Study on the inhibitory effect of deuterium-depleted water on the proliferation and invasion of cervical cancer Hela cells

Sino-US Cancer Institute, Guangdong Medical College, Dongguan 523808 , Guangdong, China

[Abstract] Objective To investigate the effect of decreasing deuterium concentration in culture medium on the proliferation and invasion of cervical cancer Hela cells.Methods Hela cells were cultured in culture medium containing different deuterium concentrations and divided into four groups: 150×10-6 , 100×10-6 , 75×10-6 and 50×10-6 . The culture medium containing 150×10-6 deuterium concentration was the control group, and the others were the experimental groups.The tetramethylthiazolium blue (MTT) method and scratch test were used to detect the changes in proliferation, migration and invasion of Hela cells with decreasing deuterium concentration in culture medium.Flow cytometry was used to detect the effect of culture medium containing different deuterium concentrations on Hela cell cycle.Western blotting and immunohistochemistry were used to detect the effect of culture medium containing different deuterium concentrations on the expression of tumor-related proteins p21 and Na+ /K+ -ATPase in Hela cells . Results The results of MTT experiment showed that the proliferation of Hela cells was significantly inhibited with the decrease of deuterium concentration in the culture medium . The inhibitory effect of the culture medium with a deuterium concentration of 50×10-6 was the most obvious. Under the same conditions, the inhibition rate reached 39.54% at 72 h compared with the control group ($P < 0.01$). The scratch test showed that the migration ability of Hela cells was significantly inhibited in the culture medium with a deuterium concentration of 75×10-6 and 50×10-6 ($P < 0.05$). After 24 h of culture in the culture medium with a deuterium concentration of 75×10-6 and 50× 10-6, the S phase of Hela cells was significantly lower than that of the control group by flow cytometry ($P < 0.01$). The expression of Hela cell tumor-related protein p21 in the culture medium with a deuterium concentration of 100×10-6 , 75×10-6 and 50×10-6 was significantly different from that in the control group ($P < 0.01$). Conclusion The decrease of deuterium concentration in the culture medium has a significant inhibitory effect on the proliferation and invasion of cervical cancer Hela cells.

[Keywords] Deuterium-depleted water; Hela; Proliferation; p21

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Research of inhibition of deuterium -depleted water for cervical cancer Hela cell proliferation and invasiveness ZHANG Li# ZHANG

Hanbin# CHEN Miao WU Yongfu HUANG Haohai ZHU Baohuaÿ

YANG Huilingÿ Sinoÿ America Cancer Research Institute, Guangdong Medical College, Guangdong Province, Dongguan 523808, China [Abstract] Objective To examine the effects of the proliferation and invasiveness in Hela cell lines by the deuterium-depleted water (DDW). Methods The Hela cell lines were cultured in the presence of media containing different con-centrations of deuterium (150×10-6 , 100×10-6 taken as the control group, the other concentrations of deuterium were taken as the experimental group. MTT and wound scratch assay were used , 75×10-6 and 50×10-6), while the 150×10-6 concentration of deuterium was to examine the effects of DDW on the proliferation and migration of Hela cells. More over, the flow cytometry was used to detect the effects of DDW on the cell cycle of Hela cell lines. Finally, the expression levels of p21 and Na+ /K+ -ATPase were assessed by using Western blot and immunohistochemistry. Results The prolif-eration of Hela cells was significantly suppressed when

culturing in three concentrations of DDW (100×10-6 , 75×10-6 and 50×10-6) medium, the inhibition effect was obvious in 50×10-6 concentration of DDW, the 72 h inhibition ratio was 39.54% (P < 0.01). The result of wound scratch assay showed that the invasiveness ability of Hela cells was signifi-cantly decreased in 75×10 -6 and 50×10 -6 concentration of DDW (P < 0.05). Cell cycle analysis revealed that DDW caused cell cycle arrest and decreased the amounts of cells in the S phase after 24 h cultivation in 75×10-6 and 50×10-6 concentration of DDW (P < 0.01). Western blot suggested that compared with the control group, DDW (100×10-6 , 75×10-6 and 50×10-6) could up-regulate the expression of p21 (P < 0.01). Conclusion The decreased of deuterium concentration in the medium can significant inhibit the proliferation and invasiveness of Hela cells.

[Key words] Deuterium-depleted water; Hela cells; Proliferation; p21 protein

Cervical cancer is the third most common malignant tumor in women worldwide and the fourth leading cause of cancer death in women. It accounts for nearly 10% of newly diagnosed cancer cases and 8% of total cancer deaths [1]. The global incidence of cervical cancer was approximately 378,000 cases per year in 1980 , and by 2010 it had increased to approximately 454,000 cases per year [2]. It is estimated that there are approximately 100,000 new cases of cervical cancer in China each year , accounting for 1/5 of the total number of new cases of cervical cancer in the world [3]. Currently, the main treatments for cervical cancer are surgery, radiotherapy, and chemotherapy.

In nature, water is a compound composed of two non-radioactive stable isotopes of hydrogen, protium (H) and deuterium (D), and oxygen. Water composed of deuterium and oxygen is called heavy water. In natural water, the ratio of deuterium to protium (D:H) is about 1:6600 , that is, the volume fraction of deuterium in natural water is 0.0139%~0.0151%[4-8]. Therefore, water with a deuterium volume fraction of less than 0.015% is called deuterium-depleted water (DDW). Since the mass difference between deuterium and protium is about 1 times and the CD bond is stronger than the CH bond and is not easy to break, changes in the content of isotopes of protium and deuterium in water will lead to significant differences in the physical, chemical and nuclear properties of water. Studies have shown that deuterium can replace protium in organisms and accumulate. As the deuterium concentration in organisms increases, the cross-linking between hydrogen bonds changes, which significantly increases the rigidity of the cytoplasm and blocks mitosis[9-12]. Drinking DDW can prevent diseases, delay aging, and activate the body's immune cells [13-14]. In recent years, foreign nuclear medicine and water physiology have made major breakthroughs in the application of DDW in the adjuvant treatment of certain cancers and other diseases [15-18]. This article will compare the effects of various culture media containing different concentrations of deuterium on the growth of Hela cell lines. 1 Materials and methods 1.1 Reagents and materials DDW (volume fraction 0.005%, 50×10-6) was purchased from Shanghai Aotequan Company; fetal bovine serum (FBS) was purchased from Gibco Company; cervical cancer cell line Hela was purchased from ATCC, USA, and maintained by the Sino-US Cancer Institute; DMEM and RPMI 1640 culture media were purchased from Gibco Company; PI dye was purchased from Beijing Solebao Company; ribonuclease A (RNaseA) was purchased from Invitrogen; MTT kit was purchased from Beijing Solebao Company ; p21 Waf1/Cip1 (12D1) Rabbit mAb was purchased from Cell Signaling; ÿ -actin (13E5) Rabbit mAb was purchased from Cell Signaling; Alexa Flour 800 goat anti-rabbit lgG (H+L) was purchased from Invitrogen; Na+ /K+ -ATPase ÿ (H-3) mouse

monoclonal lgG2b was purchased from Santa Cruz; immunohistochemical reagents UltraVision Quanto Detection System HRP Quanto & DAB Quanto were purchased from Thermo Scientific; other reagents were of analytical grade. 1.2 Cell culture and experimental grouping Cervical cancer Hela cells were cultured in 1640 medium containing 10% FBS and placed in a 37 °C incubator containing 5% (volume fraction) CO2 . DDW (100×10-6 , 75×10-6 , 50×10-6 , experimental group) and ordinary ultrapure water (150×10-6, control group) were respectively prepared into 1640 medium for Hela culture according to the volume ratio.

1.3 MTT assay to detect the effect of DDW on cell proliferation activity

After starvation culture of Hela cells with serum-free 1640 medium for 24 h , the cells were digested with 0.25% trypsin containing ethylenediaminetetraacetic acid (EDTA), counted, and seeded in a 96 -well culture plate at a density of 5×103 cells/well . 100 ÿL of ordinary 1640 medium containing 10% FBS (150×10-6 , control group) or 1640 medium containing different deuterium concentrations (100×10-6 , 75×10-6 , 50×10-6) (experimental group) was added to each well. Three replicate wells were set up in a 37°C, 5% (volume fraction) CO2 incubator for 24, 48, and 72 h. After that, 20 ÿL of MTT solution was added to each well and cultured for 4 h. Add 100 ÿL of dimethyl sulfoxide (DMSO) to each well, shake for 10 min, select ÿ 490 nm wavelength, and measure the optical density (OD) value of each well. The results are taken as the average of 3 experiments. Calculate the inhibition rate of cell proliferation by DDW . Inhibition rate = (1- OD of experimental group / OD of control group) × 100%. 1.4 Cell scratch

test Collect cells according to the method under "1.3" and add them to 6- well plates, with a density of 6×105 cells/well. Use 10% FBS ordinary 1640 medium (150×10-6 , control group) and 1640 medium containing different deuterium concentrations (100×10-6 , 75×10-6 , 50×10-6) (experimental group) for 12 h. After the cells are completely attached to the wall, use the 10 ÿL sterile pipette tip of the pipette to lightly draw a straight line in each well, measure the width of the line at the same magnification and record it as the 0 h data. After 48 h , the cells were taken out of the incubator and placed under a microscope to observe the changes in the width of the drawn lines, and photos were taken. The width of the lines was measured at the same magnification and recorded as 24 h data. The difference between the 0 h data and the 24 h data of the experimental groups (100×10-6 , 75×10-6 , 50×10-6) was compared with the difference between the 0 h data and the 24 h data of the control group (150×10-6) . Whether the difference in the effect of DDW on the invasion ability of cells was statistically significant was analyzed.

1.5 Flow cytometry detection of cell cycle Hela cells were collected according to the method in experiment

“1.3” and seeded in a culture dish with a diameter of 6 cm at a density of 1×105 cells /mL . Ordinary 1640 culture medium containing 10% FBS (150×10-6 , control group) or 1640 culture medium with different deuterium concentrations (100×10-6 , 75×10-6 , 50×10-6) (experimental group) were added and cultured for 24 h. The cells were digested and collected with 0.25% EDTA -free trypsin. Fix with 70% ethanol, incubate at 4°C overnight, wash once with PBS , centrifuge at 1000 r/min for 5 min, discard the supernatant; wash once more with PBS, centrifuge at 1000 r/min for 5 min, discard the supernatant; add 500 ÿL staining solution (500 ÿL PBS+2.5 ÿL PI+2 ÿL RNaseA) to each tube, stain in a 37°C water bath in the dark for 30 min, filter through a 60 -mesh nylon sieve, and analyze immediately with a flow cytometer. Count

1×104 cells and observe the distribution characteristics of each phase of the cell cycle.

1.6 Detection of p21 protein expression level in Hela cells by Western blot Hela cells were collected and counted according to the method in experiment “1.3” , and seeded in a 6 cm diameter culture dish at a density of 1×105 cells/mL. Ordinary 1640 culture medium containing 10% FBS (150×10-6 control group) or 1640 culture medium containing different deuterium ÿ concentrations (100×10-6 , 75×10-6 , 50×10-6) (experimental group) was added for 24 h, the culture medium was discarded, the cells were washed three times with PBS , 200 ÿL of cell lysis buffer RIPA was added for lysis, and 20 ÿL of mixed protease inhibitors were added. The cells were placed on ice for 30 min to allow for full lysis, and the cells were collected with a cell scraper. The cells were centrifuged at 4°C and 10,000 r/min for 15 min, and the supernatant was aspirated and stored in a -80°C device for later use. The protein content was determined by NanoDrop micro-volume UV spectrophotometer. The total amount of protein loaded in each well was the same, 100 ÿg. The electrophoresis stacking gel was 80 V for 50 min and the separation gel was 110 V. 150 min. After 10% denaturing polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 110 V 500 mA for 120 min. The membranes were blocked with PBST (400 mL PBS + 40 ÿL Tween 20) containing 2.5% skim milk powder at room temperature for 60 min, and rabbit anti- p21 primary antibody (1:3000) and rabbit anti -ÿ-actin primary antibody (1:3000) were added according to the antibody requirements . The membranes were incubated at 4°C overnight, renatured at room temperature for 1 h, and washed with PBST 4 times, 10 min/time , and the corresponding Alexa Flour 800 goat anti-rabbit lgG (H+L) secondary antibody was added at a certain ratio (1:10000), and incubated at room temperature in the dark for 120 min. Unbound secondary antibodies were washed away with PBST 3 times, 10 min/time, and developed with Western blot infrared fluorescence imaging. 1.7 Immunohistochemical LP method to detect changes in p21 and Na+ /K+ -ATPase protein expression

After digesting Hela cells according to the method in experiment "1.3" , 6×105 cells were added to each well of a 6- well plate in parallel. After culturing for 24 h with ordinary 1640 medium containing 10% FBS (150×10-6) and 1640 medium with low deuterium concentration (50×10-6) , the medium was removed , endogenous peroxidase was blocked with 3% H2O2 , and 10% normal goat serum was incubated. The primary antibody was added and incubated overnight at 4°C . After washing with PBS buffer (Na2HPO4 8.1 mmol/L, KH2PO4 1.5 mmol/L, NaCl 137 mmol/L, 2.7 mmol/L, pH 7.4), the secondary and tertiary antibodies were added respectively . DAB was used for color development and hematoxylin was used for counterstaining. The positive signal appeared as brown fine particles. 1.8 Statistical methods SPSS 17.0 statistical software was used for data analysis. The quantitative data were expressed as mean ± standard deviation (x ± s). One-way analysis of variance was used for comparison among multiple groups. LSD-t test was used for comparison between two groups. $P < 0.05$ was considered statistically significant. 2 Results 2.1 Effect of deuterium-depleted water on the proliferation of Hela cells The inhibition rates of Hela cells cultured in medium containing deuterium concentrations of 100 × 10-6 , 75 × 10-6 , and 50 × 10-6 for 24 h were 16.01%, 26.17% , and 33.39%, respectively; the inhibition rates for 48 h were 7.98%, 25.27%, and 34.5%, respectively ; and the inhibition rates for 72 h were 11.42%, 21.46%, and 39.54%, respectively. There were statistically

significant differences between the experimental groups and the control group ($P < 0.05$ or $P < 0.01$). See Table 1.

Table 1 Effect of deuterium-depleted water on Hela cell proliferation (x±s, n = 6)

Deuterium concentration of groups 24 h		48 h	72 h
Control group	150×10-6	0.5120±0.0755	0.7260±0.0660	0.8380±0.0481
Experimental group	100×10-6	0.4300±0.0496*	0.6680±0.0463*	0.7423±0.0582**
	75×10-6	0.3780±0.0380**	0.5425±0.0692**	0.6581±0.0733**
	50×10-6	0.3410±0.0451**	0.4755±0.0460**	0.5066±0.0903**

Note: Compared with the control group, * P < 0.05, ** P < 0.01

2.2 Effects of deuterium-depleted water on the migration and invasion ability of Hela cells

Culture medium containing 100×10-6 , 75×10-6 , and 50×10-6 DDW (experimental group) for 48 h inhibited the migration and invasion ability of Hela cells Compared with the control group (150×10-6), the inhibitory effects of 75×10-6 and 50×10-6 DDW were more obvious ($P < 0.01$). See Figure 1.

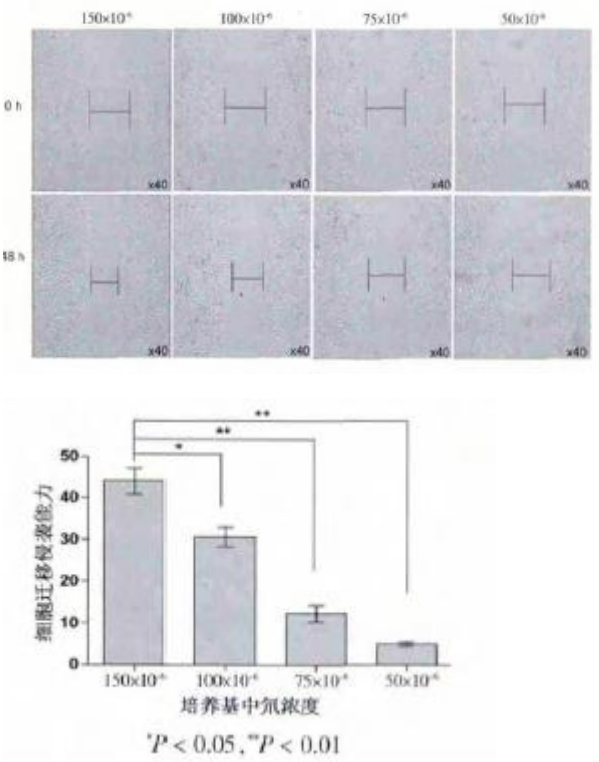


Figure 1 Effects of deuterium-depleted water on cell migration and invasion

2.3 Effect of deuterium-depleted water on Hela cell cycle

As can be seen from Figure 2 , DDW has an inhibitory effect on the cell cycle of cervical cancer Hela cells. As the deuterium concentration in the culture medium decreases, Hela cells mainly show quiescence or slight increase in G1 and G2 phases, and a gradual decrease in S phase. Among them, the inhibitory effect is more obvious when the deuterium concentration is 75 and 50 ppm , and the S phase is significantly reduced ($P < 0.01$), see Table 2.

Table 2 Hela cell cycle in each group (%, x±s, n = 3)

Group	Deuterium concentration	G1 phase	S phase	G2 phase
control group	150×10-6	48.3±0.0273	44.9±0.0364	6.7±0.0412
experimental group	100×10-6	56.0±0.0311	30.5±0.0956*	13.5±0.0271
	75×10-6	57.4±0.0378	29.7±0.0745**	12.5±0.0525
	50×10-6	58.8±0.0554	16.8±0.0632**	24.5±0.0743

Note: Compared with the control group, * P < 0.05, ** P < 0.01

2.4 Effect of deuterium-depleted water on the expression of p21 protein

After Hela cells were treated with 1640 medium containing deuterium concentrations of 100×10-6 , 75×10-6 , and 50×10-6 (experimental group) and normal 1640 medium containing deuterium concentrations of 150×10-6 (control group) for 24 h , Western blot analysis showed that DDW could significantly upregulate the expression of p21 protein in cervical cancer Hela cells (Figure 3). The grayscale values of p21 protein in Hela cells in medium containing deuterium concentrations of 100, 75, and 50 ppm (experimental group) were (0.717±0.037), (0.979±0.082), and (1.146±0.087), respectively, which were significantly different from those in the control group (0.539±0.042) (P ÿ 0.05).

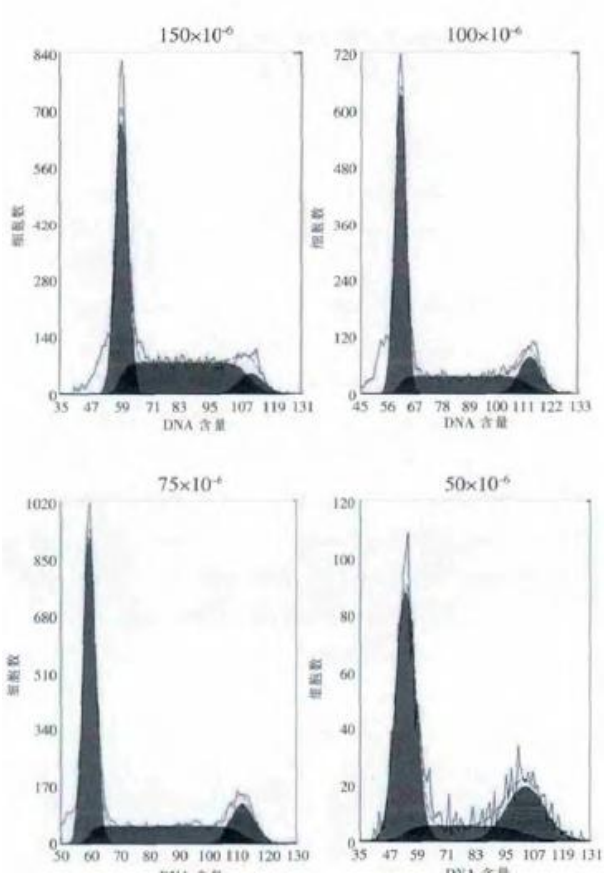
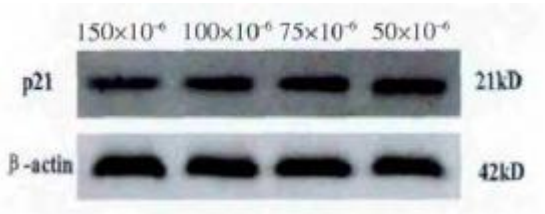
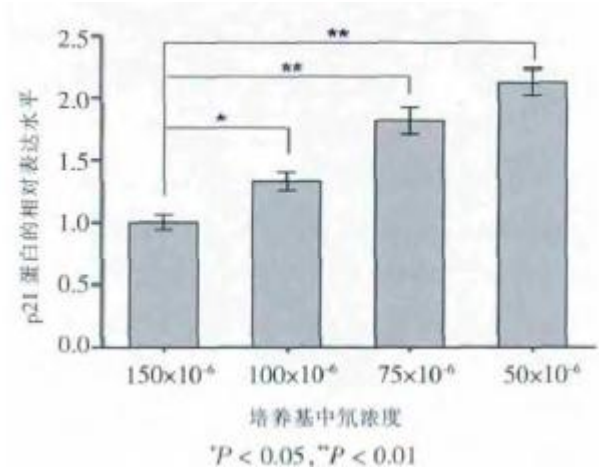


Figure 2 Effects of deuterium-depleted water on Hela cell cycle

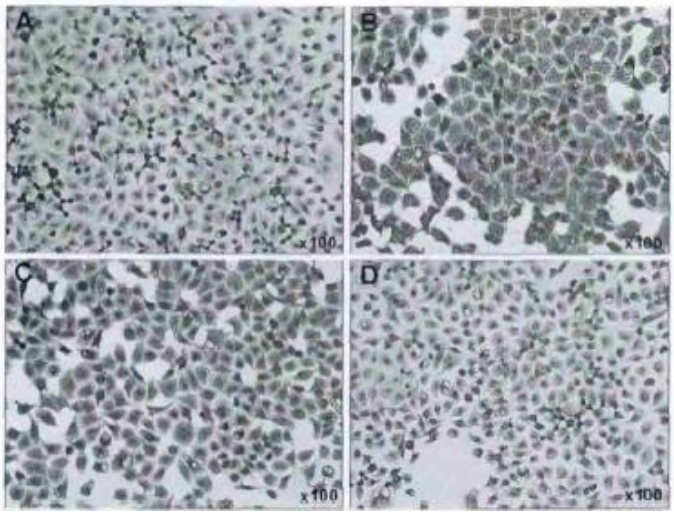




3 Expression of p21 protein detected by Western blot

2.5 Effects of deuterium-depleted water on the expression of p21 and Na+ /K+ -ATPase proteins

After Hela cells were treated with ordinary 1640 medium with a deuterium concentration of 150×10-6 and 1640 medium with a deuterium concentration of 50 × 10-6 for 24 h , the expression of p21 and Na+ /K+ -ATPase proteins was detected by immunohistochemistry . As shown in Figure 4 , after staining, it can be found that the expression of p21 protein in the 1640 medium with a deuterium concentration of 50 ×10-6 is significantly higher than that in the



A: 150×10-6 culture medium treatment, incubation with p21 antibody; B: 50×10-6 culture medium treatment, incubation with p21 antibody; C: 150×10-6 culture medium treatment, incubation with Na+ /K+ -ATPase antibody; D: 50×10-6 culture medium treatment, incubation with Na+ /K+ -ATPase antibody

Fig.4 Immunohistochemical LP method to detect the expression of p21 and Na+ / K + -ATPase protein

The deuterium concentration was 150×10-6 in normal 1640 culture medium; while the expression of Na+ /K+ -ATPase in 1640 culture medium with a deuterium concentration of 50×10-6 was significantly

lower than that in normal 1640 culture medium with a deuterium concentration of 150×10-6 . 3 Discussion In previous studies, it was found that DDW can inhibit the growth of lung cancer, nasopharyngeal carcinoma and liver cancer cells and has a positive effect on prolonging the survival of cancer patients [19-23]. However, there are no reports on the effect of low-deuterium water on cervical cancer. In this study, Hela cells were treated with culture medium with different DDW concentrations. The MTT method and scratch test showed that as the deuterium concentration in the culture medium decreased, the proliferation and invasion and metastasis ability of Hela cells were significantly inhibited. In the culture medium with a deuterium concentration of 50×10-6 , the proliferation inhibition rate reached 39.54% ($P < 0.01$) after 72 hours . The scratch test found that DDW had an inhibitory effect on the invasive ability of cervical cancer cells, and the difference was most obvious at 50×10-6 ($P < 0.01$). Western lot and immunohistochemistry were used to detect the expression of related proteins. It was found that DDW could selectively upregulate the expression level of p21 protein in Hela cells and downregulate the expression of Na+ /K+ -ATPase . The p21waf gene is one of the important downstream genes of the p53 gene. Its expression product, p21 protein, is the cell cycle inhibitory protein with the most extensive kinase inhibitory activity known to date. p21 and p53 can together form the G1 checkpoint of the cell cycle. Since DNA damage cannot pass through the G1 checkpoint without repair , the replication and accumulation of damaged DNA are reduced , thereby playing a tumor suppressor role. Sultana et al. [24] found that the sensitivity of cervical cancer patients to chemotherapy is mediated by the p53-Bax regulatory pathway, which induces cell apoptosis; early chemotherapy can promote the production of interferon ÿ by CD8+ and CD4+ positive T cells and natural killer cells in cervical cancer patients , which works through the immune system. Gyngyi et al. [25] confirmed that DDW can reduce the expression of C-myc, Ha-ras and p53 genes in rats treated with carcinogens . In this study, flow cytometry was used to detect the effects of culture medium containing different deuterium concentrations on Hela cells.

The effect on the cell cycle. As the deuterium concentration of DDW decreases, the G1 and G2 phases gradually increase, and the S phase gradually decreases. This is probably one of the molecular mechanisms by which DDW selectively inhibits the proliferation and metastasis of Hela cervical cancer cell lines, that is, through the mediation of p53 , it effectively upregulates p21 to affect the cell cycle progression and apoptosis, thereby achieving an inhibitory effect on tumor cells. Na+ /K+ -ATPase is one of the material bases for abnormal proliferation of tumor tissues. Compared with normal cells, the Na+ /K+ -ATPase activity in tumor cells is significantly increased. Studies by Li Chunxiao et al. [26] and Usta et al. [27] have shown that inhibiting the activity of Na+ /K+ -ATPase can be used as a new effective means of treating breast cancer and liver cancer. Western blot and immunohistochemistry results in this study showed that the Na+ /K+ -ATPase protein in Hela cells treated with DDW was significantly downregulated. These results indicate that reducing the volume concentration of deuterium in the cell growth environment can inhibit the proliferation and metastasis of Hela cervical cancer cell lines, and DDW has a selective anti-tumor targeted biological effect. Human papillomavirus (HPV) and smoking are the two major risk factors for cervical cancer . HPV infection is the most important factor, and almost all cervical cancers are related to HPV infection [28]. The main treatments for cervical cancer are surgery, radiotherapy, and chemotherapy. Surgical treatment is

mainly suitable for patients with early cervical cancer (stage IA to IA). Although it is traditionally believed that cervical cancer is insensitive to chemotherapy drugs and the treatment methods are mainly surgery and radiotherapy, a large number of tumor cytology research progress and clinical practice have confirmed that surgery and radiotherapy cannot completely control and eliminate subclinical and micro-metastases. Therefore, chemotherapy, as one of the effective methods for treating cancer, has received increasing attention in the comprehensive treatment of cervical cancer. In recent years, studies have shown that the growth mechanism of cancer cells is closely related to deuterium atoms. By reducing the concentration of deuterium in the body, the growth of cancer cells in the body can be treatment as a new type of non-toxic anti-cancer adjuvant therapeutic agent.

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