Inhibitory effect of low-deuterium liquor on human lung adenocarcinoma cells

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Abstract: Objective: To investigate the inhibitory effect of deuterium-depleted liquor on lung adenocarcinoma in vitro and in vivo. Methods: In vitro, the MTT method was used to detect the inhibitory effects of liquor, deuterium-depleted water (DDW) and deuterium-depleted liquor (DDA) on the proliferation of human lung cancer A549 cells; the TUNEL method and flow cytometry were used to detect the apoptosis rate of human lung cancer A549 cells induced by liquor, DDW and DDA, respectively. In vivo, a lung cancer transplant tumor model was established, and drugs were administered on the day of modeling. After 9 days, the tumor growth curve was measured. After 21 days, the mice were killed by cervical dislocation, and the body weight and tumor weight were measured. The tumor tissues and liver and lung tissues of the mice were fixed, and the pathological changes were detected by HE staining. Results: In vitro, 72h and 180mmol/L DDA could significantly inhibit the proliferation of A549 cells, with a cell proliferation rate of (70.58±2.73)%, which was significantly different from the blank group and the liquor group (Pÿ0.05). The TUNEL method was used to detect the apoptosis rate of A549 cells, indicating that DDA could promote cell apoptosis, which was significantly different from the blank group, DDW group and liquor group (Pÿ0.05). Flow cytometry detected that DDA could promote cell apoptosis. In vivo, the tumor inhibition rates of the DDA group and the DDW group were 31.014% and 27.639%, respectively, which were significantly different from the blank group (Pÿ0.05). HE staining results showed that the tumor cells in the DDW group and the low- dose DDA group were loosely arranged and surrounded by necrotic tissue. At the same time, DDW and DDA had no effect on the liver and lung tissues of nude mice. Conclusion: Low-dose DDA can inhibit the proliferation of lung adenocarcinoma A549 cells in vitro and the growth of human lung adenocarcinoma H460 nude mouse subcutaneous transplanted tumors in vivo, and induce apoptosis of cancer cells.

Inhibitory Effect of Deuterium-depleted Alcohol on A549 and H460 Cells

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Abstract Objective: To observe the inhibitory effect of deuterium-depleted alcohol on the proliferation of lung cancer cells, A549 and H460. Methods: in vitro, the inhibitory effects of alcohol, deuterium-depleted water (DDW) and deuterium-depleted alcohol (DDA) on the proliferation of A549 cells were measured by MTT assay, and the apoptosis was determined by TUNEL method and flow cytometry (FCM). in vivo, human lung cancer H460 cells were implanted into nude mice. After 9 days, a growth curve was established to reveal the growth characteristics of xenograft. After 21 days, the mice were sacrifi ced, and the tumors were weighed and calculated for tumor-suppression rate. Pathological changes of tumor tissues were observed by means of HE staining. Pathological changes of liver and lung tissues were also observed. Results: The proliferation of A549 cells was remarkably inhibited by DDA at the concentration of 180 mmol/L, when compared with the control group and the alcohol group (P ÿ 0.05). A549 cells

induced by DDA revealed an obvious apoptosis, when compared with the control group, the alcohol group and DDW group (P ÿ 0.05). Moreover, FCM assay results showed that DDA could promote apoptosis. The in vivo data obtaiend showed higher tumor inhibition rates of 31.014% and 27.639%, respectively in DDA and DDW group than in the control group (P ÿ 0.05). HE staining showed obvious necrosis and loose arrangement of tumor cells in DDA and DDW groups, but no obvious changes in lung or liver tissues were observed. Conclusion: DDA has an obvious inhibitory effect on the proliferation of lung cancer cells.

Key words: deuterium depleted water; deuterium depleted alcohol; lung cancer; inhibitory effect

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For a long time, the role of alcohol in the development of tumors has been unclear [1]. Generally, liquor itself is considered to be carcinogenic . Mufti et al. [2] proposed that in the process of chemical induction of esophageal cancer, Received Liquor appears as a promoter, but before and during the process, it inhibits the incidence of tumors. Hayashi et al. [3] believe that chronic liquor consumption promotes the formation of colon cancer induced by 1,1-dimethylhydrazine. This further confirms the viewpoint of Mufti et al. However, Yang Lianjun et al. [4] believed that low-dose ethanol can significantly induce apoptosis of HCC-9204 liver cancer cells. At present, the relationship between liquor and tumors has not been determined. In nature, water is composed of 2 hydrogen atoms and 1 oxygen atom, of which hydrogen has three isotopes: protium, deuterium and tritium. In surface water, the ratio of deuterium to protium is about 1:6600, that is, the volume fraction of deuterium in water is 0.015% [5]. Water with a deuterium volume fraction of less than 0.015% is usually called deuterium-depleted water (DDW). Regarding the study of DDW's tumor inhibition, Somlyai et al. [6] reported that DDW can inhibit the growth rate of mouse fibroblast L929 cells and cause tumor tissue regression in transplanted tumor mice. Russian researchers recently discovered that if the volume fraction of deuterium in ordinary water is reduced by 65%, it will show certain anti-tumor properties. The results of tumor inhibition experiments on mice also showed that DDW can inhibit tumor growth and prolong the survival of mice [6-8]. The research of this topic also further confirmed that DDW with a deuterium volume fraction of 0.0050% can inhibit tumor growth in vivo and in vitro [9]. Chinese liquor has a close relationship with water. Choosing the right fermentation water or Adding water to reduce alcohol content to improve the quality of wine and reduce the harmfulness of liquor to the human body has always been a concern. This experiment explored the inhibitory effects of deuterium-depleted water, liquor and deuterium-depleted liquor on the growth of human lung adenocarcinoma cells in vivo and in vitro.

1 Materials and methods

1.1 Animals and Materials 60 BALB/c nude mice, 5-6 weeks old, male, (20±2) g, were purchased from Bikai Experimental Animal Co., Ltd. [Animal Qualification Certificate No. SCXK (Shanghai)]. They were kept in the SPF animal room of the Experimental Animal Center of the School of Pharmacy of Shanghai Jiao Tong University at a room temperature of 25°C and a humidity of 40%-70%, with free access to food and water. Liquor was provided by Luzhou Laojiao Co., Ltd. 1.2 Reagents and instruments Human lung adenocarcinoma cell line H460 was provided by Institute of Cell Biology, Chinese

Academy of Medical Sciences, Shanghai; human lung cancer cell line A549 was provided by Cell Bank, Chinese Academy of Sciences. DDW (volume fraction 0.0050%) was from Shanghai Chitian Ultralight Water Bioengineering Co., Ltd.; fetal bovine serum was from Hangzhou Sijiqing Company; RPMI1640 culture medium was from Gibco, USA; MTT, TUNEL and Anexin-V kits were from Nanjing Keygen Biotechnology Co., Ltd.

Multiskan MK3 microplate reader, Thermo, USA; inverted microscope, Nikon, Japan; HF151UV CO2 incubator, Lishen Instrument Co., Ltd.; clean bench (purification efficiency 100 level), Mettler-Toledo, Switzerland; FACSCalibur flow cytometer, BD, USA. 1.3 Methods 1.3.1 Cell culture Human lung cancer cell lines A549 and H460 were cultured in RPMI1640 medium containing 10% fetal bovine serum, 5% CO2, and 37°C. In the experiment, cells were planted in culture flasks and used for experiments when 90% of the cells were confluent.

1.3.2 MTT method

A549 cells (104 cells/well) were inoculated in 96-well culture plates. They were divided into 4 groups: blank group: cultured with normal 1640 medium; liquor group: 0-300mmol/L alcohol was added to the normal medium, and different alcohol concentration gradients were set to measure the optimal alcohol concentration; DDW group: deuterium volume fraction was 0.00050%; DDA group: 0-300mmol/L alcohol was contained in the medium prepared by DDW. A549 cells were cultured in different groups for 24, 48, and 72 hours, and 50ÿL MTT reagent solution was added to continue culturing for 4 hours. After the culture medium was aspirated, 150ÿL DMSO was added to each well, and the cells were shaken for 10 minutes. The wavelength of 550nm was selected to measure the OD value of each well. Five replicate wells were set for each group.

$$Group\ cell\ profileration\ rate\ \% = \frac{OD\ Non-blank}{OD\ blank} * 100$$

Group 1.3.3

TUNEL method to detect cell apoptosis

A549 cells were cultured for 48 and 72 h in the blank group, liquor group (containing 180 mmol/L alcohol), DDW group, and DDA group (containing 180 mmol/L alcohol). Negative control group: cultured under normal conditions, and no TDT Enzyme was added during the labeling process. Positive control group: A549 cells were treated with 1000 U DNaseÿ reaction solution. The operation was performed according to the instructions of the kit, and ematoxylin was selected for counterstaining. Under optical microscopy, the cells were reduced in size and deformed; the nucleus was densely stained and lost its submorphological structure, and the nuclear chromatin was crescent-shaped, or the nucleus was broken into nuclear fragments of varying sizes, which are typical morphological changes of apoptosis. 200 cells were randomly counted for each slide, and the apoptosis rate was calculated. 1.3.4 Flow cytometric detection of cell apoptosis A549 cells were cultured for 48 and 72 h, and the blank group, liquor group, Cells in the DDW group and the DDA group. Wash twice with PBS, digest the cells with EDTA-free trypsin, and terminate the digestion after about 1 to 2 minutes. Collect the cells by centrifugation, wash twice with PBS, discard the supernatant, add 500ÿL Binding Buffer to suspend, add 5ÿL PI and mix, then add 5ÿL FITC and mix, react in the dark for 10 minutes, and detect on the machine. 1.3.5 Establishment and grouping of human lung adenocarcinoma cell transplanted tumor model in nude mice

The nude mice were randomly divided into 6 groups: blank group, low-dose liquor (CL) group (V(liquor):V(ultrapure water)=1:5.7), high-dose liquor (CH) group (V(liquor):V(ultrapure water)=1:3), DDW group, low-

dose DDA (DDAL) group (V(liquor):V(DDW)=1:5.7), and high-dose DDA (DDAH) group (V(liquor):V(DDW)=1:3), with 10 mice in each group. The DDW group, DDAL group, and DDAH group drank sterilized DDW in advance, and the other 3 groups were raised normally. After 2 weeks, the human lung cancer cell line H460 was collected and the density was adjusted to 1×107 /mL. 0.2mL of the above cell suspension (containing 2×106 cells) was taken with a 1mL syringe and injected subcutaneously into the right axilla of the nude mouse to complete the nude mouse tumor inoculation. 1.3.6 Administration and sample collection The mice were gavaged on the day of modeling, and 0.2 mL was administered once a day. After 25 days, the tumor-bearing nude mice were killed by cervical dislocation after blood collection. The subcutaneous solid tumor masses were removed, the fascia was removed, and the masses were weighed using an electronic balance. The tumor mass and tumor inhibition rate of each group were calculated. The mouse lungs, livers, kidneys, spleens, and tumors were stored at -70°C for subsequent pathological and protein detection.

1.3.7 Observation of tumor growth and tumor inhibition effect The model was established for 10 days, and the longest diameter (a) and shortest diameter (b) of the tumor were measured every 3 days . The tumor volume (V) was calculated according to formula (2), and the tumor growth curve was drawn. The tumor was removed 24 hours after the last intragastric administration, and the tumor inhibition rate was calculated according to formula (3).

V=ab2 / 2

(2)

$$Group\ tumor\ inhibition\ rate\ \% = \frac{V\ control\ group - V\ experimental}{V\ Control\ group} * 100$$

(3)

1.3.8 HE staining After

blood collection, the animals were killed by cervical dislocation, and the tumor, liver, and lung tissues were fixed in 4% neutral formaldehyde, dehydrated, embedded in paraffin, sliced 4 ÿm thick, stained with HE, and sealed. Pathological observation was performed under light microscopy. 1.4 Statistical analysis SPSS statistical software package was used for data analysis, and one-way analysis of variance was used to compare the differences between the two groups. P < 0.05 was considered statistically significant.

2 Results and analysis

2.1 Effects of different concentrations of alcohol on A549 cell proliferation

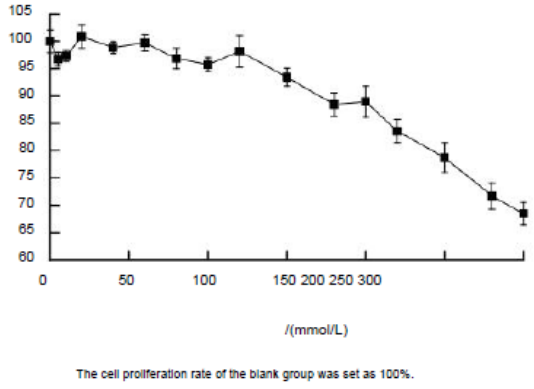


Figure 1 Relationship between different alcohol concentrations and A549 cell proliferation

The cell proliferation rate of the blank group was set as 100%.

Figure 1 Relationship between different alcohol concentrations and A549 cell proliferation

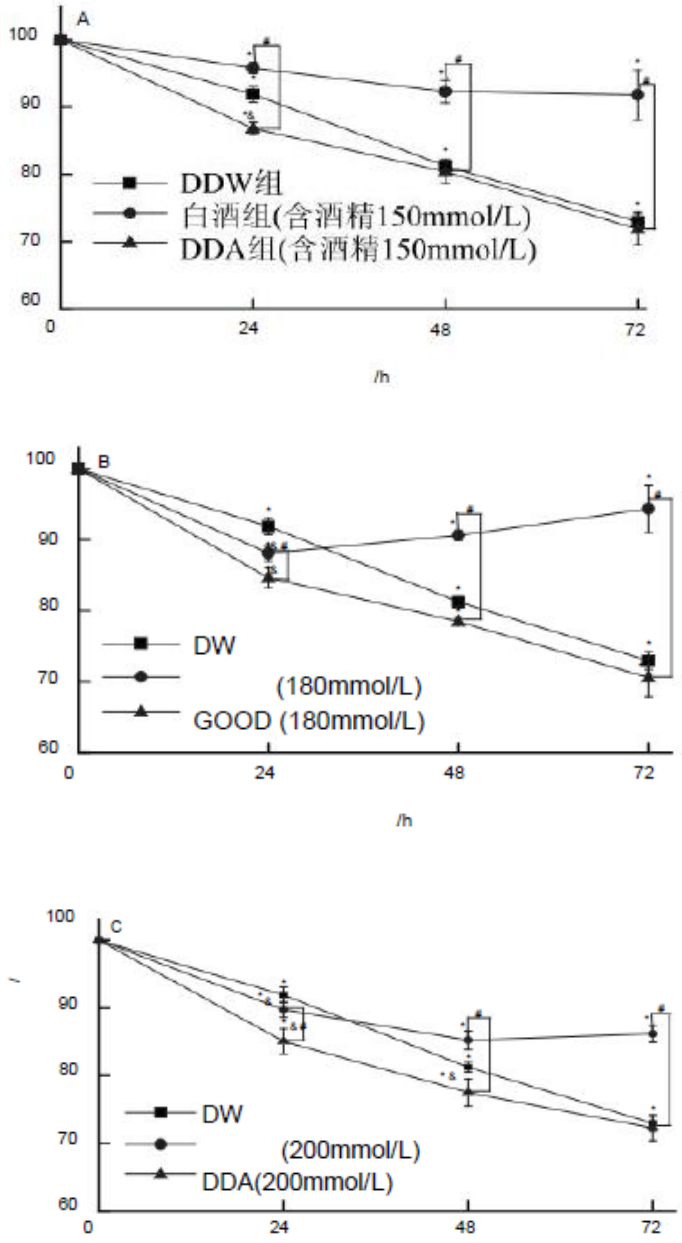
Fig.1 Effect of alcohol on the proliferation of A549 cells

It is known that high doses of liquor can damage cells. This study first determined the dosage of liquor for in vitro experiments. As shown in Figure 1, at alcohol concentrations of 180 and 200 mmol/L, the cell proliferation rates were almost the same, 88.429% and 88.943% respectively. When the alcohol concentration exceeded 200

mmol/L, cell proliferation was inhibited as the alcohol concentration increased. Therefore, the alcohol concentration range of MTT in vitro was determined to be 150-200 mmol/L. Within the above initial range, 150, 180, and 200 mmol/L were selected respectively.

Three different alcohol concentrations were used to further determine the optimal cell proliferation inhibition concentration.

As shown in Figure 2A, compared with the blank group, the cell proliferation of the DDW group, the liquor group (containing 150mmol/L alcohol) and the DDA group (containing 150mmol/L alcohol) was inhibited ($P < 0.05$). Among them, at 72h, the DDA group (cell proliferation rate was (71.88±2.47)% compared with the liquor group (cell proliferation rate was (91.81±3.70)%), the difference was statistically significant ($P < 0.05$); there was no significant difference between the DDA group and the DDW group (cell proliferation rate was (72.96±1.22)%). As shown in Figure 2B, compared with the blank group, the cells in the DDW group, the white wine group (containing 180mmol/L alcohol) and the DDA group (containing 180mmol/L alcohol) The proliferation was inhibited (Pÿ0.05). Among them, at 72h, the DDA group (cell proliferation rate was (70.58±2.73)%) was compared with the white wine group (cell proliferation rate was (94.36±3.31)%), and the difference was statistically significant (Pÿ0.05). There was no significant difference between the DDA group and the DDW group (cell proliferation rate was (72.96±1.22)%). As shown in Figure 2C, compared with the blank group, the cell proliferation in the DDW group, the liquor group (containing 200mmol/L alcohol) and the DDA group (containing 200mmol/L alcohol) was inhibited ($P < 0.05$). Among them, the difference between the DDA group and the liquor group was statistically significant ($P < 0.05$). At 48h, there was no significant difference between the DDA group (cell proliferation rate was (77.50±2.00)%) and the DDW group (cell proliferation rate was (81.25±0.72)%).



The cell proliferation rate of the blank group was set as 100%; *. Significant difference compared with the blank group (Pÿ0.05); &. Significant difference compared with the DDW group (Pÿ0.05); ÿ. There was a significant difference compared with the DDA group (Pÿ0.05).

2 Effects of three different alcohol concentrations on the proliferation of A549 cells

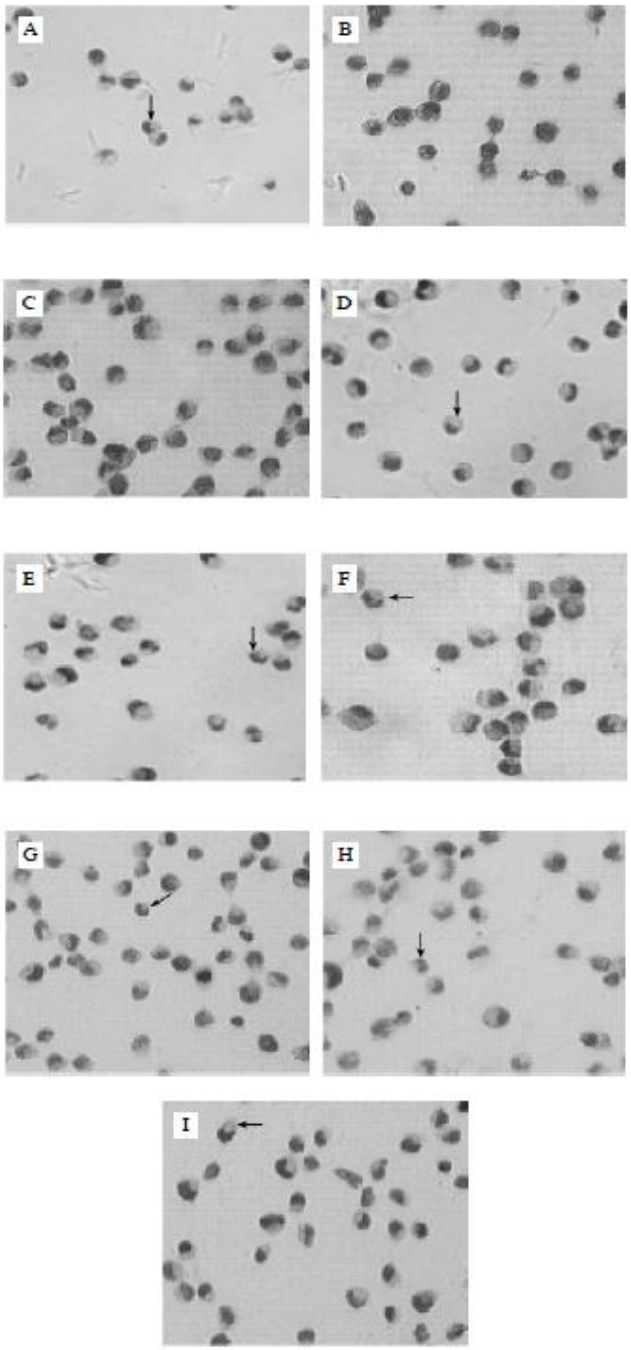
Fig.2 Effect of alcohol concentration on the proliferation of A549 cells

Compared with the group containing 180mmol/L alcohol, the group containing 150mmol/L alcohol and the group containing 200mmol/L alcohol, the former had an inhibitory effect on cell proliferation between the latter two groups. Therefore, this study selected 180mmol/L alcohol concentration for subsequent in vitro experiments.

2.2 TUNEL assay to detect the effect of DDA on apoptosis of A549 cells TUNEL assay showed that DDW, DDA and liquor had an effect on A549 cells.

After 48 and 72 h, the cells showed typical features of apoptosis (Figure 3).

The apoptosis rate of cells in the blank group was (8.468±1.203)%, and that in the positive control group The apoptosis rate of cells was (36.762±2.733)%. Compared with the blank group, the DDA group could significantly induce cell apoptosis. There was no significant statistical difference (Pÿ0.05). Compared with the alcohol group, the DDA group significantly promoted cell apoptosis (Pÿ0.05). Compared with the alcohol group, cell apoptosis was more significant (Pÿ0.05). The apoptosis rate increased among the groups with the same concentration of liquor (Figure 4).

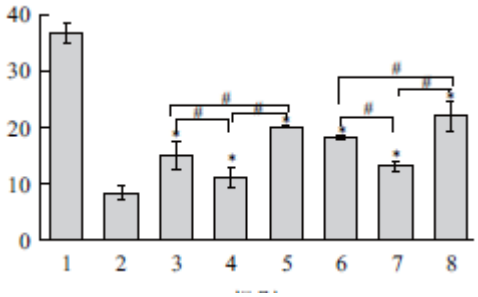


A. Positive control group; B. Negative control group; C. Blank group; D. DDW group (48h);

E. DDA group (48h); F. Baijiu group (48h); G. DDW group (72h); H. DDA group (72h);

I. Liquor group (72 h). Arrows indicate individual apoptotic cells in each group, except for the negative control group. Figure 3 TUNEL 3 TUNEL assay to detect DDA-induced cell apoptosis (×400)

Fig.3 DDA-induced apoptosis of A549 cells evaluated by TUNEL method (×400)



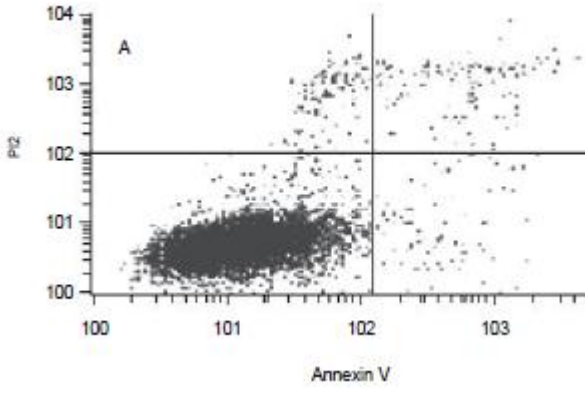
1. Positive control group; 2. Blank group; 3. DDW group (48h); 4. Liquor group (48h);

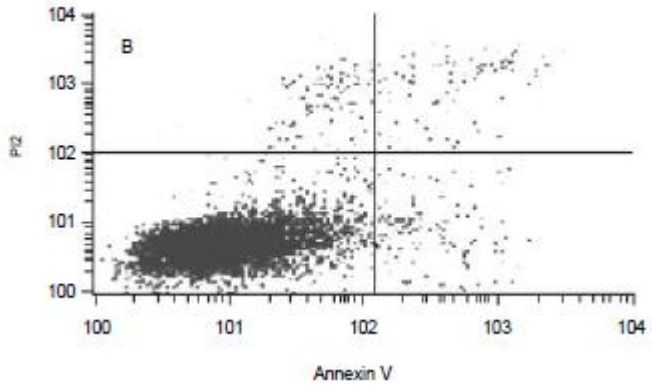
5. DDA group (48h); 6. DDW group (72h); 7. Baijiu group (72h); 8. DDA group (72h). *. There were significant differences compared with the blank group (Pÿ0.05); #. There were significant differences among the groups (Pÿ0.05).

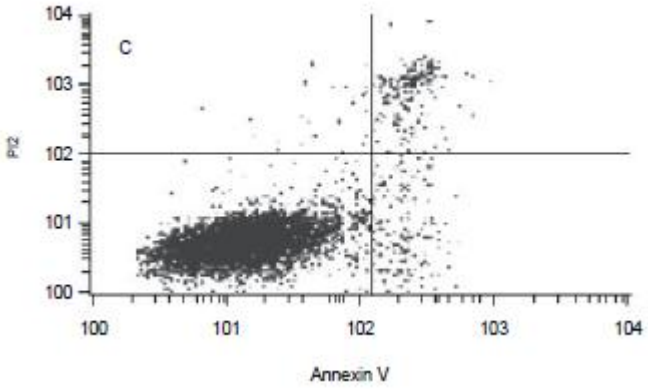
Figure 4 shows the TUNEL data Fig.4 Plot of the TUNEL data

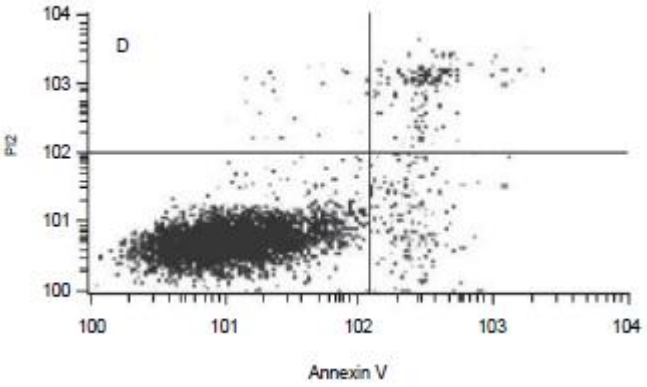
2.3 Flow cytometry detection of DDA-induced apoptosis in A549 cells

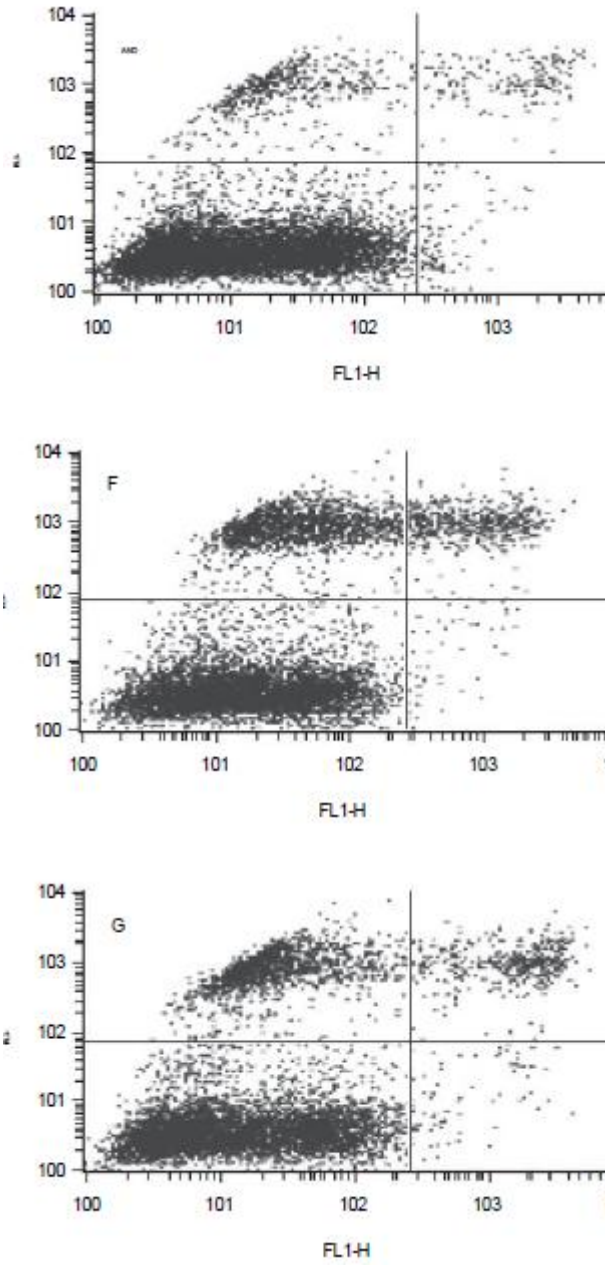
A549 cells were cultured with DDW, DDA and liquor respectively for 48 hours. After collection and processing, cell apoptosis was detected by computer, as shown in FIG 5.











A. Blank group; B. DDW group (48h); C. Liquor group (48h); D. DDA group (48h); E. DDW group (72h); F. white wine group (72h); G. DDA group (72h).

Figure 5 Flow cytometry detection of DDA-induced cell apoptosis

Fig.5 DDA-induced apoptosis of tumor cells evaluated by FCM assay

Table 1 Flow cytometry detection of DDA-induced cell apoptosis (x±s)

Table tumor 1 DAA-cells induced evaluated by apoptosis FCM of tumor cells evaluated by FCM able 1 DAA-induced apoptosis of

%

Group	48h	72 hours
Blank Group	2.23±0.21	1.45±0.20
DDW Group	2.64±0.17	2.58±0.15
Liquor Group	4.59±0.23	6.99±0.39
DDA Group	4.04±0.14	5.80±0.54

As shown in Table 1. after 48 hours of action. the apoptosis rate of cells in the blank group was

As shown in Table 1, after 48 hours of action, the apoptosis rate of cells in the blank group was (2.23±0.21)%, the apoptosis rate of DDW group was (2.64±0.17)%, The apoptosis rate of the liquor group was (4.59±0.23)%, and the apoptosis rate of the DDA group was (4.59±0.23)%. (4.04±0.14)%. After 72h of treatment, the apoptosis rate of each group was similar to that of each group at 48h. There was no significant change in mortality rate.

2.4 Inhibition of lung cancer xenograft growth by DDA

There was no significant difference in the physiological state of nude mice in each group before treatment. As shown in Figure 6, After 9 days of intragastric administration, the tumor volume of each group increased, except for the CH group and the blank group. Except for the DDAL group, the growth rate of tumors in other groups was significantly slow. The tumor growth was the slowest in the DDW group, followed by the DDAH group and As shown in Table 2, the tumor inhibition rate of the DDAL group was 31.014%, and that of the DDW group was The tumor inhibition rate was 27.639%, which was statistically significant compared with the blank group. The CH group has a promoting effect on the growth of transplanted tumors.

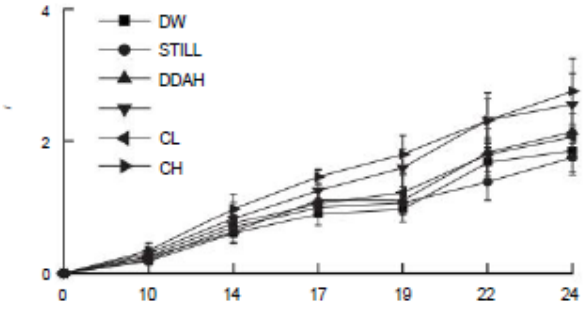


Figure 6 Growth curve of lung cancer transplanted tumor Growth curve of lung cancer transplanted tumor

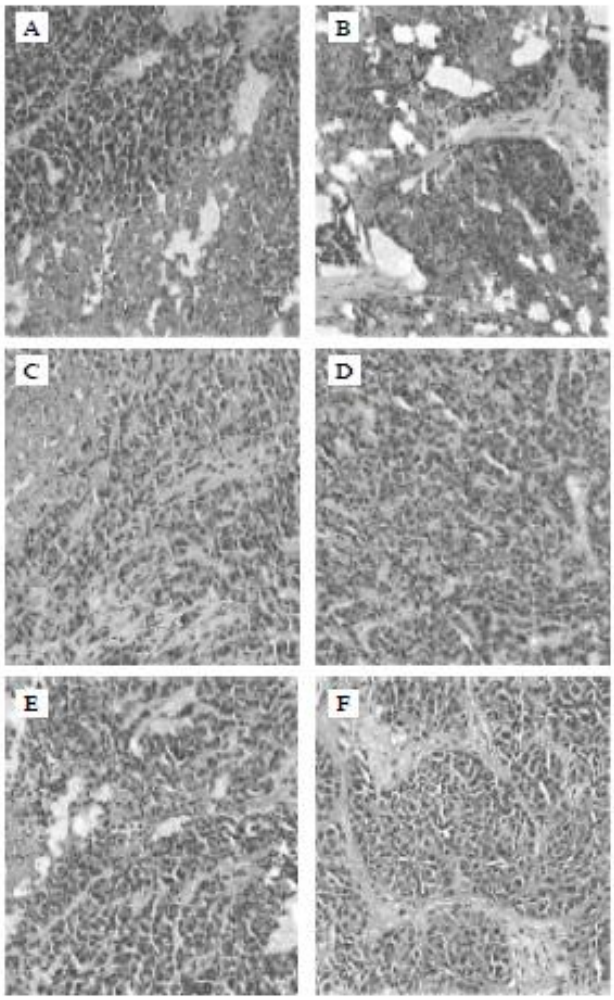
Fig.6 Growth curve of xenograft with lung cancer cells

Table 2 Volumes and inhibitory rates of H460 cells in nude mice d (x±s) inhibitory Volumes rates and of inhibitory H460 cells rates in of nude H460 mice cells (in nude mice (x±s)

Group		Tumor volume/cm3 Tumor inhibition rate/%	
Blank Group	10	2.56±0.56	
DDW Group	10	1.85±0.13	27.639*
DDAL group	10	1.77±0.23	31.014*
DDAH group	9	2.14±0.66	16.531
CL Group	10	2.05±0.13	19.584
CH Group	10	2.75±1.26	ÿ7.64

Note: *. There are significant differences compared with the blank group (Pÿ 0.05).

2.5 Observation of pathological changes in transplanted tumors, lungs, and liver tissues by HE staining



A. DDW group; B. DDAL group; C. DDAH group; D. blank group; E. CL group; F. CH group. Same below.

Figure 7 Pathological changes of tumor tissue detected by HE staining Pathological changes of tumor tissue detected by HE staining (×100)

As shown in Figure 7, the tumor cells in the blank group were densely arranged and the tissues grew actively (Figure 7D). In comparison, in the DDW group and the DDAL group, there was necrotic tissue around the tumor cells, and the number of tumor cells was significantly lower than that in the blank group (Figure 7A, B). Compared with the blank group, the tumor cells in the CL group and the DDAH group were more loosely arranged, and there were necrotic tumor cells (Figure 7C, E). In the CH group, the tumor cells were closely arranged, and

the sections showed that there were blood vessels in the tumor tissue, and the cancer cells grew vigorously (Figure 7F).

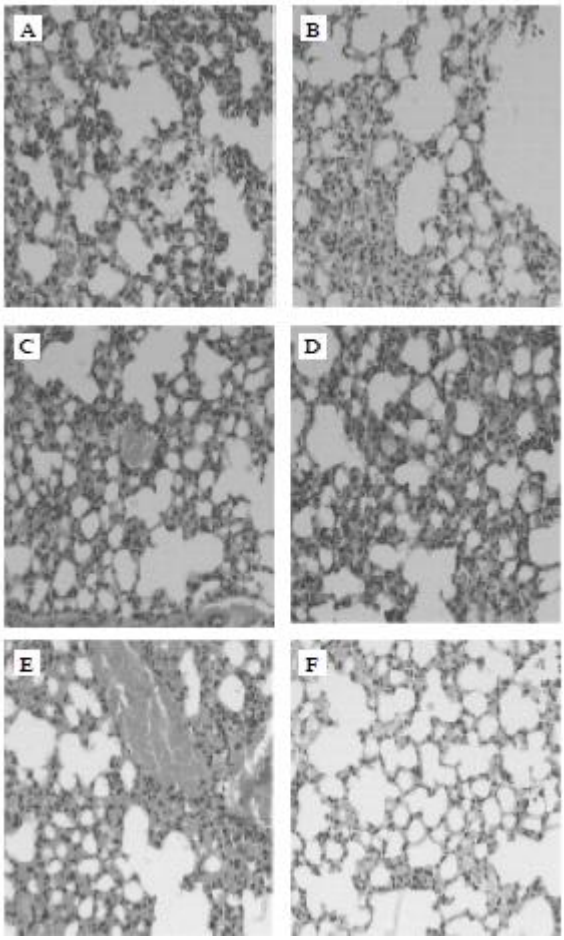


Figure 8 Pathological changes of lung tissue detected by HE staining Pathological changes of lung tissue detected by HE staining (×100)

As shown in Figure 8, HE staining of nude mouse lung tissues showed that the lungs of mice in the blank group were normal without tumor lesions, and the lungs had complete alveoli, alveolar ducts and alveolar sacs, and the intervals between alveoli and alveoli were normal (Figure 8D). Compared with the blank group, the alveolar structures of the other groups showed no pathological changes (Figures 8A-F).

Figure 9 HE staining of nude mouse liver tissue showed that the liver structure of mice in the blank group was normal, the hepatocyte morphology, size and structure were normal, the hepatocytes were neatly arranged, and the boundaries between cells were clear (Figure 9D). Compared with the blank group, there was no pathological change in the liver structure of the other groups (Figure 9A-F).

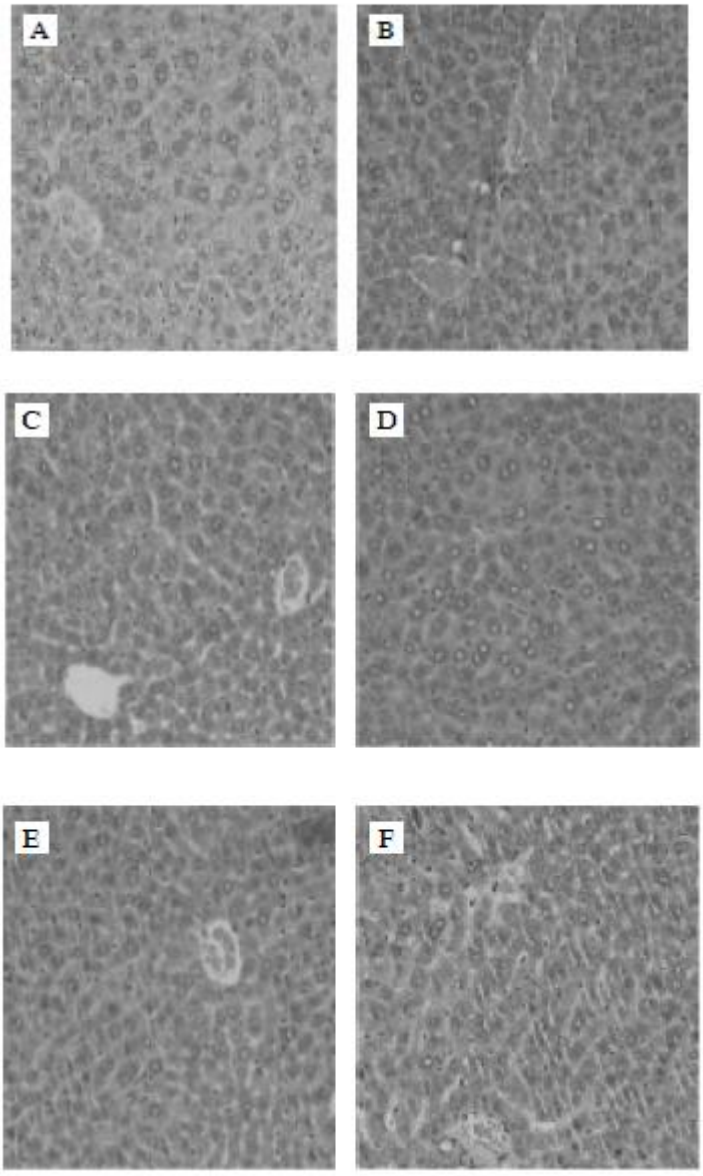


Figure 9 Pathological changes of liver tissue detected by HE staining Pathological changes of liver tissue detected by HE staining (×100)

3 Discussions

Experimental studies have confirmed that low-dose low-deuterium liquor can Significantly inhibited the growth of human lung adenocarcinoma tumor cells. MTT results showed that compared with the same concentration of liquor group, low-

deuterium liquor could significantly inhibit the growth of lung cancer cell A549. TUNEL assay showed that low-deuterium liquor, low-deuterium water and liquor could all induce apoptosis of A549 cells. Among them, low-deuterium liquor induced apoptosis more significantly than the liquor group. At the same time, the apoptosis rate increased over time. Flow cytometry assay further confirmed that low-deuterium liquor, low-deuterium water and liquor could all induce apoptosis of A549 cells. Compared with the normal group, low-deuterium liquor induced more apoptosis. It is currently believed that the possible mechanisms by which alcohol inhibits tumor cell growth are: 1) causing damage to cell chromosomes, DNA breaks, and inhibition of the function of DNA repair enzymes [10] ; 2) causing damage to the intracellular electron transport chain, impairing the function of ATP synthesis, stimulating lipid peroxidation, and causing changes in the fluidity of intracellular biological membranes [11] ; 3) intermediates produced by alcohol metabolism, potentially causing cell damage and affecting cell proliferation. Existing studies have shown [9] that deuterium-depleted water works mainly by inducing apoptosis of tumor cells. In vitro, deuterium-depleted water and alcohol jointly promote apoptosis of tumor cells. In in vivo experiments, it was found that high-dose liquor promoted tumor growth, while low-dose liquor inhibited tumor growth. Existing studies have pointed out [12] that long-term alcohol intake can enlarge tumors and increase the levels of angiogenic factors and vascular endothelial growth factor (VEGF). The study also found that low-dose deuterium-depleted liquor can significantly inhibit the growth of nude mouse transplanted tumors, and there is a significant difference compared with the blank group, and the tumor inhibition effect is better than other experimental groups. Pathological observation showed that the tumor tissue cells in the deuterium-depleted liquor group were loosely arranged, and necrotic tumor cells were found. The results of in vivo experiments showed that the combined use of deuterium-depleted water and liquor had a synergistic effect and further inhibited the growth of tumor cells.

Pathological sections of liver and lung showed that mice in the low-deuterium water group and the low-deuterium liquor group The liver and lung tissues were normal with no obvious pathological changes, which is consistent with the findings of Kovacs et al. [13]

The conclusion is consistent. The future research direction of anti-tumor drugs will be high efficiency and low toxicity. Therefore, deuterium-depleted water is worthy of further in-depth research.

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