Chinese Journal of Applied Physiology, 2017, 33(1)

In vitro experiment on the effect of low deuterium environment on the growth of gastric cancer cells*

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ÿAbstractÿ Objective: To study the effect of low deuterium environment on the proliferation of human gastric cancer cells (SGC-7901) and to preliminarily explore its related mechanisms. Methods: Gastric cancer cells SGC-7901 were cultured in RPMI-1640 medium prepared with distilled water containing different deuterium concentrations (experimental group: 25 ppm; control group: 150 ppm). The proliferation rate, cell cycle and apoptosis of the two groups of cells were detected at different time points, and the expression of proliferating cell nuclear antigen protein (PCNA) in the two groups of cells was detected by Western blot. Results: The proliferation rate of SGC-7901 cells in the low deuterium environment was about 10% lower than that in the control group. The low deuterium environment also had a significant inhibitory effect on the scratch healing ability and colony formation ability of the cells (P ÿ 0.05). Flow cytometry results showed that compared with the control group, the proportion of cells in the G1 phase of the low-deuterium group increased (P < 0.01), while the proportion of cells in the S phase decreased (P < 0.05). There was no significant difference in the ratio of early apoptosis and late apoptosis between the two groups. Western blot results showed that the expression of PCNA in gastric cancer cells cultured in a low-deuterium environment was significantly decreased. Conclusion: Low-deuterium environment can inhibit the growth of gastric cancer cells, which may be related to the arrest of gastric cancer cells in the G1 phase and the downregulation of their PCNA expression in a low-deuterium environment. [Keywords] Low-deuterium environment; Gastric cancer; Proliferation; PCNA; Cell cycle; Apoptosis [Chinese Library Classification Number] R453.9 ÿArticle numberÿ1000-6834(2017) 01-001-05

[Document Identification Code] A [DOI] 10.12047/j.cjap.5448.2017.001

Deuterium depleted environment inhibits the growth of gastric cancer cells: in vitro study

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【ABSTRACT】Objective: To examine whether deuterium depleted environment may affect the biological features of human gastric cancer cells(SGC-7901) and explore the possible underlying mechanisms. Methods: SGC-7901 cells were cultured in RPMI-1640 me-dium prepared with distilled

water of different deuterium concentrations(experimental group: 25ppm deuterium ; control group: 150ppm deuterium) . Assays on cellular proliferation, cell cycle and apoptosis were conducted at different time points and comparison. The pro-tein expression of proliferating cell nuclear antigen (PCNA) was measured using Western blot. Results: In contrast to 150ppm group, the proliferation rate of SGC-7901 cells in 25ppm deuterium was decreased by 10% as indicated by the CCK-8 assay. Wound healing a-bility and the colony formation ability of these cells were also significantly suppressed (P ＜ 0. 05) . Flow cytometry analysis further re-vealed that exposure to 25ppm significantly increased the ratio of cancer cells at G1 phase (P ＜ 0. 01) while decreased the ratio at S phase (P ＜ 0. 05) compared to the 150ppm group. There was no significant difference in apoptosis between the two groups. Down-regulated expression of PCNA was also identified in cancer cells treated with 25ppm deuterium. Conclusion: Deuterium depleted environment inhibited the proliferation of gastric cancer cells, which may be attributed to the down-regulation of PCNA and cell cycle arrest at G1 phase.

【KEY WORDS】 deuterium depleted environment; gastric cancer; proliferation; PCNA; cell cycle; apoptosis

Gastric cancer is one of the most common malignant tumors in China. According to relevant survey data in 2008, its incidence and mortality rate ranked third and fourth among malignant tumors [1, 2]. At present, in addition to conventional treatments, people are constantly exploring new methods to treat gastric cancer. Deuterium is a stable isotope of hydrogen that exists in nature. The deuterium/hydrogen (D/H) ratio is about 1:6600. The deuterium content in natural water is about 0.0150% (150 ppm). Water with a deuterium content below 150 ppm is usually called deuterium-depleted water (DDW) [3, 4]. Relevant studies have shown that the content of deuterium in mouse tissues and plasma is unevenly distributed (more in plasma than in tissues) [5]. The deuterium content in drinking water has an important impact on the growth and development of organisms. Deuterium can maintain the growth of normal cells, while a low-deuterium environment has an inhibitory effect on the synthesis of tumor cell proteins and nucleic acids, and cell division.

[6, 7]. With the gradual deepening of deuterium research, the adverse effects of high concentrations of deuterium on organisms have also been gradually reported. It has been found that reducing the deuterium content in natural water by 65% has the effect of inhibiting the growth of various tumor cells [8]. Subsequently, related clinical trials have also proved that the combined use of deuterium-depleted water on the basis of conventional anticancer drugs can effectively prolong the survival of patients with lung cancer, breast cancer, and prostate cancer [9-11]. However, there are few reports on the effect of low-deuterium environment on the proliferation of gastric cancer cells. Therefore, this study preliminarily explores the effect of low-deuterium environment on the proliferation of human gastric cancer cells (SGC-7901) and its related mechanisms.

1 Materials and methods

1.1 Materials and Grouping

Deuterium-depleted water was prepared from tap water by distillation [11]. The deuterium content was adjusted to 25 ppm (experimental group) and 150 ppm (control group), but the concentration of 18 O in the water was kept constant (0.198%). SGC-7901 cells were provided by the Department of Gastroenterology, General Hospital of PLA, RPMI-1640 medium was purchased from Gibco, and mouse anti-human PCNA

monoclonal antibody was purchased from Abacm. 1.2 Cell proliferation assay SGC-7901 cells in the logarithmic growth phase were seeded in 96-well plates at a concentration of 1 × 103 cells/100 ÿl, with 5 replicate wells in each group. The cells were added to RPMI-1640 medium prepared with 50 ppm, 100 ppm, and 150 ppm DDW, respectively, and placed in an incubator at 37°C and 5% CO2 for 48 h. The 96-well plates were taken out, 10 ÿl of CCK-8 reaction solution was added to each well, and incubated at 37°C in the dark for 1 h. The OD value of each group was detected at a wavelength of 450 nm using an enzyme reader. According to the above preliminary experimental results, there was no statistically significant difference in the OD450 values of cells cultured in deuterium environments of different concentrations at 48 h (50 ppm, 0.439 ± 0.013; 100 ppm, 0.433 ± 0.016; 150 ppm, 0.435 ± 0.013; n = 5). Therefore, this experiment used 25 ppm and 150 ppm as the deuterium concentrations used in the experimental group and control group for subsequent experiments. The cells were cultured in RPMI-1640 medium prepared with 25 ppm DDW and 150 ppm DDW for 24 h, 48 h, 72 h, and 96 h, and the OD values of each group were detected at a wavelength of 450 nm using an enzyme marker. 1. 3 Cell scratch assay SGC-7901 cells in the logarithmic growth phase were seeded in 6-well plates at a concentration of 5 × 105 cells/ml and cultured conventionally until the cells reached 95% to 100% confluence. Use a 10 ÿl pipette tip to scratch in a straight line perpendicular to the bottom of the 6-well plate. The cells were then washed 3 times with PBS to remove the scratched cells. RPMI-1640 culture medium prepared with 25 ppm DDW and 150 ppm DDW were added to the experimental group and the control group, respectively, and cultured in an incubator at 37°C and 5% CO2. The cell scratch assay was used to detect the migration ability of the two groups of gastric cancer cells. Pictures were taken at 0 h, 24 h, and 48 h of culture, and the width of the scratches of the two groups of cells at different time points were measured with the same ruler. The scratch width at 0 h was set as 1, and the width at 48 h was set as 2.

The relative width of the scratch at 24 h and 48 h was calculated. 1. 4 Colony formation assay SGC-7901 cells in the logarithmic growth phase were inoculated at 500 cells/well in 6-well plates, with 3 replicate wells in each group. The experimental group and the control group were added with RPMI-1640 medium prepared with 25 ppm DDW and 150 ppm DDW, respectively, and placed in an incubator for 14 days, until colonies were formed visible to the naked eye. 2 ml of 4% paraformaldehyde fixative was added to each well and fixed at 4°C for 20 min. The fixative was discarded, crystal violet stain was added for 30 min, the floating color was washed off with deionized water, and the cells were dried in the air. Pictures were taken under a microscope and the number of colonies in each well was statistically analyzed. 1. 5 Cell cycle detection The experimental group and control group cells were cultured with RPMI-1640 medium prepared with 25 ppm DDW and 150 ppm DDW for 48 h, respectively. The cells of the two groups were collected and their concentration was adjusted to 2 × 106 cells/ml. The cells were fixed with 70% ethanol at 4ÿ for 2 h. The cells were centrifuged at 1 000 r/min for 5 min and washed twice with PBS. The supernatant was discarded and 1 ml RNAse solution was added, which was placed at 37ÿ for 30 min. After the solution was cooled to room temperature, 0.1 ml PI staining solution was added to each centrifuge tube, stored in the dark and detected by flow cytometry. 1. 6 Cell apoptosis detection The cells in the experimental group and control group were cultured with RPMI-1640 medium prepared

with 25 ppm DDW and 150 ppm DDW for 48 h, respectively. The culture supernatant and cells of each group were collected on ice, centrifuged at 2 000 r/min for 3 min at 4°C using a low-temperature centrifuge, and the cells were washed once with 4°C PBS. The cells were washed with binding buffer and the concentration was adjusted to (1-5) × 106 cells/ml. 100 ÿl of cell suspension was taken and 5 ÿl of Annexin V fluorescent dye was added, and incubated at room temperature for 10-15 min. The cells were washed with binding buffer and resuspended with 200 ÿl of binding buffer. 5 ÿl of PI staining solution was added, stored at 4°C in the dark, and detected by the machine.

1.7 PCNA protein detection SGC-7901 cells in the logarithmic growth phase were inoculated in a 6 cm cell culture dish at a density of 3 × 105 cells/ml. The experimental group and control group cells were cultured in RPMI-1640 medium prepared with 25 ppm DDW and 150 ppm DDW, respectively, for 48 h. The cells were collected and lysed with RIPA lysis buffer. The cells were centrifuged at 12 000 r/min for 10 min at 4°C. The supernatant was taken and the protein was quantified by BCA method. 100 ÿg protein was loaded per well, and the concentrated gel was run at 60 V for 40 min and the separation gel was run at 100 V for 90 min until the blue band reached the bottom of the gel. After protein electrophoresis separation, the membrane was transferred to 100 V for 2 h. The membrane was blocked with 10% skim milk powder (TTBS) at room temperature for 2 h and the membrane was washed twice for 5 min each time. Add PCNA primary antibody (1: 500) and ÿ-actin primary antibody (1: 1000) and incubate at 4ÿ overnight. Wash the membrane with TTBS 3 times, 10 min each time. Add secondary antibody (1: 1000) of appropriate dilution and incubate at room temperature for 2 h. Discard unbound secondary antibody and wash the membrane with TTBS 3 times, 10 min each time. Finally, develop in a dark room.

1.8 Statistical analysis

The experimental data are expressed in the form of mean ± standard deviation (x±s). SPSS 13.0 was used to analyze the data using Student's t test or

Two-way analysis of variance (ANOVA) test, using

GraphPad Prism 5.0 was used to analyze and process the data.

2 Results

2.1 Effect of low-deuterium environment on the proliferation of gastric cancer cells CCK-8 assay to detect the effect of low-deuterium environment on the proliferation of human gastric cancer cells

Compared with the 150 ppm group, the 25 ppm deuterium group The OD450 value of cells decreased significantly at 48 h . Cultured in 25 ppm and 150 ppm deuterium concentration environment for 24 h, 48 h, Effect of 25 ppm deuterium environment on the proliferation of gastric cancer cells at 72 h and 96 h The inhibition rate was significantly different from that of the control group ($P < 0.05$, P ÿ 0.01, Table 1).

2.2 Effect of low-deuterium environment on the migration ability of gastric cancer cells

The width of cell scratches in the 25 ppm group at 24 h and 48 h were 0.790 ± 0.017, 0.569 ± 0.047, compared with the 150 ppm group (0.607 ± 0.022, 0.419 ± 0.034), the difference was statistically significant Significance (P ÿ 0.01, Figure 1)

Tab. 1 Cell proliferation was inhibited in deuterium depleted environment ($\bar{x} \pm s$, $n = 3$)

Group	OD_{450}			
	24 h	48 h	72 h	96 h
150 ppm	0.309 ± 0.009	0.312 ± 0.007	0.552 ± 0.008	0.915 ± 0.050
25 ppm	0.270 ± 0.016**	0.282 ± 0.010**	0.525 ± 0.008**	0.824 ± 0.039*

*$P < 0.05$, **$P < 0.01$ *vs* 150 ppm group

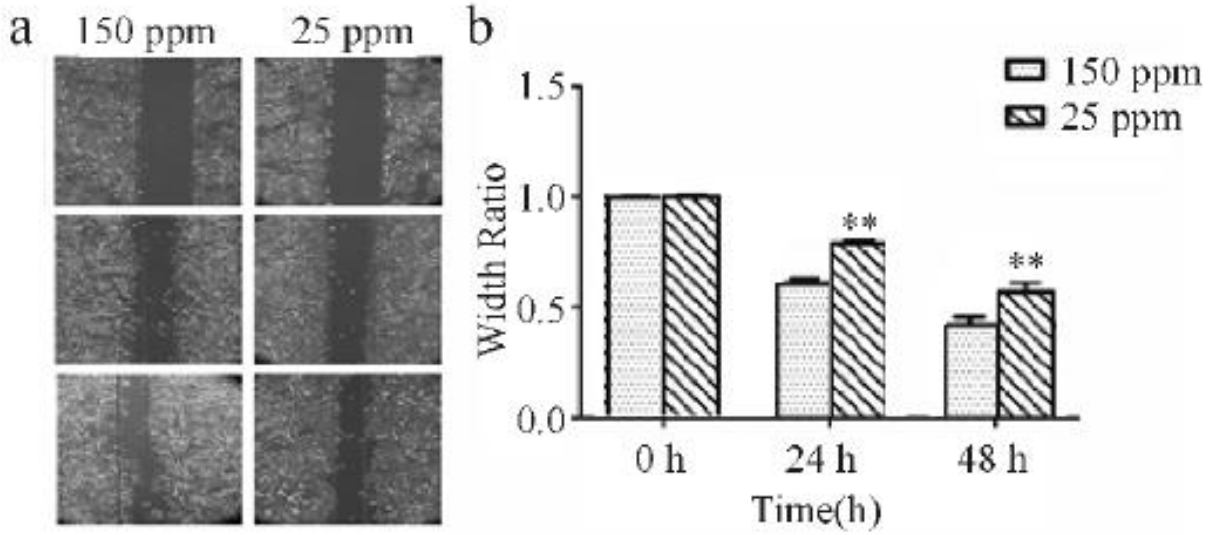


Fig. 1 Effect of deuterium depletion on wound healing ability of gastric cancer cells ($\bar{x} \pm s$, $n = 3$)

** $P < 0.01$ *vs* 150 ppm group

2.3 Effect of low-deuterium environment on colony-forming ability of gastric cancer cells Colony formation assay to detect the effects of long-term deuterium-depleted environment on gastric cancer cells The number of colonies in the experimental group and the control group were 234 ± 19, 327 ± 13, 25 ppm low deuterium environment cell colony count The levels of leukocytes in the 42 samples of the control group were significantly lower than those in the control group (P < 0.01, Figure 2).

2.4 Effects of low-deuterium environment on the cell cycle of gastric cancer cells

Effect of low-deuterium environment on the cell cycle of gastric cancer cells detected by flow cytometry Compared with the control group, the cell cycle of the experimental group cells was arrested at G1/S phase, as shown by a decrease in the ratio of S phase cells (25 ppm,27ÿ 98% ± 3ÿ 77% ; 150 ppmÿ39ÿ 83% ± 1ÿ 99% ; P ÿ 0.05) The ratio of cells in G1 phase increased (25 ppm, 57.81% ± 5ÿ 63%; 43ÿ 77% ± 6ÿ 176%; P ÿ 0ÿ 01).

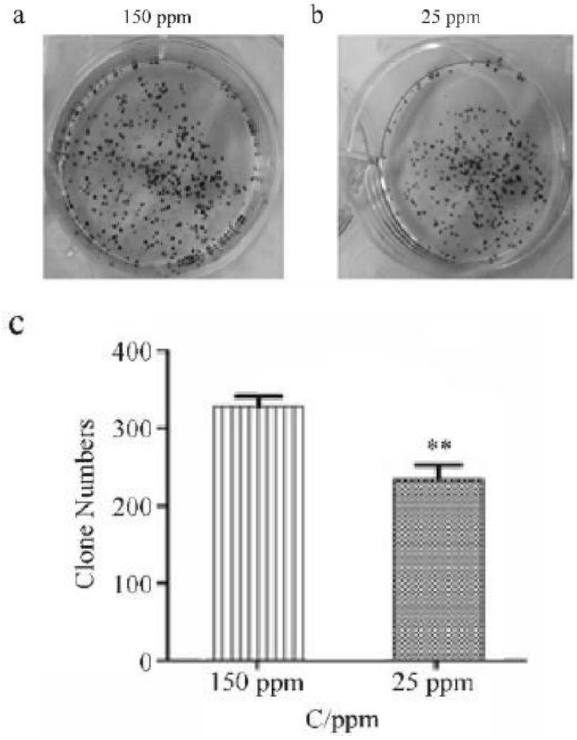
There was no statistically significant difference in the ratio of cells in the early and late stages (Figure 3).

2.5 Effect of low-deuterium environment on apoptosis of gastric cancer cells

Flow cytometry detection of early apoptosis of gastric cancer cells in a deuterium-depleted environment The early apoptosis and late apoptosis of the cells in the experimental group and the control group

The death rates were 1.593% ± 0.206% and 1.290% ± The rates of late apoptosis were 1.907% ± 1.068% and 0.053% respectively. ÿ

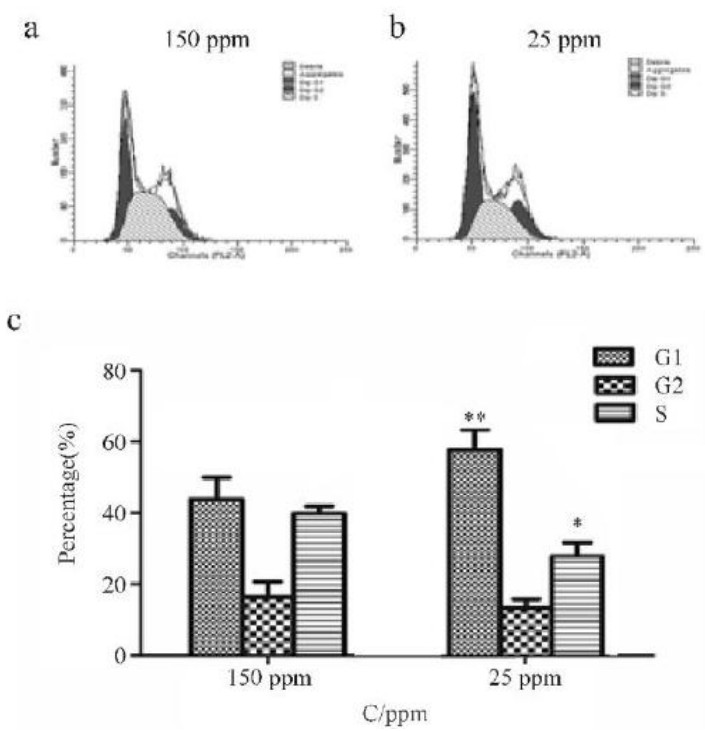
1. 757% ± 1. 097%, the difference in the ratio of early and late apoptosis between the two groups There was no statistical significance. The difference was 3.500% ± 1.274% and 3.047% ± 1.111% respectively. There was no statistical significance (Figure 4).



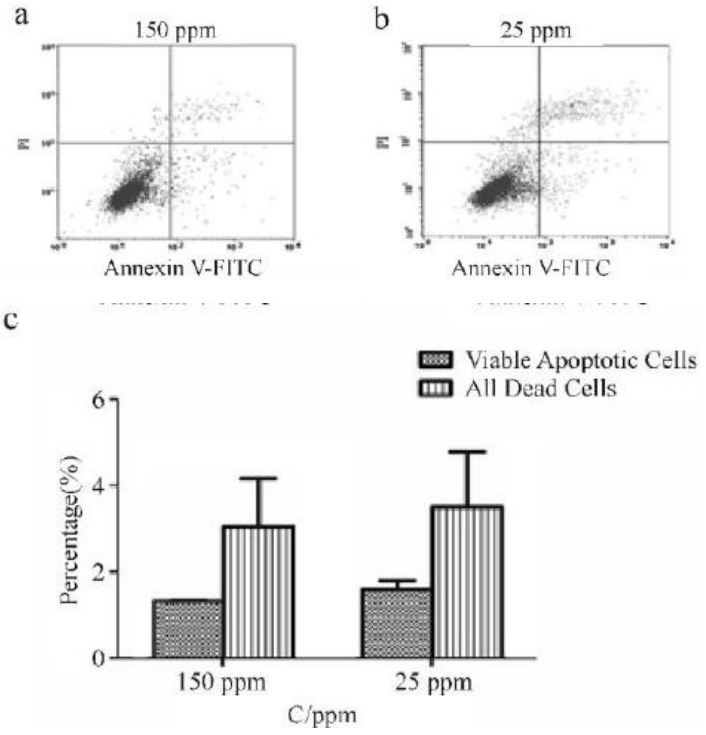
Figÿ 2 Deuterium depletion inhibited colony formation of gas-tric cancer cells(xÿ± sÿn = 3) P ÿ 0ÿ 01 vs 150 ppm group

2.6 Effect of low-deuterium environment on PCNA protein expression in gastric cancer cells PCNA is a good indicator reflecting the proliferation status of tumor cells.

The results showed that the expression of PCNA protein in gastric cancer cells in the experimental group was higher than that in The control group was significantly lower, and the difference was statistically significant (P ÿ 0.05, Fig. 5) ÿ



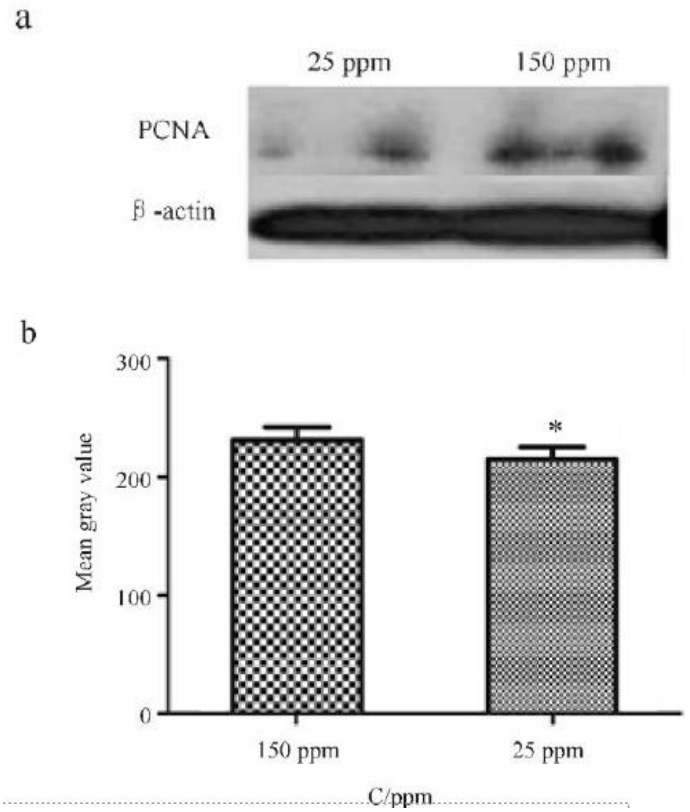
Figÿ 3 Deuterium depletion arrested cell cycle of gastric cancer cells at G1 phase(xÿ± sÿn = 3) * P ÿ 0ÿ 05ÿÿÿP ÿ 0ÿ 01 vs 150 ppm group



Figÿ 4 Apoptosis of gastric cancer cells remained unchanged in deuterium depleted environment(xÿ± sÿn = 3)

3 Discussion

At present, the clinical treatment effect of gastric cancer is far from satisfactory. In recent years, more and more research results have shown that reducing the deuterium content in water can inhibit the growth of various tumor cells [12, 13]. However, there are few reports on the effect of deuterium-depleted water on the proliferation of gastric cancer cells. This study found that a deuterium-depleted environment can inhibit the growth of gastric cancer cells by downregulating the expression of PCNA and blocking the cell cycle. Since the gastric mucosa can directly contact deuterium-depleted liquids (such as deuterium-depleted water), this discovery is expected to become a new strategy for the treatment of gastric cancer. Related studies have shown that the inhibition rate of deuterium-depleted water on the growth of gastric cancer cells is as high as 20%, and its inhibitory effect varies at different action time points. Cong et al. [8] proposed that short-term exposure to a low-deuterium environment (50 ppm) can inhibit the proliferation of lung cancer cells, and then the cells resume normal growth. Then, as the exposure time increases, the proliferation of lung cancer cells is inhibited again. This study found that low-deuterium environments with concentrations of 50 ppm and 100 ppm could not inhibit the proliferation of gastric cancer cells. Low-deuterium environments in the concentration range of 25 ppm to 105 ppm have no toxic side effects on organisms [10, 17]. Therefore, this study reduced the deuterium concentration to 25 ppm and observed its inhibitory effect on the proliferation of gastric cancer cells. Compared with other related studies, the inhibition rate of low-deuterium environment on gastric cancer cell proliferation in this study was about 10%, which may be related to the cell line and experimental design used by the researchers. Unlike other studies, this study strictly controlled the content of other elements (such as 18O, etc.) in water when preparing low-deuterium water, so as to exclude the influence of other factors other than deuterium on the proliferation of gastric cancer cells. Because the content of 18O and other trace elements and electrolytes in water will change when deuterium-depleted water is prepared by distillation, 150 ppm natural water was selected instead of distilled water as the control group of the experiment.



Figÿ 5 Expression of proliferating cell nuclear antigen (PC-NA) in gastric cancer cells(xÿ± sÿn = 4) a: Western blot analysis of PCNA in cells exposed to 25 ppm and 150 ppm deuterium for 48 h; b: Mean gray value obtained by densitometric analysis of the results of Western

Blot analysis P ÿ 0ÿ 05 vs 150 ppm group This paper preliminarily explored the related mechanisms of low deuterium environment inhibiting the proliferation of gastric cancer cells, such as cell cycle and apoptosis. It was

found that low deuterium environment can make gastric cancer cells stay in the G1 phase. Related studies have also shown that cell division and proliferation are closely related to changes in the intracellular environment and the content of deuterium [14, 15]. In addition, the increase in the D/H ratio in cells can induce cells to enter the S phase [16], which is consistent with the above research results. This experiment revealed that the low deuterium environment can reduce the ratio of gastric cancer cells in the S phase.

This may be related to the fact that the low-deuterium environment changes the growth environment of gastric cancer cells, affecting Affects the intracellular D/H ratio, thereby adjusting cell cycle-related signals The source induces a series of physiological changes in cells. Studies have shown that deuterium-depleted water can induce cell apoptosis, which may be related to downregulation of It is related to the expression of C-myc, Ha-ras and P53 genes, but its specific role is The mechanism of action has not yet been determined [17].

It has been observed that low-deuterium environment induces apoptosis of gastric cancer cells, which may Could be related to the cell lines used in this study. PCNA is a protein in the nucleus of cells in the S phase of the cell proliferation cycle. The antigen expressed by DNA replication, repair and expression of cell cycle regulatory proteins are closely related Therefore, it is often used as an indicator to assess tumor progression and clinical prognosis. This study found that a 25 ppm low-deuterium environment can downregulate gastric The expression of PCNA protein in cancer cells is consistent with the experimental group in this study. This is consistent with the results that gastric cancer cells are arrested in the G1 phase, but further research is needed. It is necessary to study its related mechanisms in more depth. In summary, the growth of gastric cancer cells cultured in a deuterium-depleted environment The cycle can be effectively blocked and the growth of gastric cancer cells is inhibited. This may be related to the low deuterium environment, which can downregulate the expression of PCNA and block This finding is related to the inhibition of gastric cancer cell proliferation. This study provides a theoretical basis for the new treatment strategy for gastric cancer.

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