



Research Article

Exploring the anticancer potential of deuterium-depleted water: effects on tumor cell proliferation, migration, and apoptosis

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Abstract: Cancer persists as one of the primary global causes of death, highlighting the pressing demand for innovative therapeutic methods. Deuterium-depleted water (DDW) has emerged as a potential anticancer agent due to its ability to influence cellular processes. This study explores the inhibitory impacts of DDW with diverse concentrations on the proliferation of lung (A549) and liver (HepG2) cancer cells and the migration of breast (MCF-7) cancer cells. It also examines its apoptosis effects on A549 and MCF-7 cancer cells. Using the CCK-8 assay, real-time cell analysis, fluorescence microscopy, and wound-healing assays, the impact of DDW on tumor cells was systematically evaluated. The CCK-8 assay demonstrated that DDW significantly inhibited tumor cell growth, a finding corroborated by real-time cell growth curves. Fluorescence microscopy revealed that DDW triggered apoptosis and necrosis in A549 and MCF-7 cells. In addition, the wound - healing assays demonstrated that, compared with the control group, DDW at concentrations of 0 ppm, 30 ppm, 60 ppm, and 100 ppm significantly weakened the migration ability of MCF-7 cells, and all these differences were statistically significant ($P < 0.05$). These results suggest that DDW effectively suppresses tumor cell proliferation and migration while promoting apoptosis. This study suggests that DDW may have potential to inhibit tumor cell proliferation as an adjuvant therapy, and it provides a solid basis for subsequent research on the mechanisms and possible therapeutic applications of DDW in cancer care.

Keywords: deuterium-depleted water; tumor cells; cancer adjuvant therapy; proliferation inhibition; apoptosis induction; migration suppression

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1 Introduction

Cancer remains one of the primary causes of mortality, with nearly 10 million deaths in 2020^[1]. The existing cancer treatment modalities encompass surgery, cellular immunotherapy, radiation therapy and chemotherapy. Although chemotherapy and radiotherapy can increase the survival rate of patients, they are accompanied by various side effects-such as hair loss, diarrhea, fatigue, anorexia, and anemia-posing significant challenges for patients^[2]. Surgery can be curative for localized cancers, but it is not suitable for metastatic cancer, and does not remove all cancer cells^[3,4]. Cellular immunotherapy targets cancer cells specifically with the immune system, but may lead to severe immune-related side effects^[5]. And stem cell transplantation is a medical procedure, but has a high risk of complications, and requires a suitable donor^[6]. Despite notable advancements in cancer treatment research, numerous cancer types still lack effective treatment options. Therefore, exploring novel treatment approaches and

methodologies is of critical importance. As clinical practice progresses, adjuvant therapies are not only vital components of cancer treatment but also provide novel perspectives and innovative strategies for oncology management^[7]. Adjuvant therapies are typically administered following primary treatments like surgeries or chemotherapy. Their purpose is to strengthen the elimination of cancer cells and lower the likelihood of tumor recurrence^[8]. Recent research has indicated that deuterium-depleted water (DDW), when used as an adjuvant, can remarkably improve the effectiveness of chemotherapy. Additionally, DDW has been demonstrated to inhibit tumor cell growth through oxidative stress mechanisms, suggesting its potential as an adjuvant therapy^[9,10].

DDW refers to water with a deuterium content significantly lower than that found in natural water. Deuterium, which is an isotope of hydrogen, typically presents in water at approximately 150 parts per million (ppm). DDW generally has a deuterium content below 120 ppm, with some formulations achieving even

lower levels^[11]. DDW production methods include desalination, distillation, and catalytic exchange^[12,13]. Clinical studies have shown that daily consumption of DDW at concentrations ranging from 25 ppm to 125 ppm can replace normal water and create a deuterium-depleted environment within the body^[14]. Recently, DDW has drawn substantial attention from the scientific community because of its potential biological effects on cancer cells. Research indicates that DDW significantly impacts cell metabolism, DNA synthesis, and cell proliferation in certain cell types^[15-18]. In oncology, preliminary experiments suggest that DDW can potentially hinder the growth, migration, and multiplication of cancer cells^[13]. However, these specific mechanisms underlying these effects and their biological implications remain poorly understood, particularly regarding their roles in different types of tumor cells.

Recent investigations have demonstrated that DDW is capable of suppressing the proliferation and migration of diverse cancer cells, including lung cancer, colorectal cancer, and breast cancer, by accelerating apoptosis, autophagy, and senescence^[19-21]. Furthermore, the use of DDW in adjuvant therapy effectively reduced the required doses of chemotherapy drugs while achieving equivalent therapeutic outcomes, thereby minimizing the side effects associated with chemotherapy^[10]. Cancer is among the most prevalent and lethal diseases globally^[22]. For instance, Lung cancer is a major global health concern, ranking among the most common and fatal diseases. Liver cancer has a high mortality rate because of difficulties in early detection and a high recurrence rate. In females, breast cancer ranks as the most commonly occurring form of cancer^[23-26]. Previous research has shown that DDW can impede the growth of numerous cancer cell types, including breast cancer cell, lung cancer cell, glioma cell, stomach cancer cell, leukemia cell, and prostate cancer cell lines^[19, 21, 27-29]. However, the specific experimental data is still very scarce and the underlying mechanisms are still unclear. The research has progressed to a point where effects of DDW on different cancer types have been observed. Moreover, there is a need to explore optimal treatment protocols and investigate potential therapies with DDW in cancer. Understanding the biological effects of DDW on tumor cells is of great significance since it could provide new insights and approaches for cancer treatments. To fill this gap, this research conducts *in vitro* experiments. The goal is to systematically assess the impacts of DDW on the biological characteristics of A549, MCF-7 and HepG2 cancer cells, including growth, proliferation, and apoptosis. Such investigations not only contribute to elucidating the potential mechanisms of DDW in cancer treatment but also provide a theoretical foundation and experimental evidence for creating innovative adjuvant treating cancer approaches.

2 Materials and methods

2.1 Materials

DDW with deuterium concentrations of 100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm were provided by China academy of engineering physics. Fetal bovine serum (FBS) was purchased from Biochannel Co., RPMI 1640 medium was obtained from GIBCO. The human lung normal cell line (BEAS-2B), human lung adenocarcinoma cells (A549), human liver cancer cells (HepG2), and human breast cancer cells (MCF-7) were sourced from Zhong Qiao Xin Zhou Co., Ltd.

(China). The CCK-8 assay kit was purchased from Abbkine Scientific Co., Ltd. (China). The *in-situ* cell detection kit was acquired from Beyotime Biotech Co., Ltd. (China), the microplate reader was acquired from Tecan Austria GmbH, and the MuviCyte™ live-cell imaging system was procured from PerkinElmer, Inc.

2.2 Cell culture

RPMI 1640 medium, with 10% FBS, was used to culture A549, MCF-7, and HepG2 cells. The cultivation was carried out in a humid environment with 5% CO₂ at a temperature of 37 °C. In the experimental groups, 5 mL of RPMI 1640 medium was prepared using DDW with different deuterium concentrations: 100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm. For the control group, A549, BEAS-2B, MCF-7, and HepG2 cells were maintained in RPMI 1640 medium prepared using distilled water, and the culture conditions were kept identical to those of the experimental groups.

2.3 CCK-8 assay for cell proliferation

Cell proliferation was measured by the CCK-8 assay. A549 cells (at a density of 1×10^4 cells/well), MCF-7 cells (5×10^3 cells/well), and HepG2 cells (5×10^3 cells/well) were individually plated into 96-well plates, with five replicates for each type of cell. Each well was treated with 100 µL of DDW at preset concentrations (100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm) or control water and cultured for 6, 12, 24, 48 and 72 h. After that, 10 µL of the CCK-8 reagent was added to each well, and the plates were further incubated for 1 hour in an incubator maintained at 37 °C with a 5% CO₂ atmosphere. The optical density was measured at 450 nm by using a microplate reader. Every experiment was replicated thrice. The formula for calculating the proliferation rate is presented in Equation 1:

$$\text{Proliferation rate (\%)} = \frac{(\text{OD of experimental group} - \text{OD of blank group})}{(\text{OD of control group} - \text{OD of blank group})} \times 100\% \quad (1)$$

2.4 Real-Time cell analysis for cell proliferation

Cells underwent cultivating in RPMI 1640 medium with 10% FBS and incubated at 37 °C in an environment with 5% CO₂ until they achieved an appropriate confluence level. The cell density was adjusted to 1×10^4 cells/well. These cells were then inoculated into 96-well plates suitable for the real-time cell analysis system. Experimental groups were set with cells treated with medium configured with DDW at preset concentrations (100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm), while the control group was set with cells treated with medium configured with distilled water. Each group included at least three replicate wells. The 96-well plates were placed in the real-time cell analyzer, and cell index (CI) values were recorded every hour for 24 h. Cell adherence and growth on electrodes were measured with the real-time cell analyzer, and CI values were generated to reflect proliferations. The MuviCyte™ software was utilized to process and analyze the data, plot proliferation curves, and compare CI changes among different groups, aiming to evaluate the impacts of DDW on cell proliferation.

2.5 Wound healing assay

Cells underwent cultivating in RPMI 1640 medium with 10% FBS. They were maintained at 37 °C in an environment with 5%

CO₂ until the cell confluence reached 70%–80%. To initiate the experiment, a sterile 200 µL pipette tip was employed to make a vertical scratch of a uniform width in the center of each well. Subsequently, the cells were rinsed three times with phosphate-buffered saline (PBS) to eliminate any loosely attached cells and debris. Experimental groups were set with medium configured with DDW at preset concentrations (100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm), while the control group was set with cells treated with medium configured with distilled water. All the plates were then placed in an incubator at 37 °C with a 5% CO₂ atmosphere. Images of the scratch area were captured every 6 h using the MuviCyte™ live-cell imaging system. Wound closure rates and evaluated cell migration were analyzed with analysis software in the MuviCyte™ live-cell imaging system.

2.6 In situ fluorescence assay for apoptosis detection

Cells were placed and cultivated in 96-well plates. The cells were then washed one time with PBS before adding DDW at preset concentrations (100 ppm, 80 ppm, 60 ppm, 40 ppm, 30 ppm, 20 ppm, and 0 ppm) for the experimental groups, while the control group received serum-free RPMI 1640 medium. Subsequently, the plates were incubated at 37 °C in an atmosphere containing 5% CO₂ for 24 h. Post incubation, 195 µL of Annexin V-FITC binding solution and 5 µL of Annexin V-FITC were added to each well. These components were gently blended. Then, 10 µL of propidium iodide (PI) staining solution was introduced and gently mixed as well. The samples were incubated in the dark at room temperature, ranging from 20 °C to 25 °C, for a duration

of 10–20 min, followed by placement on ice for observation. An inverted fluorescence microscope was used to observe the fluorescence. In this process, Annexin V-FITC gave off green fluorescence, and PI produced red fluorescence. V-FITC and PI were jointly used to analyze the cell conditions.

2.7 Statistical analysis

GraphPad Prism software was employed for the statistical analysis of the data. One-way ANOVA was applied to conduct comparisons among multiple groups. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Influence of DDW on the proliferation of lung cancer cells (A549)

The impacts of diverse concentrations of DDW on A549 cells proliferation were evaluated by using the CCK-8 assay at 6, 12, 24, 48, and 72 h. Distilled water containing a deuterium concentration of 150 ppm was set as the control group. At 6 and 12 h, DDW demonstrated a notable suppressive impact on the growth of A549 cells (Fig. 1). With the passage of time, the inhibitory effects of higher deuterium concentrations (≥ 30 ppm) decreased. However, DDW at concentrations of 20 ppm and 0 ppm consistently showed prominent inhibitory effects on A549 cell proliferation throughout the duration of the experiment. This result suggests that the DDW, especially at lower concentrations, effectively

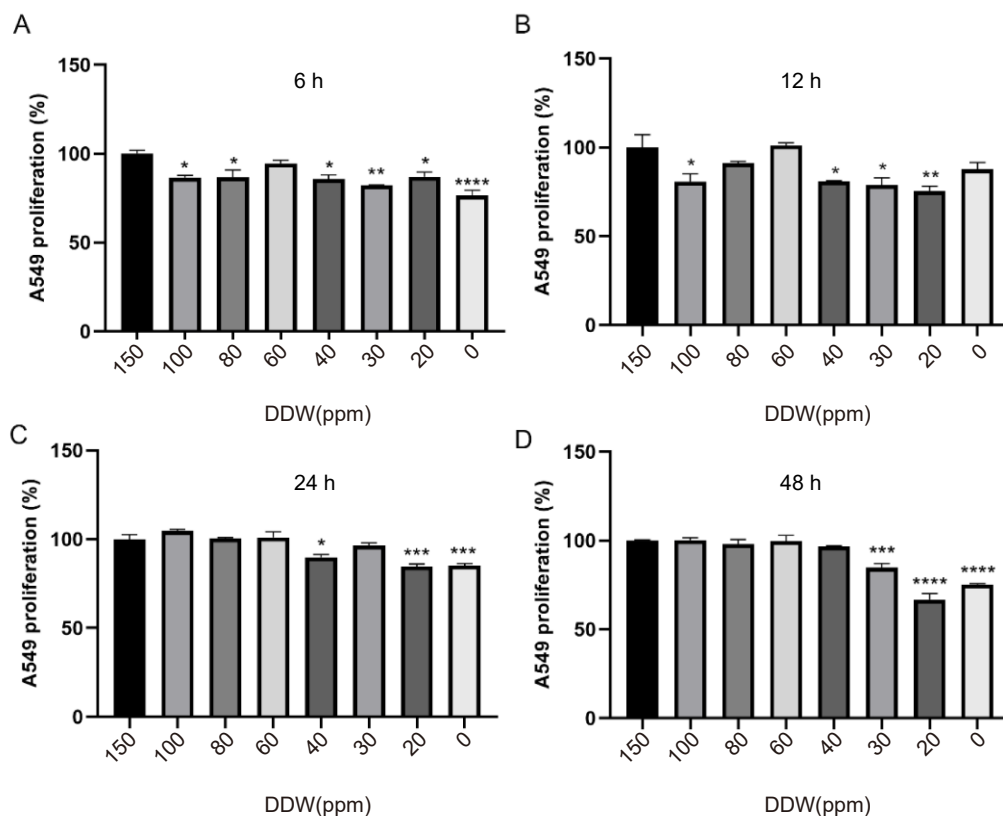


Figure 1 Influence of DDW on the proliferation of lung cancer cells (A549). The growth of A549 cells was gauged by the CCK-8 assay over 72 h. The data were shown as the mean $\pm$ standard error of measurement (SEM) which were derived from three separate independent experiments. The statistical significance was determined by comparing the experimental groups with distilled water (150 ppm) in RPMI 1640 medium as the control group. The significance levels were marked as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. The same below.

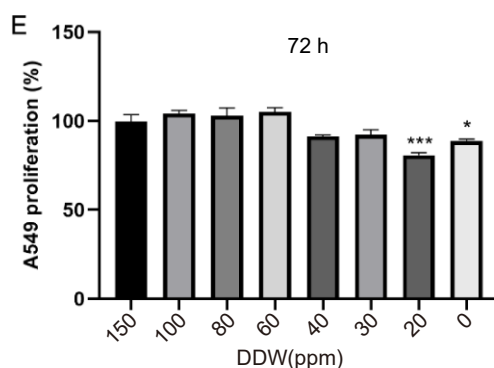


Figure 1 (Continued).

inhibits the proliferation of A549 cells, with more pronounced effects observed at earlier time points.

Contrary to its inhibitory effects on A549 tumor cells, when normal human lung cells (BEAS-2B) were treated with deuterium-depleted water (DDW) for 6, 24, 48, and 72 h, no substantial effect on their growth was observed compared to the control group (Fig. 2). This outcome implies that DDW selectively inhibits the proliferation of tumor cells (A549) while sparing the normal lung cells.

3.2 Real-Time monitoring of the effects of DDW on A549 cell proliferation

In order to delve deeper into the impacts of DDW on the proliferation and viability of A549 cells, a real-time cell analysis

system was employed to monitor the cell growth curves. The biological effects of DDW were assessed by tracking cell proliferation and survival in real time. The real-time proliferation of A549 cells treated with DDW and normal water was monitored over a 24 h period (Fig. 3). At the initial time point (0 h), the CI of both groups was similar and remained at baseline levels. With the passage of time, the growth rates of the DDW-treated groups were found to be reduced in comparison to the control group exposed to regular water. These results demonstrate that DDW slows down the growth rate of A549 cells and inhibits their proliferation. This further supports the anticancer potential of DDW.

3.3 DDW promotes apoptosis in lung cancer cells (A549)

In situ fluorescence detection was performed to analyze

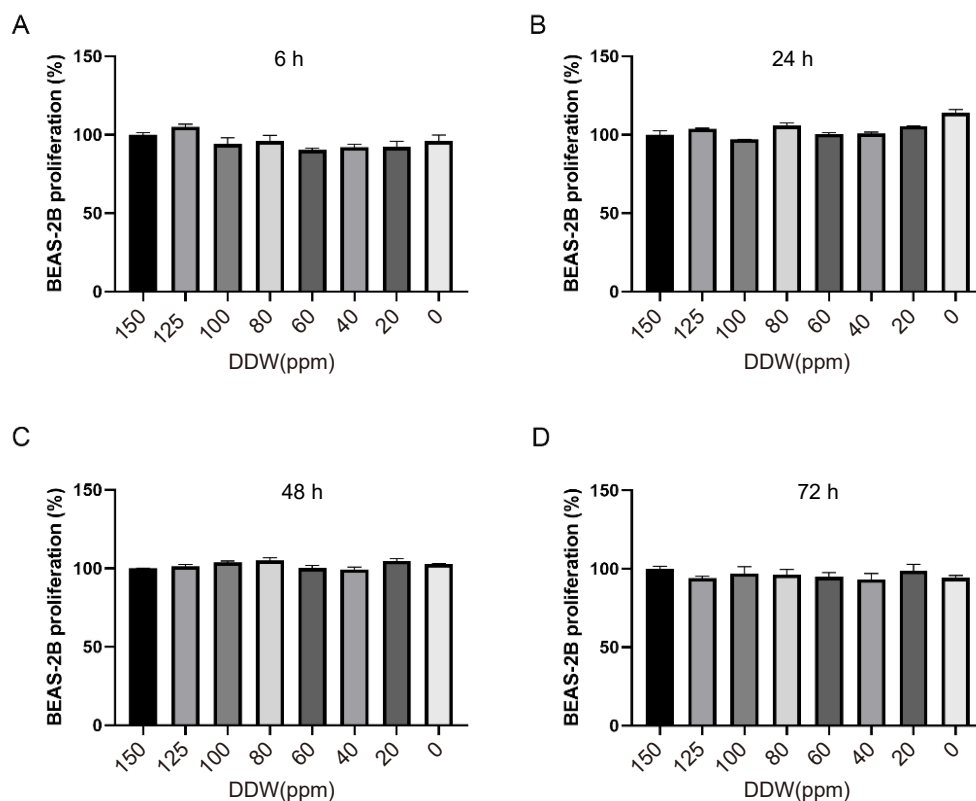


Figure 2 Effects of DDW on the proliferation of normal human lung cells (BEAS-2B). The growth of BEAS-2B cells was evaluated through the CCK-8 assay over a 72-h period. Results were presented as a percentage relative to the growth of cells cultured in normal water which served as the control group. Data are expressed as the mean $\pm$ SEM, based on three independent experiments.

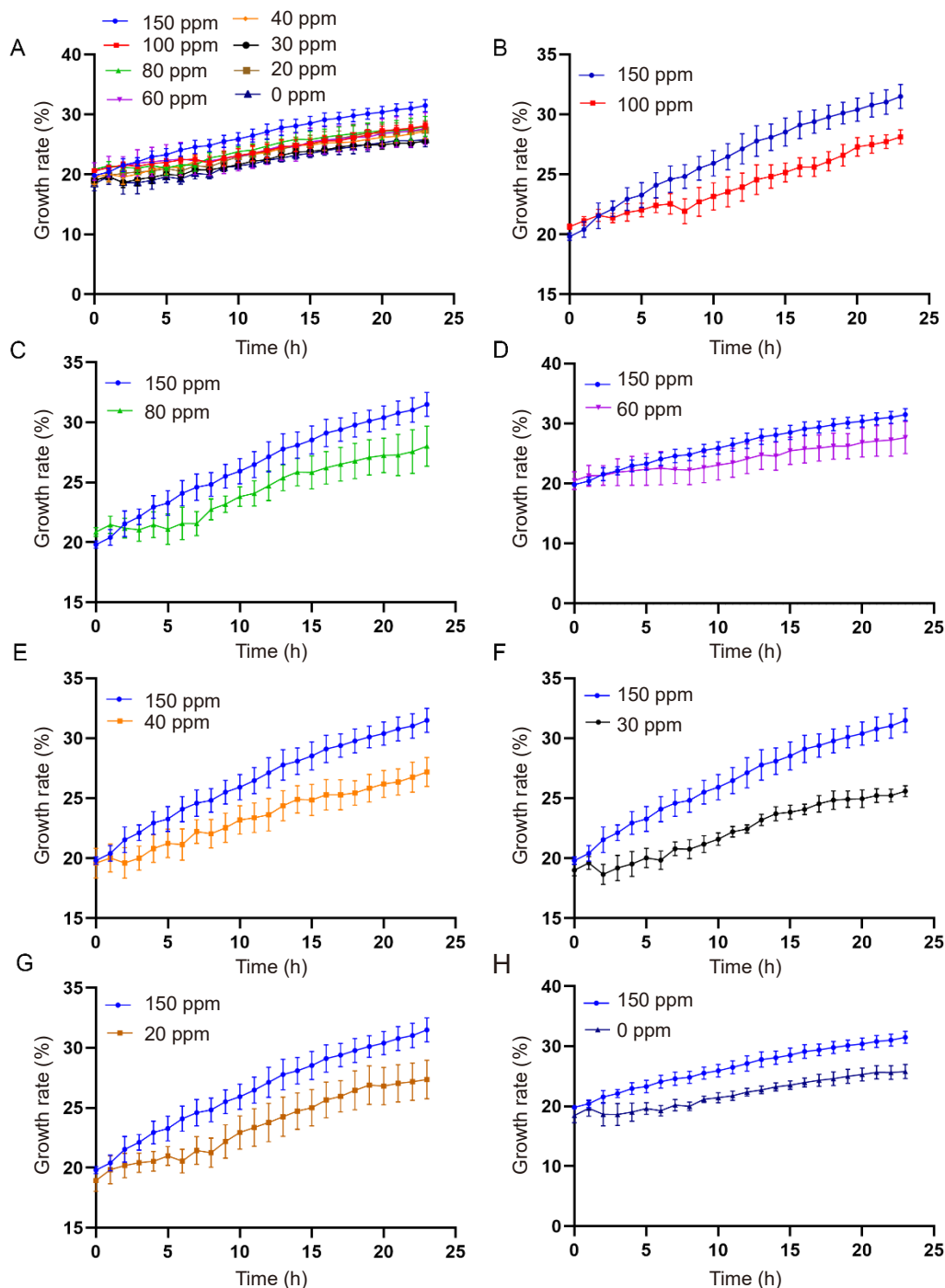


Figure 3 DDW reduces the survival rate of lung cancer cells (A549). Real-time cell analysis was performed to monitor the growth of A549 cells exposed to DDW and normal water over a 24 h period. The results indicate that DDW treatment significantly inhibited A549 cell proliferation, as shown by the lower CI compared with the control group.

apoptosis in A549 cells. FITC (fluorescein isothiocyanate) was used to label apoptotic cells, presenting green fluorescence, while PI was used to label necrotic cells, presenting red fluorescence. The experimental group was set with A549 cells treated with DDW and the control group was set with A549 cells treated with normal water. The experimental result demonstrated that the intensities of both green and red fluorescence in the DDW-treated groups were significantly enhanced contrasted with the control group (Fig. 4). Specifically, green fluorescence intensities in the DDW-treated groups were markedly increased, indicating significant rises in the

number of apoptotic cells. Additionally, the red fluorescence intensities were also elevated, which implied a rise in the quantity of necrotic cells. These findings suggest that DDW promotes both apoptosis and necrosis in A549 cells, thereby inhibiting tumor cell growth.

3.4 Influence of DDW on the growth of liver cancer cells (HepG2)

The impacts of diverse concentrations of DDW on the growth of HepG2 tumor cells were evaluated by using the CCK-8 assay at

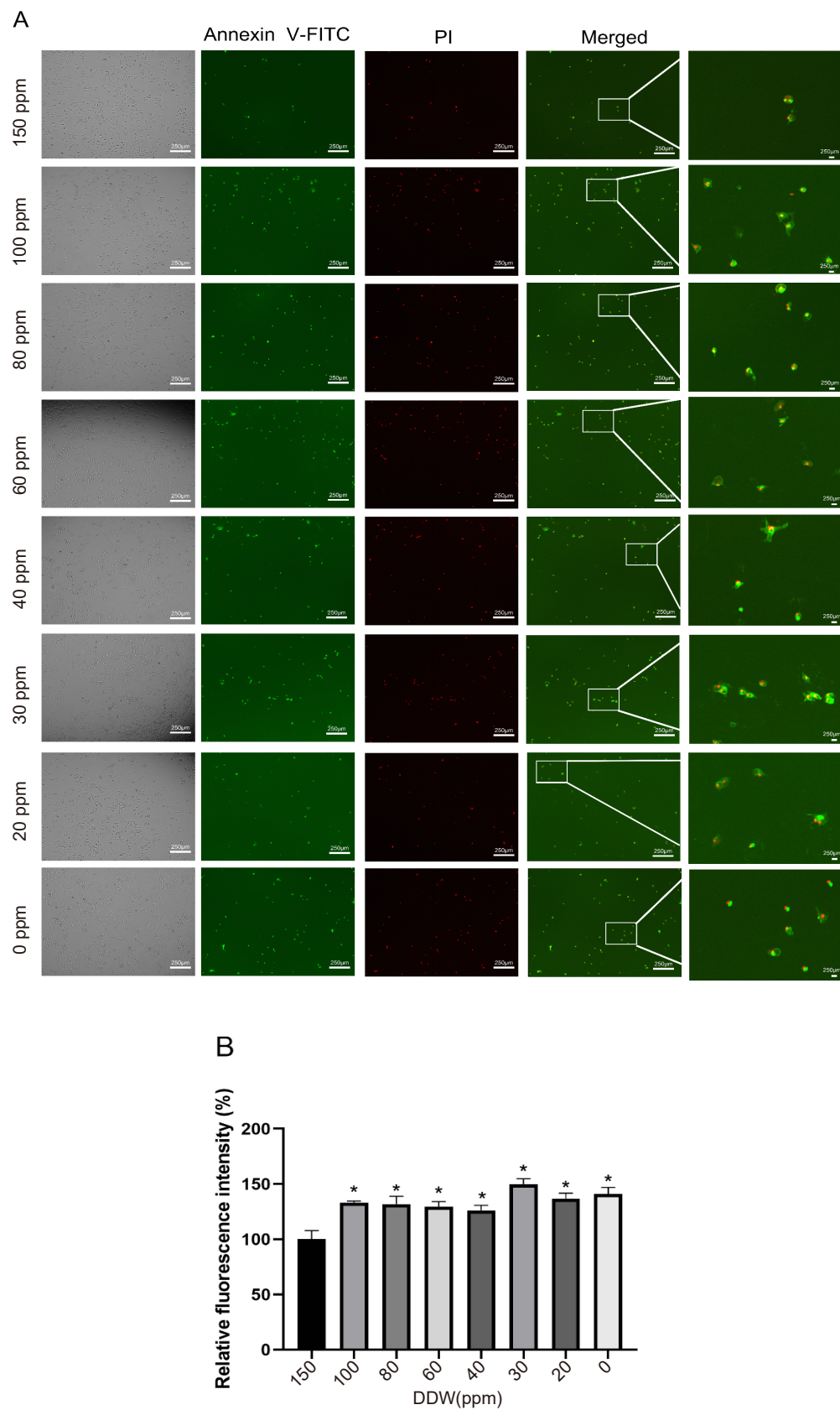


Figure 4 DDW promotes apoptosis in lung cancer cells (A549). Apoptosis in A549 cells treated with DDW at concentrations of 0 ppm, 20 ppm, 30 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm, as well as normal water (150 ppm), was detected using *in situ* fluorescence staining. FITC was used to label apoptotic cells (green fluorescence), and PI was used to label necrotic cells (red fluorescence). The scale bar was set at 250 μ m. Values are expressed as mean $\pm$ SEM, and these are based on three separate experiments.

6, 12, 24, 48, 66 and 72 h. Normal drinking water with a deuterium concentration of 150 ppm was used as the control group. DDW exhibited no significant inhibitory effect on HepG2 cell proliferation at 6, 12, 24, 48, or 66 h. However, at 72 h, the inhibitory effects of DDW became prominent (Fig. 5). Statistical significances were found between the DDW-treated groups and the control group at 72 h. These outcomes imply that while DDW has postponed inhibitory effect on the proliferation of HepG2 cells, its impact becomes significant over longer treatment durations. This finding supports the potential value of DDW in cancer treatment, especially for liver cancer, with prolonged exposure.

3.5 Real-Time monitoring of the impacts of DDW on HepG2 cell growth

To delve deeper into the impacts of DDW on the proliferation and survival of HepG2 cells, a real-time cell analysis approach was

utilized. This method enabled continuous monitoring of cell proliferation and survival to gauge the biological consequences of DDW based on bioelectrical impedance technique (BIT). The real-time proliferation of HepG2 cells treated with DDW and normal water was monitored over 36 h, and CI were recorded every 2 h (Fig. 6). At the initial time point (0 h), the CI of both groups was similar, remaining at baseline levels. With the passage of time, the growth rates of the DDW-treated groups were notably lower compared to the control group. These outcomes demonstrate that DDW can impede the proliferation of HepG2 cells and reduces their survival rate.

3.6 Influence of DDW on the proliferation of MCF-7 breast cancer cells.

MCF-7 cell proliferation was assessed by the CCK-8 assay to evaluate the effects of various DDW concentrations at 6, 12, 24,

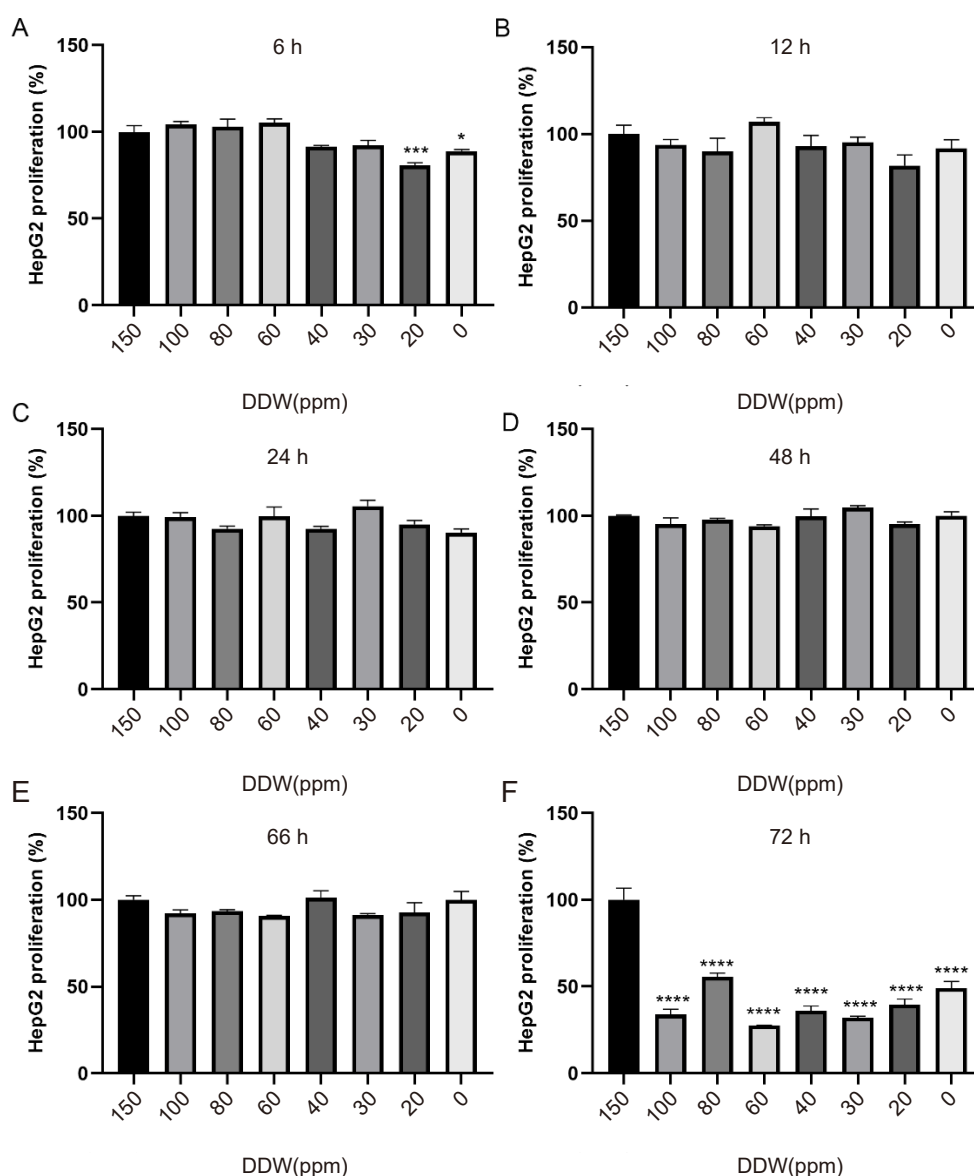


Figure 5 Influence of DDW on the growth of liver cancer cells (HepG2). The growth of HepG2 cells treated with various concentrations of DDW was measured using the CCK-8 assay over a 72-h period. The data were shown as the mean $\pm$ SEM, based on three independent experiments. Statistical significance was ascertained by contrasting the DDW-treated groups with the control group cultured in 1640 medium containing 150 ppm DDW.

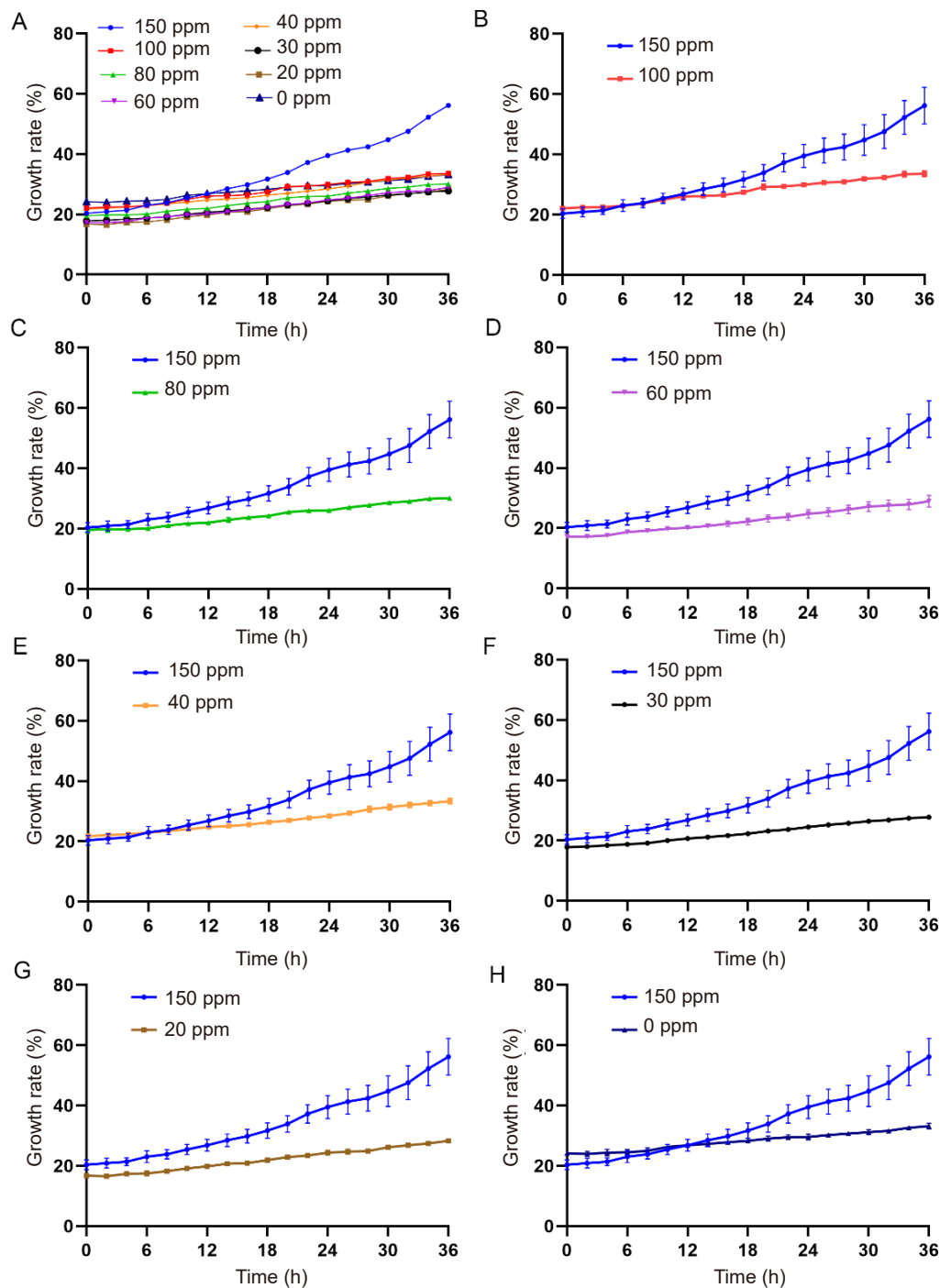


Figure 6 Real-Time monitoring of the impacts of DDW on HepG2 cell growth. Real-Time cell analysis was employed to track the growth of HepG2 cells incubated with DDW and those cultured with normal water over a 36-h period. The findings reveal that, in contrast to the control group using normal water, the growth rate of HepG2 cells treated with DDW decreased significantly.

48, and 72 h. MCF-7 cells treated with normal drinking water with a deuterium concentration of 150 ppm was used as the control group. DDW demonstrated substantial inhibitory influences on the growth of MCF-7 cells at 6 and 12 h (Figs. 7A-B). With the passage of time, the inhibitory effects of groups with higher deuterium concentrations diminished. However, DDW at deuterium concentrations of 20 ppm and 0 ppm maintained significant inhibitory effects throughout the experiment (Figs. 7C-E). These findings suggest that DDW, particularly at lower deuterium concentrations, effectively impairs the growth of

MCF-7 cells with the passage of time.

3.7 DDW inhibits the migration ability of MCF-7 cells

In this research, a wound-healing assay was utilized to investigate the impacts of varying concentrations of DDW on the migratory capacity of MCF-7 cells. Cells were incubated in media prepared with DDW at various deuterium concentrations of 0 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm, and the wound-healing rates were analyzed using The MuviCyte™ live-cell imaging system and its built-in analysis software. The

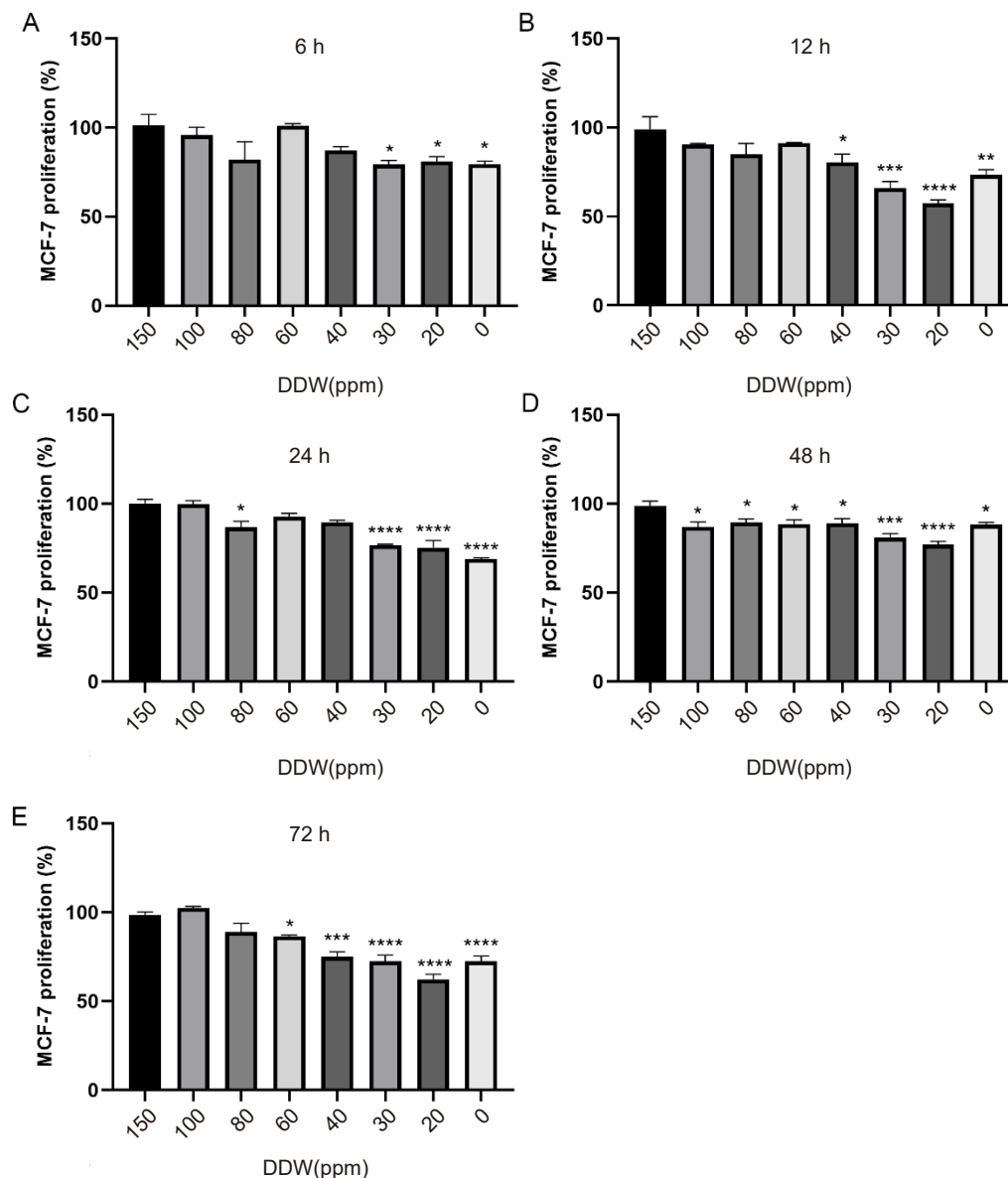


Figure 7 Influence of DDW on the proliferation of MCF-7 breast cancer cells. The growth of MCF-7 cells treated with different concentrations of DDW was measured using the CCK-8 assay over a 72-h period. Data were shown as the mean $\pm$ SEM derived from three separate experimental runs. Statistical significance was ascertained by making comparisons between the low concentration of DDW groups and the control group cultured distilled water.

percentages of wound closure in MCF-7 cells treated with DDW at different concentrations showed significant differences over a 72-h period (Fig. 8).

3.8 DDW promotes apoptosis in MCF-7 cells

To analyze apoptosis in MCF-7 cells, *in-situ* fluorescence detection was carried out. Apoptotic cells were labeled with green fluorescence using FITC, and necrotic cells labeled with red fluorescence using PI. The experimental group was set with MCF-7 cells treated with DDW and the control group was set with MCF-7 cells treated with normal water. In contrast to the control group, the intensities of both the FITC green fluorescence and PI red fluorescence in the DDW-treated groups increased by more than 25% (Fig. 9). Specifically, the green fluorescence intensity was significantly increased in the DDW-treated groups, indicating a rise in the number of apoptotic cells. Similarly, the red fluorescence intensity was also elevated, suggesting a rise in the number of necrotic cells. These outcomes indicate that DDW may

impede the growth of MCF-7 cells by triggering apoptosis.

4 Discussion

In this study, the impacts of DDW on lung, liver, and breast cancer cells were systematically evaluated using CCK-8 proliferation assays, wound-healing experiments, real-time cell analysis, and *in situ* fluorescence detection. This study is the first to reveal that DDW significantly inhibits the growth of HepG2 cells. The outcomes of CCK-8 assay indicate that after 72 h, the inhibitory impact of DDW on liver tumor cells is particularly pronounced compared to lung cancer cells and breast cancer cells. With its high metabolic activity, the liver is an essential organ for metabolic processes in the body^[30], which may lead to liver cells being more sensitive to DDW. Studies have shown that changes in deuterium concentration in the blood can create isotopic gradients in the body, directly participating in the citric acid cycle, thereby affecting liver cell growth and division^[31]. Furthermore, liver cells

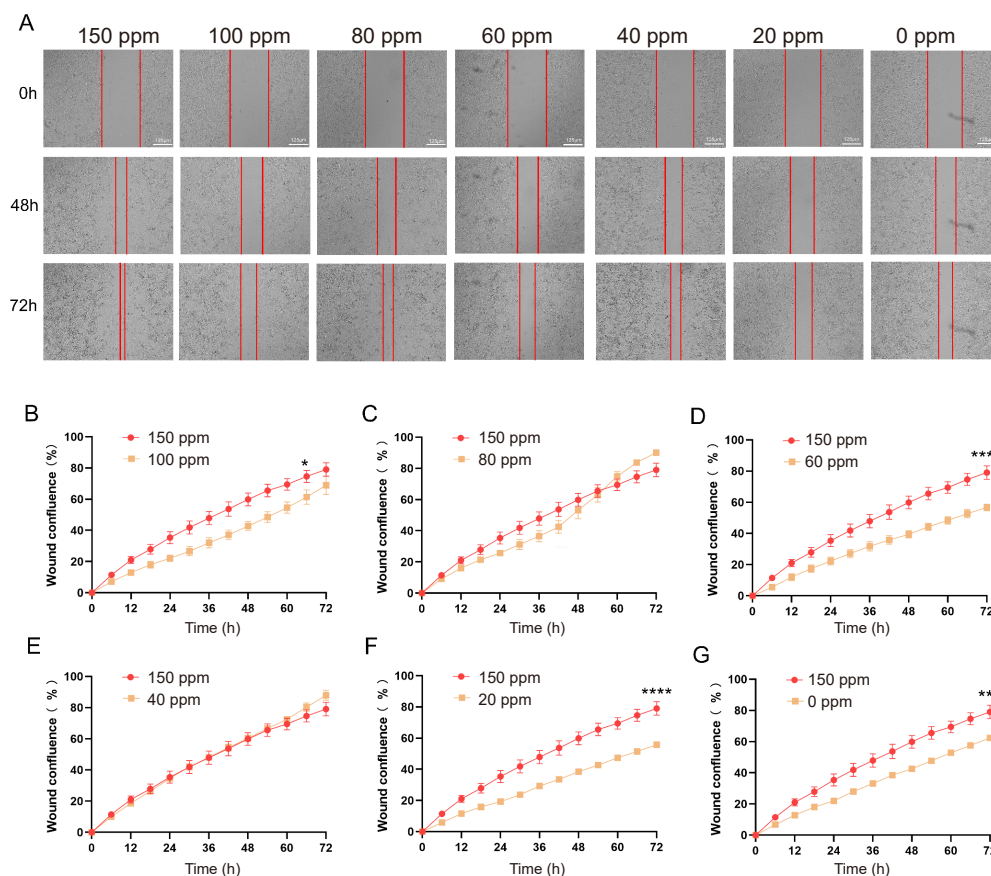


Figure 8 DDW inhibits the migration ability of MCF-7 cells. (A) wound-healing assay analyzing was conducted to evaluate the migratory potential of MCF-7 cells that were exposed to DDW at deuterium concentrations of 0 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm. Scale bar: 125 μm. (B-G) Statistical analysis of wound-healing rates using The MuviCyte™ live-cell imaging system's built-in analysis software. The 150 ppm group was served as the contrast group. The data are reported as the mean ± SD, with values from three independent experiments.

are of great significance in regulating cell growth and necrosis^[32]. Particularly in liver cancer cells, DDW may increase oxidative stress within the cells through isotopic effects, altering the cell cycle and apoptosis pathways, thereby interfering with cell proliferation and survival, which enhances its inhibitory effect on liver cancer cell growth. Although previous studies have explored the effects of DDW, there are few comprehensive comparisons of the impacts of DDW across different cancer types. Our research addresses this gap by evaluating the effects of DDW among lung, liver, and breast cancer cells, thereby highlighting the tumor-specific responses of DDW. Besides, the results demonstrate that DDW has no impact on normal cells, which is in accordance with previous studies^[33]. DDW might impede the growth of tumor cell growth in A549, MCF-7, and HepG2 cells lines by meddling with the cell cycle progression or triggering apoptosis. This aligns with previous studies that suggest that a deuterium-depleted environment can impact cell metabolism and proliferation^[19,27,33,34]. However, the study went a step further by providing dynamic, real-time data on cell proliferation through continuous real-time cell analysis, which allows researchers to observe the long-term effects and dynamics of DDW on cancer cells. This approach offers new insights into how DDW may influence cell adhesion, growth and division with the passage of time, aspects that have not been thoroughly explored in prior studies. Results from the wound-healing assay indicated that DDW notably decreased the migratory potential of MCF-7 cells. Cell migration is a vital stage in tumor invasion and metastasis. While previous research has

shown that DDW can influence cell migration, the specific mechanisms by which DDW interferes with tumor cell invasion and metastasis remain poorly understood^[35-37]. Our study suggests that DDW's inhibitory effects on migration may be an important aspect of its potential application in preventing tumor progression, an area that warrants further investigation. *In situ* fluorescence analysis revealed increased fluorescence intensity in DDW-treated A549 cells and MCF-7 cells, further demonstrating the suppressive effects of DDW on cell viability. The enhanced fluorescence likely reflects a rise in apoptosis and a decline in cell viability. This discovery is in line with earlier research indicating that DDW can encourage cell apoptosis^[20,37,38]. Notably, this study also adds to the understanding of how DDW-induced apoptosis might be an essential mechanism through which DDW impedes cancer cell survival.

Although DDW exhibits notable inhibitory effects on tumor cell proliferation, the study reveals that its effects are not strongly dose-dependent. The results show that not all concentrations of DDW significantly inhibit tumor cell proliferation, and the lowest concentration of deuterium ions does not necessarily produce the strongest effects. In our study, the 60 ppm group shows a significantly lower inhibitory effect on A549 cells compared to the 80 ppm group. Similarly, in the wound-healing assay of MCF-7 cells, concentrations of 60 ppm, 30 ppm, and 20 ppm show stronger inhibitory effects than the 0 ppm group. In addition, the fluorescence results showed that different concentrations of DDW had no dose-dependent effect on the apoptosis of A549 and

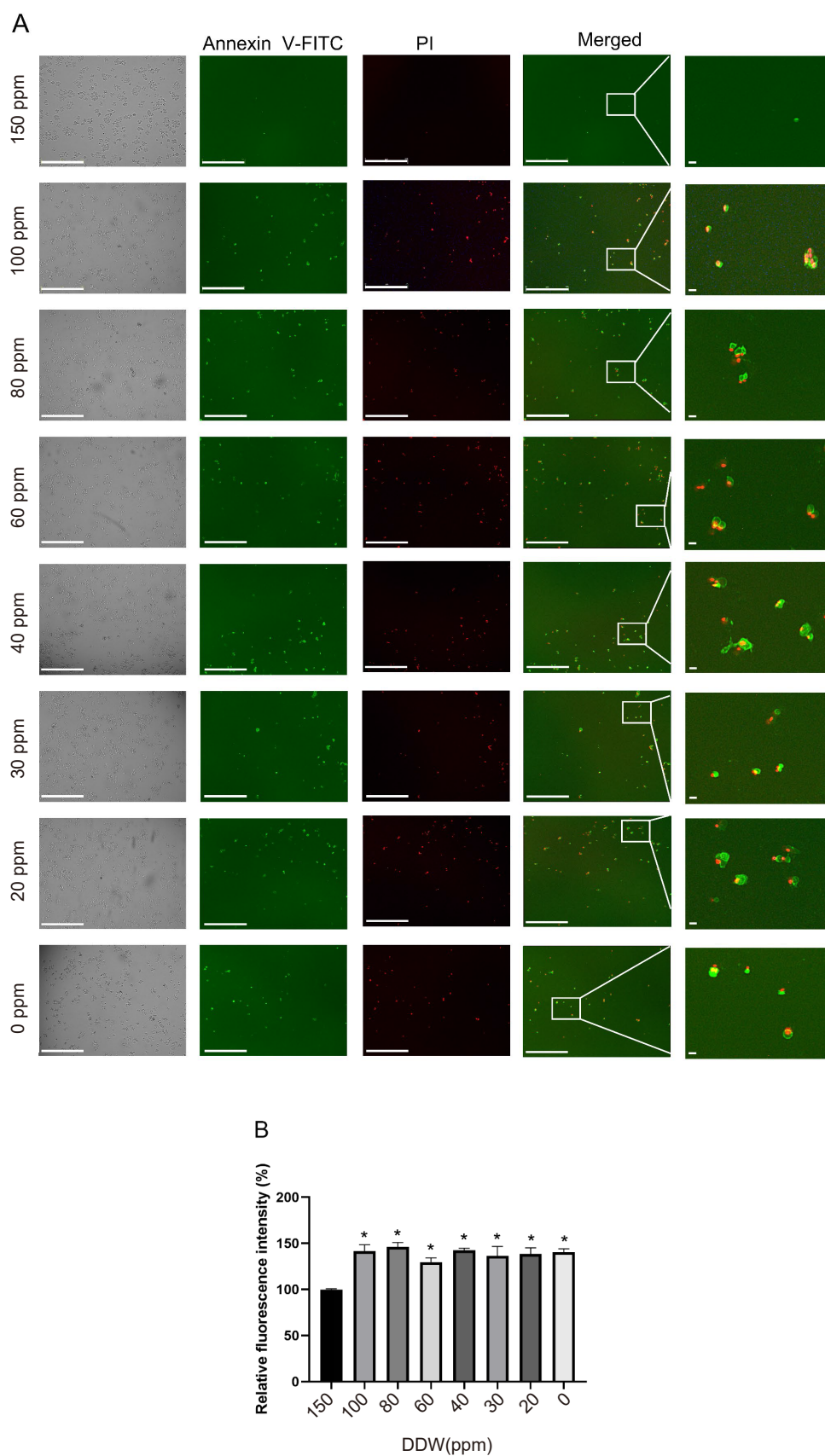


Figure 9 DDW promotes apoptosis in MCF-7 cells. Apoptosis was detected in MCF-7 cells exposed to different concentrations DDW. FITC was used to label apoptotic cells (green fluorescence), while PI was used to label necrotic cells (red fluorescence). The scale bar in the images is 125 μ m. The data are expressed as the mean values $\pm$ SEM from separate experimental trials.

MCF-7 cells. Although deuterium shares chemical similarities with hydrogen, its role in cellular processes is limited. Studies show that most cellular biochemical reactions, particularly those involved in energy conversion and metabolism, depend on hydrogen atoms^[39]. While deuterium's larger mass may impact cellular hydration or metabolic balance, its effect on key cellular reactions is relatively minimal^[41]. Thus, within a certain concentration range, DDW may not induce significant changes in cellular processes, leading to a lack of pronounced dose-dependent inhibition. In addition, tumor cells have a high degree of metabolic flexibility and can compensate for external factors by activating alternative metabolic pathways^[40,41]. Even though DDW may disrupt tumor cell glycolysis, such as the Warburg effect, tumor cells often adapt by adjusting mitochondrial respiration or initiating other pathways to sustain growth^[42,43]. As a result, low concentrations of DDW do not produce a significant dose-dependent inhibitory impact on the proliferation of tumor cells.

Interestingly, drinking water with isotopic compositions of hydrogen (^2H) and oxygen (^{18}O) significantly different from natural water can induce stress responses in the body. Under normal conditions, there is a natural $^2\text{H}/^1\text{H}$ gradient among different tissues and organs^[44]. This gradient may inhibit biochemical processes in cells, including growth, division, and migration. In this study, the observed inhibition of tumor cell proliferation by DDW may be attributed to oxidative stress responses induced by isotopic shock, which in turn affect cell proliferation. Previous studies on isotopic gradients in human biological fluids have confirmed that drinking DDW significantly reduces these gradients^[45]. Moreover, studies suggest that DDW may inhibit tumor growth by interfering with glutathione metabolic pathways^[10]. Under standard circumstances, the deuterium content in the water of the mitochondrial matrix is less than that in the intermembrane water. This difference leads to the formation of a positive deuterium gradient across the inner mitochondrial membrane. This increases the mitochondrial membrane potential, which promotes the production of reactive oxygen species (ROS). As a result, oxidative stress occurs in mitochondria and cells^[10,46]. Additionally, Gyöngyi and Somlyai^[47] found that DDW treatment reduced the expression levels of C-myc, P53, and Ha-ras genes in different organs in nude mice. Salomonsson and Brändén^[48] hypothesized that DDW induces stress signals through cell cycle mechanisms, thereby promoting apoptosis in tumor cells. Therefore, the deuterium content in intracellular water can be significantly reduced through drinking DDW, which may alter the metabolic state and function of the cells, thereby inhibiting the growth of tumor cells. Although current research is still in its early stages and more clinical trials are necessary to confirm its efficacy, the potential of DDW in treating cancer has gradually been recognized. With the progress of further studies, DDW is expected to become a promising adjunctive therapy in modern medicine.

The role of natural products in adjunct disease therapy is increasingly recognized, serving as an important complement in various biological systems, including the extension of lifespan and enhancement of resistance to oxidative stress^[49]. The findings of this research indicate that DDW curbs the proliferation, growth, and migration of tumor cells, highlighting its potential as an adjuvant therapeutic strategy to enhance the effectiveness of existing treatments and mitigate adverse effects. However, it is crucial to conduct additional *in vivo* investigations with animal models to assess the safety and effectiveness of DDW in cancer treatment. Prior research has confirmed that DDW can impede

the growth of cancer cells in mice, but the precise mechanism underlying this effect remains to be delved into^[29]. These animal experiments provide more comprehensive insights into its biological effects, optimal dosing, and potential toxicities. Moreover, exploring the effects and mechanisms of combining DDW with existing therapies, such as chemotherapy and radiotherapy, through both animal studies and clinical research may offer new strategies for cancer treatment.

5 Conclusion

This study investigates the effects of DDW on A549, MCF-7, and HepG2 tumor cell proliferation. The results from CCK-8 assays, real-time analysis, wound-healing assays, and *in situ* fluorescence assays reveal that DDW inhibits the proliferation of all three tumor cell types to varying extents. Additionally, DDW does not significantly affect BEAS-2B cells, highlighting its specificity for tumor cells. These findings suggest that DDW could serve as an adjuvant therapeutic strategy for cancer treatment and support the potential application of DDW as a supplementary treatment in cancer care. However, this study has several limitations, including the reliance on *in vitro* experiments, which may not fully reflect the *in vivo* environment. Additionally, varying concentrations and treatment durations may affect tumor cell responses, requiring further optimization. The specific molecular mechanisms underlying anticancer effects of DDW still need to be fully explored. Overall, the study offers promising evidence regarding the application of DDW in treating cancer approaches.

Conflicts of interest

The researchers assert that there are no recognized financial conflicts of interest that might have seemingly affected the work detailed in this article.

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