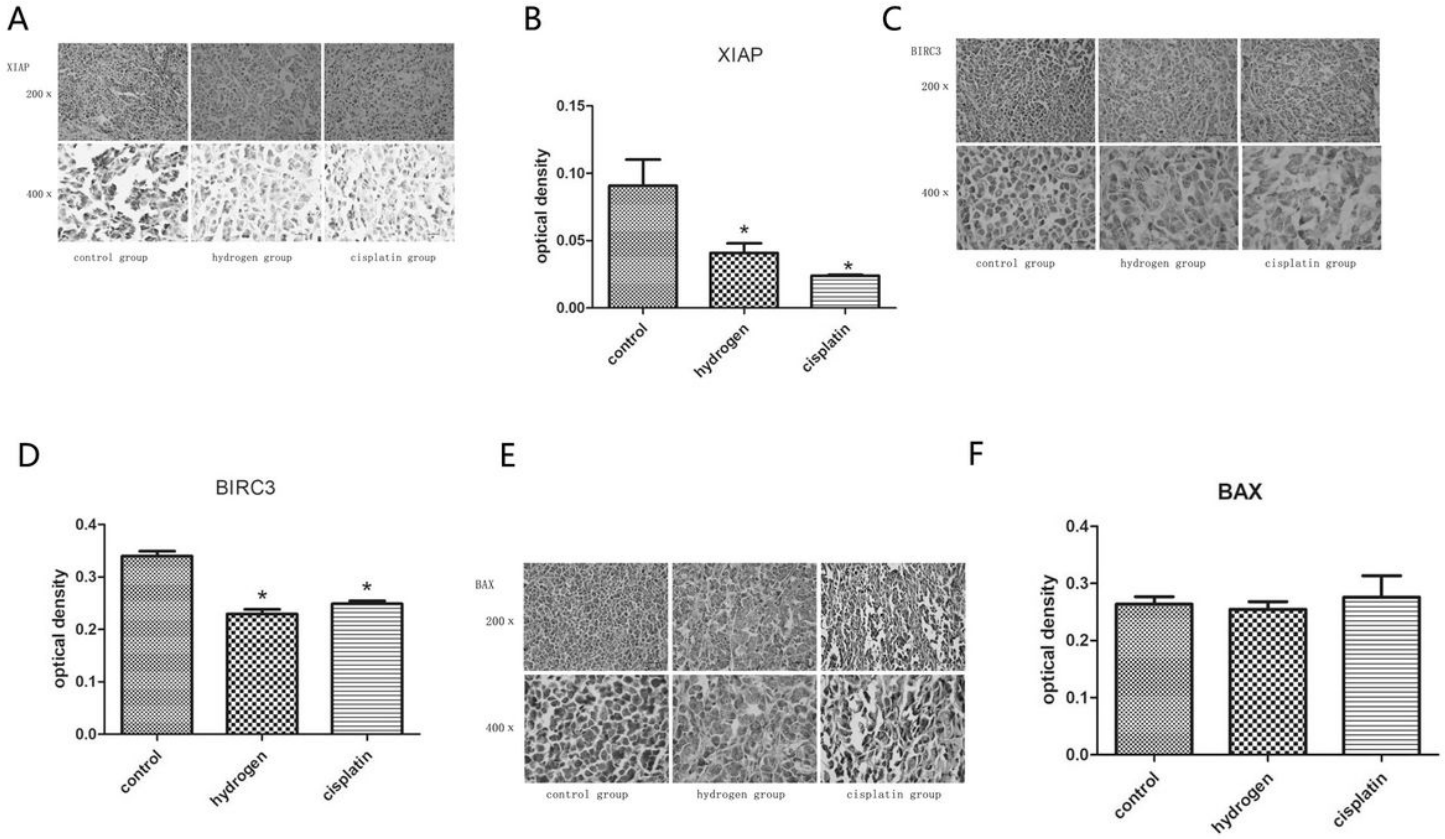
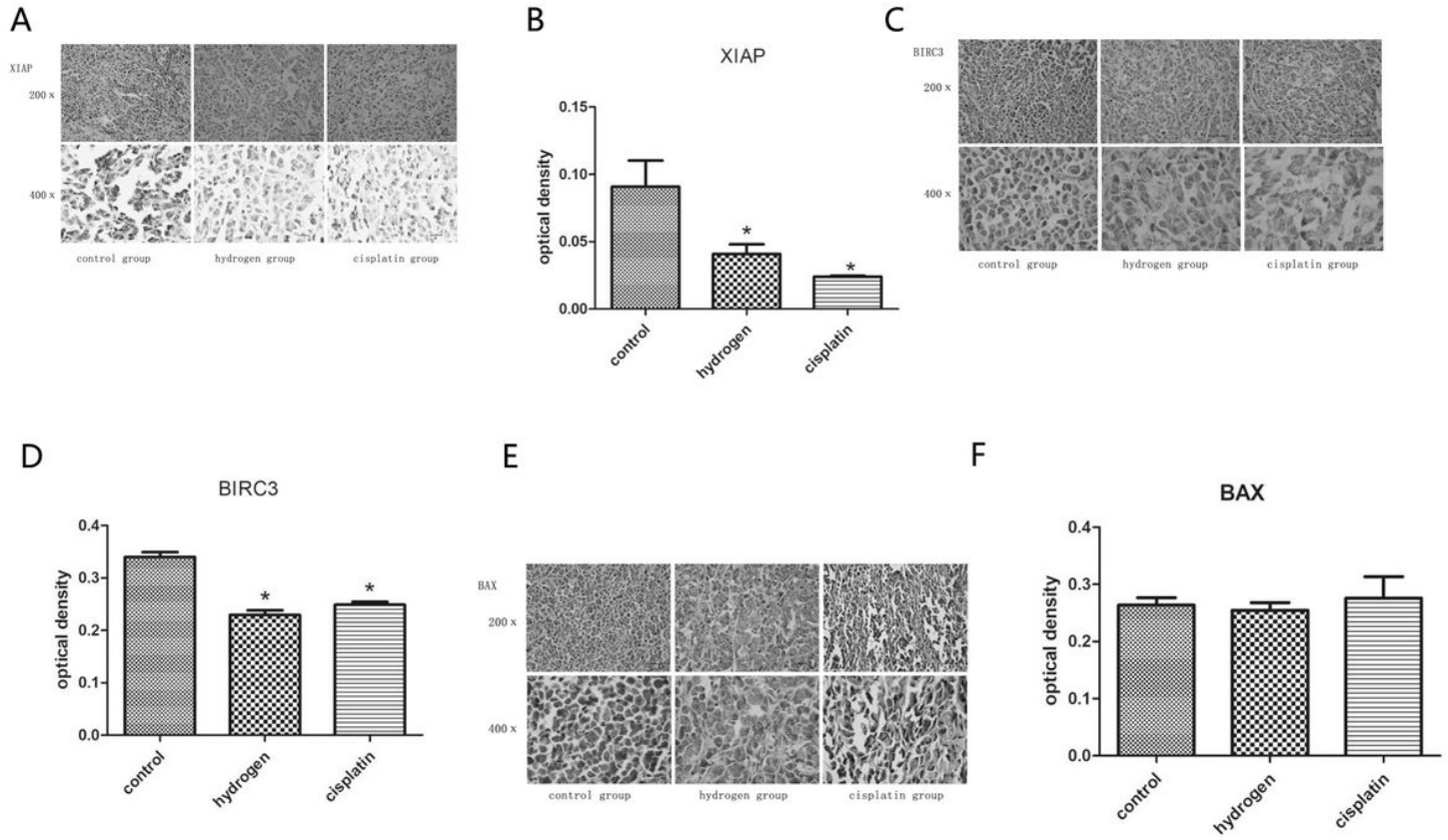


Figure 4

The result of immunohistochemistry. A: Immunohistochemistry was used to detect the expression of XIAP. B: The bar graphs represents the
$x \pm sofXIAPopticaldensity.\ \square\ n=3\ \square\ \cdot P<0.05 compared \rightarrow controlgroup\ \square .\ C{:}\ Im\mu nohis \rightarrow chemistrywasused \rightarrow \det ectthe\exp ressionofBIR$
x±s of BIRC3 optical density.（n=3）* P<0.05 compared to control group）. E: Immunohistochemistry was used to detect the expression of bax. F: The bar graphs represents the`x±s of bax optical density.（n=3）* P<0.05 compared to control group）



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The result of immunohistochemistry. A: Immunohistochemistry was used to detect the expression of XIAP. B: The bar graphs represents the $x \pm sofXIAPopticaldensity.\ \ n = 3\ \ \cdot P < 0.05compared \rightarrow controlgroup\ \ .\ C{:}\ Im\mu nohis \rightarrow chemistrywasused \rightarrow$ det*ectthe*exp*ressionofBIR* x±s of BIRC3 optical density.n=3* P<0.05 compared to control group. E: Immunohistochemistry was used to detect the expression of bax. F: The bar graphs represents the`x±s of bax optical density.n=3* P<0.05 compared to control group