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Inhibitory effect of deuterium-depleted water on lung cancer cell proliferation and its possible mechanism

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[Abstract] Objective: To investigate the inhibitory effect of deuterium-depleted water (DDW) on the proliferation of human lung cancer cells in vitro and in vivo and its possible mechanism.Methods : The inhibitory effect of DDW on the proliferation of lung cancer A549 cells and normal human embryonic lung fibroblasts HLF-1 was detected by MTT assay, the apoptosis of A549 cells was detected by TUNEL assay, and the changes of cell cycle were detected by flow cytometry. A BALB/c nude mouse model of human lung cancer H460 cell transplantation was established, and the growth of transplanted tumors in nude mice was observed after drinking deuterium-depleted water for 60 days. Results: Compared with the control group, DDW with a volume fraction of 0.0025%, 0.0050% and 0.0105% had a significant inhibitory effect on the proliferation of A549 cells at 10 h of culture ($P<0.05$), and then the inhibitory effect disappeared. After 48 h, the inhibitory phenomenon gradually appeared, and the inhibitory effect was significant at 72 h ($P<0.05$). Under the same conditions, DDW with different volume fractions had no significant inhibitory effect on normal human embryonic lung fibroblast HLF-1. TUNEL staining showed that A549 cells under the action of 0.005% DDW showed apoptosis, and the apoptotic rate was significantly higher than that of the control group [(25.38 ±3.90)% vs (10.87 ±1.11)%), $P<0.05$]. Flow cytometry showed that the number of S phase cells of A549 cells increased significantly after DDW treatment ($P<0.05$). After drinking DDW, nude mice bearing human lung cancer H460 transplanted tumors could significantly improve their quality of life, and the tumor inhibition rate reached 30.08%. Conclusion: The inhibitory effect of DDW on lung cancer cell proliferation is limited to a certain dose range, and has the characteristics of fluctuation and time period; its mechanism may be related to the induction of S phase arrest and apoptosis of lung cancer cells. [Keywords] Deuterium-depleted water; Lung

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Inhibitoryeffectofdeuterium-depletedwateronproliferationoflungcarcinoma cellsandthepossiblemechanism

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[Abstract] Objective:To explore the invitro and invivoinhibitory effects of deuterium-depleted water (DDW) on the prolife ration of human lung carcinoma cells, and to explore the possible mechanism. Methods: The inhibitory effect of DDW ontheproliferationofhumanlungcarcinomaA549 cells and human embryoniclung fibro blast HLF-1 cells was examined by MTT assay;apoptosisofA549 cells was examined by TUNEL; and cell cycle was analyzed by flowcytome-try. Mouse model ofl ungcarcinoma was established by inoculating human lung carcinoma H460 Cells into BALB/cnude mice, and the growth of implanted tumors was observed after DDW treatmentfor60 d.Results: Compared with control group, A549 cellstreatedwith0.0025%, 0.0050% or 0.0105% DDW showedsignificantlydecreasedproliferation10 h after treatment (P<0.01). The inhibitory effects of DDW gradually disappeared, but appeared 48 hlater again, with the inhibitory effects at 72h being significant (P<0.05).DDW at the same dosages showed no inhibition on the proliferationofHLF-1 cells(P>0.05).TUNEL assay verified the apoptosis of DDW-treatedA549 cells, andtheapopto-sisrateofDDW-treatedA549 cells was significantly higher than that of control group([45.30 ±4.21] % vs[22.25 ± 0.30] %, P<0.01).CellsinSphaseweresignificantlyincreasedinDDW-treatedA549 cells compared with those in the control group (P<0.05).The life quality of H460 cell-inoculated nude mice treated with DDW was greatly improved, with the tumorinhibitionratebeing30.08%.Conclusion:DDW can inhibit the prolife ration of lung cancer cells within A certain range of dosage and inafluctuating pattern; its mechanism might be associated with induction of apoptosis and cell cycle arrest in Sphase of tumor cells.

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Water (H2O) is composed of 2 hydrogen atoms and 1 oxygen atom. Hydrogen atoms have 3 nuclides: hydrogen (H) with a mass of 1, also known as "protium", combines with oxygen atoms to form light water (H2O); heavy hydrogen (D) with a mass of 2, also known as deuterium, combines with oxygen elements to form heavy water (D2O); tritium (T) with a mass of 3, also known as tritium, is extremely small and can be ignored. In ordinary water, the ratio of deuterium to protium (D/H) is about 1:6,600, that is, the volume fraction of deuterium in water is 0.015%. Usually, water with a deuterium volume fraction of less than 0.015% is called deuterium-depleted water (DDW). Due to the different masses of deuterium and protium, these two stable nuclides of hydrogen are The difference in physical and chemical properties between [2-3] In living organisms, deuterium the two has been discussed in the early days. The radionuclide effect produced by [4-5] Early research replacing hydrogen has been studied. It has been found that drinking 25% to 30% heavy water can improve the Expect [6] survival of ascites tumors. The mortality rate of mice taking 30% heavy water and receiving 60Co radiation is Compared with the control group, a significant reduction However, high concentrations of in water content significantly reduced the survival time of mice, and even caused the death of mice [10]. It Death . Gross et al. was found that high concentrations of heavy water can cause mitochondria to stagnation of the

splitting process. Since high concentration of heavy water can cause adverse reactions to the human body, its clinical use is limited. So far, there are few studies on deuterium-depleted water. [11]

The Deuterium-depleted water can inhibit the growth of mouse fibroblast L929 cells growth rate of deuterium-depleted water and the regression of tumor tissue in transplanted tumor mice were reduced by 65%. Russian It was found that if the deuterium in ordinary water researchers recently found that the score was reduced by 65%, which showed certain anti-tumor properties, inhibited tumor growth, and prolonged the survival of mice. This study intends to use in vitro cell culture and nude mouse transplanted tumor models to observe the inhibitory effect of deuterium-depleted water on the proliferation of human lung adenocarcinoma cells and the effect on the survival of transplanted tumor nude mice, and explore the possible mechanism of the above-mentioned effects of deuterium-depleted water.

1 Materials and methods

1.1 Reagents and experimental animals

Deuterium-depleted water (volume fractions of 0.0025%, 0.0050%, and 0.0105%) was provided by Shanghai Chitian Ultralight Water Bioengineering Co., Ltd. Fetal bovine serum was purchased from Hangzhou Sijiqing Company, MTT kit was purchased from Nanjing Keygen Biotechnology Co., Ltd., RPMI1640 culture medium was purchased from GIBCO, and ÿ-MEM (containing RNA and DNA) was purchased from Gino. Human lung adenocarcinoma cell lines A549 and H460 and human embryonic lung fibroblast HLF-1 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. TUNEL. Cell in situ detection kit was purchased from Nanjing KeyGen Biotechnology Development Co., Ltd. Flow cytometer (2000FCA) was purchased from the United States

Product of BD.

BALB/c nude mice, male, (20 ± 2) g, purchased from China Science The animals were obtained from Shanghai Slake Laboratory Animal Co., Ltd. [Animal Certificate No. SCXK(ÿ)2003-0003] and maintained in the SPF animal room of the Experimental Center of Shanghai Longhua Hospital. 1.2 Cell culture A549 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum, 5% CO2 , and 37°C. HLF-1 cells were cultured in ÿ-MEM medium containing 10% fetal bovine serum, 5% CO2 , and 37°C. Cells in the DDW experimental group were added with 6 ml of culture medium prepared with deuterium-depleted water (volume fraction of 0.0025%, 0.0050%, and 0.0105%) and cultured in a 37°C incubator. The control group was cells cultured in normal RPMI1640 culture medium.

1.2 MTT assay to detect the proliferation activity of tumor cells MTT kit was used to assay the proliferation of A549 cells.

Cells (1 × 10 cells/well) cells/well), HLF-1 cells (5 × 10

were inoculated into 96-well culture plates, 100 ÿl per well, and 3 replicates were set up. After culturing for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 48, and 72 h, 50 ÿl of MTT solution was added to each well and cultured for 4 h. The culture medium was aspirated, and 150 ÿl of DMSO was added to each well. The cells were shaken for 10 min, and the wavelength of 550 nm was selected to measure the optical density (D) of each well. The results were taken as the average of 3 experiments. The inhibition rate of DDW on cell proliferation was calculated, and the inhibition rate (%) = (1 - D of experimental group/D of control group) × 100%.

1.3 TUNEL method

To detect the apoptosis rate of A549 cells A549 cells cultured with 0.005% deuterium-depleted water for 48 and 72 h were collected, A549 cells cultured with normal culture medium were used as the control group, and A549 cells treated with DNaseÿ reaction solution were used as the positive control. The cells were treated according to the instructions of the kit. Under optical microscopy, brown particles appeared in the nucleus or brown particles appeared in the cytoplasm due to nuclear DNA overflow were positive cells, i.e. apoptotic cells. 200 cells were randomly counted for each slide, and the apoptosis rate was calculated. 1.5 Flow cytometry to detect cell cycle After A549 cells were cultured in 0.005% deuterium-depleted water for 10 and 72 h, they were digested with trypsin and collected. They were fixed with 95% ethanol, washed once with PBS, centrifuged at 1,000 × g for 5 min, and the supernatant was discarded. They were washed once again with PBS and the supernatant was discarded. 1 ml RNase A was added, and the cells were incubated in a 37°C water bath for 10 min, centrifuged at 1,000 × g for 5 min, and the supernatant ÿl PI staining was added and filtered through a 60-mesh nylon mesh at was discarded. 20,000 14°C for 10 min, and the cells were immediately analyzed. 1.6 Establishment and grouping of lung cancer cell transplanted tumor animal model.

The nude mice were randomly divided into 2 groups, 8 in each group. The control group Drink sterilized normal drinking water, 0.005% deuterium-drank DDW freely for 14 days, and depleted water. Adjust the then human lung cancer cells H460 were injected into concentration to 1 × 10 /ml, 0.2 ml (containing 2 × 10 cells The tumor was inoculated by injecting it into the right axilla of nude mice. 1.7 .

Observation on the inhibition of transplanted tumor growth by drinking deuterium-depleted water Nude mice were inoculated with cancer cells and observed until the 60th day after drinking DDW water. The indicators of quality of life of tumor-bearing nude mice included activity level, reaction force, appetite, skin color, etc. After the observation, the tumor-bearing nude mice were killed by pulling the cervical vertebra, and the subcutaneous solid tumor masses were peeled off, the fascia was removed, and the weights were weighed separately using an electronic balance. The tumor weight and tumor inhibition rate of each group were calculated. Tumor inhibition rate (%) = (tumor weight of control group - tumor weight of experimental group) / tumor weight of control group × 100%.

1.8 Statistical analysis SPSS statistical software package was used for data analysis and inter-group comparison Analysis of variance was used, and the differences between the two groups were compared using q test.

2 Results

2.1 Inhibition of A549 cell proliferation by DDW

MTT assay was used to detect the effect of DDW on A549 cell proliferation. The results showed that compared with the control group, the volume fraction of DDW group increased significantly after 10 h of culture. The inhibitory rates of A549 cells in deuterium-depleted water with a volume fraction of 0.0025%, 0.0050% and 0.0105% were 29.61%, 31.07% and 30.10%, respectively, with statistically significant differences ($P<0.05$, Figure 1A, 1B, 1C). The inhibitory effect disappeared over time; after 48 hours, the inhibitory effect began to appear again and lasted until 72 hours; compared with the control group, the inhibitory rates of A549 cells in deuterium-depleted water with a volume fraction of

0.0025%, 0.0050% and 0.0105% were 15.89%, 24.77% and 13.56%, respectively, with statistically significant differences in deuterium-depleted water with a volume fraction of 0.0050% compared with the control group (P<0.05, Figure 1B).

2.2 DDW has no inhibitory effect on the proliferation of human embryonic lung fibroblast HLF-1 cells.

The MTT method was used to detect the effect of DDW on the proliferation of HLF-1 cells. The results showed that in the DDW group, the inhibition rates of 0.0025%, 0.0050% and 0.0105% deuterated water on HLF-1 cells were 2.53%, 6.82% and 8.38% after culturing for 10 h, respectively; the inhibition rates after culturing for 72 h were 4.64%, 4.92% and 4.23%, respectively; compared with the control group, there was no statistically significant difference in proliferation inhibition (P>0.05, Figure 2).

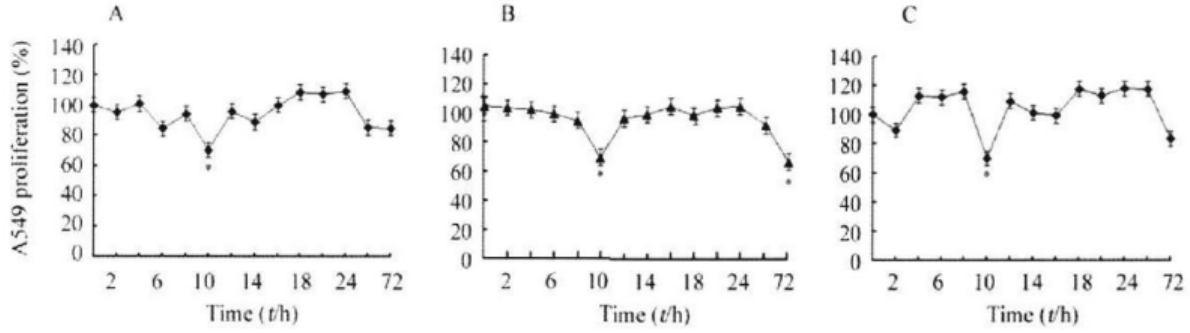

图 1　不同体积分数的低氘水对 A549 细胞增殖的抑制作用

Fig. 1　Inhibitory effect of different volume fractions of DDW on proliferation of A549 cells

A: 0.0025% DDW; B: 0.0050% DDW; C: 0.0105% DDW; * $P<0.05$ *vs* 0 h

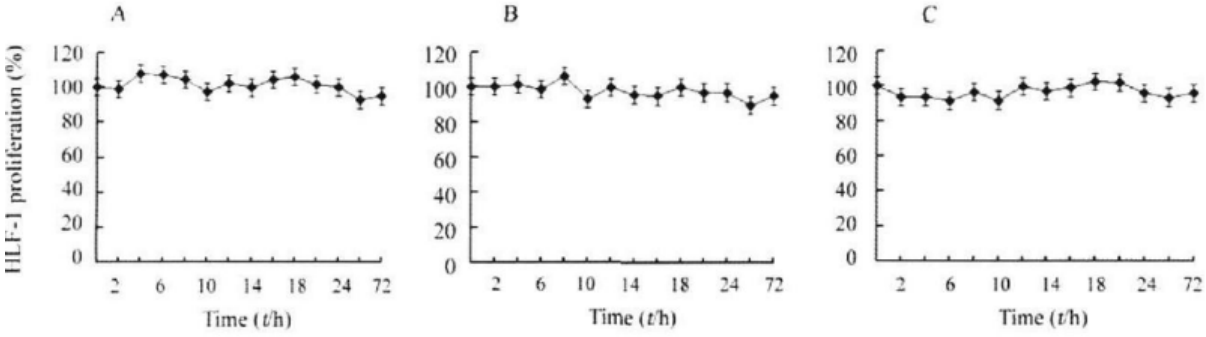

图 2　不同体积分数的低氘水对正常人 HLF-1 细胞增殖的抑制作用

Fig. 2　Inhibitory effect of different volume fractions of DDW on proliferation of normal human HLF-1 cells

A: 0.0025% DDW; B: 0.0050% DDW; C: 0.0105% DDW

2.3 Effect of DDW on apoptosis of A549 cells TUNEL assay showed

that after 0.005% DDW acted on A549 cells for 48 and 72 h, the cells showed typical apoptosis characteristics: The cell volume decreased, and brown particles appeared in the nucleus or in the cytoplasm due to the overflow of nuclear DNA (Figure 3). The apoptosis rate of cells was calculated. The apoptosis rate of cells in the control group was (10.87 ± 1.11)%, and the positive control group was (10.87 ± 1.11)%.

The apoptosis rate of cells in the control group was (46.14 ± 2.82)%, the apoptosis

rate after DDW treatment for 48 h was (31.39 ± 2.54)%, and the apoptosis rate after DDW treatment for 72 h was (25.38 ± 3.90)%. DDW treatment significantly increased the apoptosis rate of A549 cells ($P<0.05$). 2.4 Effect of DDW on the cell cycle of A549 cells The results of flow cytometry showed that the cell cycle of A549 cells changed significantly after DDW induction. Compared with the control group, the number of cells in the G0 /G1 phase gradually decreased, the number of cells in the S phase increased, and the number of cells in the G2 /M phase decreased. After DDW treatment of A549 cells for 10 h, the number of cells in the S phase was (38.47 ± 0.29)%, and after 72 h of treatment, the number of cells in the S phase increased significantly to (44.03 ± 0.35)%, which was statistically significant compared with (32.65 ± 0.78)% in the control group ($P < 0.05$; Figure 4, Table 1).

2.5 Effect of DDW on the quality of life of tumor-bearing nude mice The nude mice in the model group had reduced activity, slow response to external stimuli, reduced daily food intake, liked to gather in groups, and had poor skin gloss.

The nude mice in the DDW group had good activity and were more responsive to external stimuli. The daily food intake was normal and the skin color was ruddy, indicating that the quality of life of nude mice in the DDW group was better than that in the model group.

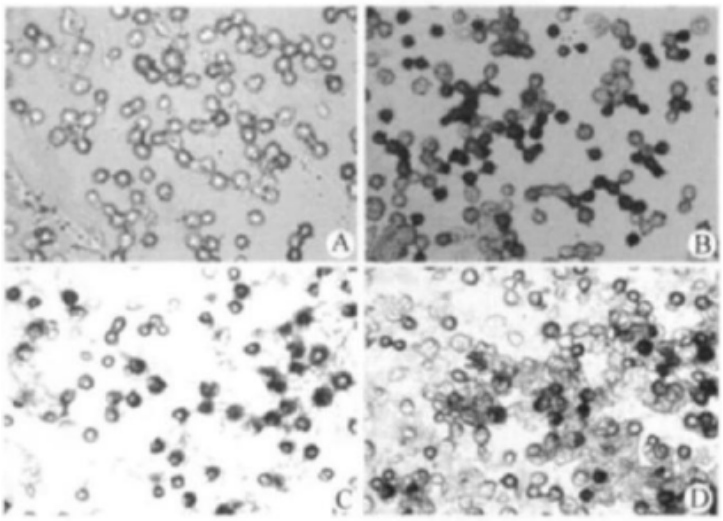

Fig.3 TUNEL assay to detect DDW-induced apoptosis of A549 cells (×400)

A:ControlA549 cells;B:Positive control cells; C:DDW treated for48 h; D:DDW treated for 72 h

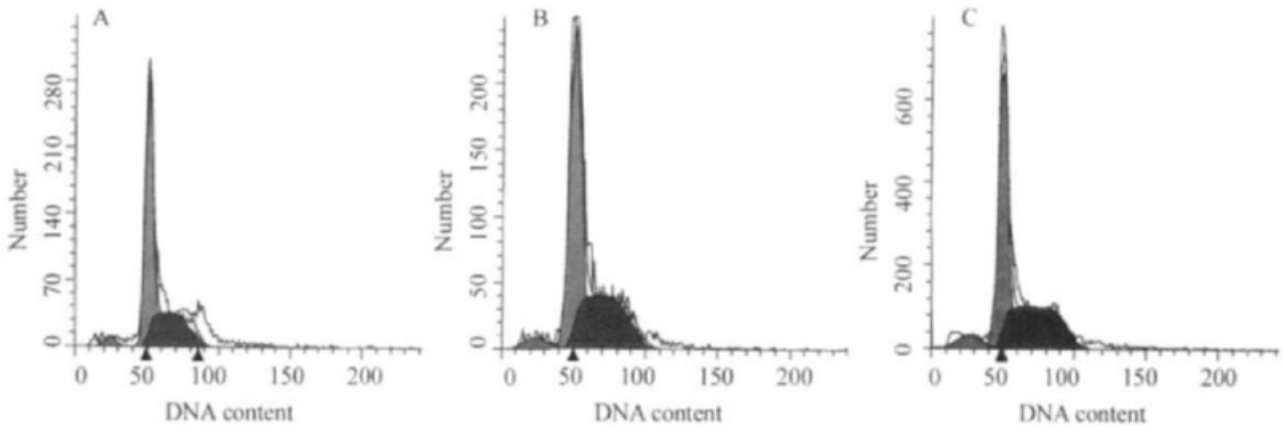

Fig.4 Effect of DDW on cell cycle of A549 cells by FCM

A:Controlgroup;B:treatmentfor10 h;C:72 h

Table 1 Changes in the cell cycle of A549 cells after DDW treatment (x±s)

Time(t/h)	G0-G1	S	G2-M
0	60.11 ±2.15	32.65 ±0.78	7.24 ±1.37
10	58.63 ±2.40	38.47 ±0.29 '	2.90 ±0.08 '
72	55.23 ±1.47	44.03 ±0.35 '	0.75 ±0.01 '

' P<0.05 vs0 h

2.6 Inhibitory effect of DDW on nude mouse transplanted tumors The control group and the treatment group each had 8 nude mice, which were killed after 60 days. The transplanted tumors were removed and weighed. The average tumor weight of the control group was (10.64 ± 0.83).

The average tumor weight of the treatment group was (7.36 ± 0.78) g, and the tumor inhibition rate of the treatment group was 30.08%. The results showed that DDW has the effect of inhibiting the growth of transplanted tumors.

3 Discussion

Lung cancer is one of the most common malignant tumors in the world. The mortality rate of lung cancer ranks first among all cancers and is increasing at a rate of 0.5% per year worldwide. However, the treatment effect is still Although chemotherapy regimens are being optimized unsatisfactory, and the survival time of patients has not been significantly improved. The results of this study [16] show that DDW Therefore, it is necessary to find drugs that can improve the therapeutic effect. [17] has an inhibitory effect on the ÿ proliferation of lung cancer cells. The results of MTT assay show that DDW has an inhibitory effect on the proliferation of A549 cells.

The most obvious inhibitory effect on proliferation occurred 10 hours after the start of cell culture, and DDW with different deuterium volume fractions had a significant inhibitory effect on the proliferation of A549 cells, and then the inhibitory effect disappeared. After 48 hours, the inhibitory phenomenon gradually appeared. At 72 hours, the difference in the inhibitory effect of 0.005% DDW on the proliferation of A549 cells was statistically significant. Under the same conditions, DDW had no significant inhibition on the growth of normal embryonic lung fibroblasts HLF-1 cells. The

results suggest that DDW has certain characteristics in its effect on lung cancer cell proliferation: the effective dose is limited to the volume fraction range of 0.0025% to 0.0105%; the inhibitory effect is volatile and time-dependent, with the peak of inhibition at about 10 hours, and then the effect disappears, and the second inhibition peak appears at 72 hours. In the 1990s, some scholars proposed that the natural abundance of deuterium can be expressed by volume fraction. Initiate and maintain normal cell proliferation. The researchers cultured L929 cells in 0.003% deuterium-depleted water medium and found that deuterium is essential for cell division, and the hysteresis period of cell division is longer than 5 to 10 hours. Plant germination experiments showed that the inhibitory effect of deuterium-depleted water was most obvious 5 to 6 days after the start of plant germination. The effects seen in the early stage were not observed for 10 to 20 days. The researchers also used the fronds of seaweed to observe the effects of low-deuterium water on plant cells. They found that when the fronds were placed in a medium containing low-deuterium water, the respiration, photosynthesis, intracellular and extracellular membrane potentials, and pH changes of plant cells all showed the same situation in the first 30 minutes: the plants showed the physiological state of being placed in the dark, the respiration of the cells became active, photosynthesis stopped, the intracellular pH was alkaline, and the extracellular pH tended to be acidic; these effects appeared within 30 minutes after treatment and then gradually disappeared. These special experimental phenomena are very similar to the characteristics of DDW observed in this study. Facts show that cell division is very sensitive to changes in deuterium concentration, and the presence of naturally abundant deuterium is necessary for cell division. It is speculated that there is probably a mechanism in cells (including plants and animals) to recognize changes in deuterium concentration. The cell cycle regulatory system can recognize changes in the D/H ratio, and when this ratio reaches a certain threshold, it triggers cell division. When cells are cultured in deuterium-depleted water, the low concentration of D content delays the cells from reaching the required D/H threshold, showing a growth inhibitory effect. However, after millions of years of evolution, higher animals and plants have a very complete regulatory system in their bodies. Changes in deuterium concentration will cause cell stress responses, and cells will quickly adapt to the low-deuterium environment through their own regulatory mechanisms. For example, the H on the plasma + -ATPase + membrane of algae cultured , thereby affecting the D/H ratio of the cell and regulating in deuterium-depleted water is activated, and the excretion of H inhibits the growth of cells.

Apoptosis is a form of cell death that occurs by initiating the cell's own internal death mechanism. Many anti-tumor drugs achieve the purpose of treating tumors by inducing cell-specific apoptosis. This study found that A549 cells showed significantThe cell proliferation was blocked in the S phase. The results of TUNEL assay showed that the apoptosis rate of A549 cells increased significantly after DDW treatment for 48 h. This suggests that the apoptosis mechanism is involved in the inhibition of A549 cell proliferation by DDW. DDW may inhibit the malignant growth of cancer cells by inducing cell S phase arrest and tumor cell apoptosis.

The molecular mechanism of DDW promoting apoptosis signal transduction pathways needs further study. Although tumor cells, like normal cells, also show adaptability to changes in deuterium concentration, it is likely that tumor cells have a higher growth rate and need to consume more deuterium. Finally, it was observed that DDW can not only inhibit

tumor cell growth in vitro and induce apoptosis of lung cancer cells, but also inhibit tumor growth in vivo and improve the quality of life of nude mice with transplanted tumors. This provides a new idea for the adjuvant treatment of lung cancer with deuterium-depleted water.

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