# Decreasing Deuterium Concentration of Water: A Possible Tool in Colon Cancer Therphy

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# Abstract

Purpose Recent studies show that the depletion of naturally occurring deuterium can result in tumor regression in both in vitro and in vivo models. In the present paper we report the inhibitory effect of different concentrations of DDW discretely and in combination with 5-FU on HT29 colon cancer cells and the possible mechanisms underlying these effects.

Methods HT29 cells were treated with decreasing deuterium concentrations of DDW individually, 5-FU alone and both from 24 to 72 h. Cellular cytotoxicity of the cells were determined by the MTT proliferation assay. The morphology and structure of the cells were observed by scanning electron microscopy (SEM).

Results We observed the changes in the cell cycle by flow cytometry and alterations in the cellular antioxidant enzymes by transmission chemiluminescence. Treatment with DDW especially in concentrations of 50 and 75ppm alone imposed inhibitory effects on the growth of the cell line and DDW augmented 5-FU inhibitory effects on HT29 cell lines. The cell cycle analysis revealed that DDW caused the cell cycle arrest in the G1/S transition, reduced the number of the cells in the S phase and significantly increased the population of cells in the G1 phase in HT29 cells. We observed that DDW induced the cellular antioxidant defense system. Under SEM examination, the control cells showed smooth surface and more extracellular matrix, while the DDW exposed group showed a rough surface and more microvillus on the cell surface.

Conclusions We conclude that DDW is a promising new adjuvant anti-cancer agent with the potential for future clinical applications.


# Introduction

Colon cancer, which is the result of uncontrolled growth of the epithelial cells in the large intestine, rectum and appendix, is the third most common cancer and the second most lethal cancer in the world [1, 2]. Colon cancer has an extensive epidemiological range and is prevalent in advanced countries, including North America [1–3]. A study in Iran shows that there are 10 to 15 people per 100,000 populace, which is close to developing countries. Colon cancer common in the north and northwest of the country [4]. Depending on the stage of the cancer, various therapies, including the removal of the parts damaged by colonoscopy or surgery, radiation therapy, hormone therapy, and radiotherapy, are used. Despite the advances in chemotherapy, the average survival rate for the patients with advanced colorectal cancer remains extremely poor, thus new agents are needed to establish an effective therapeutic strategy against colorectal cancer [5–7]. There is great interest in developing new preventive and anti-tumor agents that are more effective and less toxic. It has recently been suggested that deuterium-depleted water (DDW) may play a potentially beneficial role in cancer prevention [8]. Deuterium-depleted water (DDW), also known as light water, is water which has a lower concentration of deuterium than occurs naturally (less than 150 ppm). In nature, the ratio between deuterium and hydrogen (D/H) in ordinary water is

approximately 1:6600 [9–13]. Thus, the mass difference between hydrogen and deuterium leads to differences in the physical and chemical behaviors of the two stable isotopes.

In vitro studies have shown that deuterium evacuation causes apoptosis, affects the proto-ontogenesis and tumor-suppressing genes, and undermines the excessive expression of genes that are exposed to carcinogens [14]. A retrospective study

revealed that administration of DDW as an adjuvant increases the life span of mice with ascites tumors[14]. The studies indicated that DDW significantly decreases the growth rate of the cell lines in vitro with the main effects on the tumor cell line remission and inhibits the tumor growth in mice [15]. A recent study among lung cancer patients showed that DDW as an adjuvant to the conventional chemoradiotherapy contributed to the survival [15] and recently Kovacs et al in a study yielded promising results for using DDW as adjuvant therapy to cancer conventional regimen[16]. However, it is yet to be clearly determined the cytotoxic behaviors and mechanism underlying the anti-tumor effect of DDW. In this study, we investigated the in vitro effects of DDW on the growth of the human colon cancer line to determine the nature of DDW effect alone and in combination with a conventional cytotoxic drug and the possible mechanisms of these effects.

## Search Methods

Dulbecco's modified eagle medium (DMEM), fetal bovine serum, phosphate-buffered saline (PBS), trypsin-EDTA solution ( 0.25% trypsin, 1mM EDTA), dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide (MTT) were purchased from Sigma-Aldrich, DDW was provided from Atomic Energy Organization of Iran (Tehran-Iran). The HT29 cell lines were purchased from Pasteur Institute of Iran.

# Cell culture

The human colon carcinoma HT29 cell line was maintained in Dulbecco's modified eagle medium (DMEM) at 37˚C in 5% CO2. For in vitro studies, the cells were seeded in 25 ml cell culture bottles and grown in complete medium. Once cells reached 80% or greater confluence, 0.25% trypsin was used to remove them from the flask. The cells were conveyed to 96-well microtitration plates with a seeding density of 5000 cells per well in 100µL DMEM medium containing 10% FBS and 2mM L-glutamine. The plates were incubated in humidified air containing 5% CO2 at 37°C. The next day, when the cells were entered to the logarithmic phase of growth, exposure period was started by adding DMEM prepared with water containing deuterium concentrations of 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm and 150 ppm, alone or in combination with various concentrations of 5-FU(1,8,16,32and 64µM). Each treatment was run triplicate.

# Cell proliferation assay test

The cytotoxic effects and cellular growth inhibition of DDW, alone and in combination with 5-FU were measured in the HT29 cells every day for 3day by the 3-(4, 5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium-bromide (sigma) colorimetric assay. The MTT proliferation assay is based on the ability of mitochondrial dehydrogenase in viable cells to convert the MTT reagent into a soluble blue formazan dye. At the end of the culture period, 50µl of the MTT reagent was added, and the cells were incubated for 4h at 37˚C. After the removal of the culture medium, the cells were lysed with dimethyl sulfoxide (DMSO) to determine the amount of formazan product. The absorption was measured by a microplate reader (Biotech, Korea) at 570 nm, and the results were expressed as a percent decrease in the cell viability compared with the controls [17]. Each cell sample was measured three times, and the mean was reported.

# Flow cytometry analysis of the cell cycle

The cells were conveyed to 6-well microtitration plates with a seeding density of 300,000 cells per well in 1000 µL. The cells were harvested by trypsinization after DDW alone or combined with 5-Fu treatment, washed twice with PBS and then re-suspended in ice-cold PBS and fixed with ethanol. After removing the ethanol, the cells were washed in PBS. The cells were then centrifuged, and the cell pellets were re-suspended in propidium iodide (PI)/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/ml RNase Aand 10 µg/ml PI) and incubated at room temperature for 30-45min. The colored cell suspensions were introduced into the analyzers for specific analysis.

# Antioxidant enzymes assay

Catalase (CAT) and superoxid dismutase (SOD) were determined in the HT29 cells hemolyzates by colorimetric methods [18, 19].

# 2–5. Evaluation of morphological changes induced in DDW and drug-treated cells

In order to evaluate the morphological changes of the cells treated with DDW, these cells were treated with DDW at 75ppm for one month and were subsequently treated with the 5-FU for 24h. The treated cells were analyzed by SEM.

## Results

# DDW inhibits the growth rates of HT29 cells

To determine the optimal DDW treatment in the HT29 human colon carcinoma cell line, the effect of DDW alone or together with 5-FU was assessed using the MTT cell proliferation assay. The effect of increasing the concentration of 5-FU with $P < 0.0089$ and the effect of various concentrations of deuterium in DDW with $P < 0.0001$ have been reported to significantly decrease the cell survival rate.

Figure 1 shows the survival percentage of the cells growth in the cells treated with different deuterium concentrations of DDW alone. It illustrates significant differences in the surviving fractions of the cell

groups assessed and there is a statistically significant difference in the survival of the HT29 cells when co-treated with 5-FU and different concentrations of DDW.

The surviving fractions of 5-FU-treated groups in combination with DDW at 75 ppm and 100 ppm (Fig. 1A, 1B, and 1C) were significantly decreased compared with 150ppm as a control and there was a significant positive correlation between 5-FU cytotoxity and deuterium depletion in the HT29 cell line, when used together.

Also the highest difference in the percentage of cell survival in the treated samples was observed in 75 and 100ppm concentrations of DDW in comparison with the control samples (DDW 150ppm). In Fig. 1A-E, DDW with 75ppm deuterium significantly induced reductions in the surviving fraction of 5-FU-treated group in comparison to control (12.19%, 8.68%, 27.98%, 15.1% and 28.57 respectively in the 1,8,16,32and 64μM concentrations of 5-FU ) while these reductions in survival fraction of 5-FU-treated(with the above mentioned concentrations) groups in combination with 100ppm DDW, were 20.82%,21.93%, 28.20%, and 27.98% respectively.

The effects of DDW with different deuterium concentrations individually and with 5-FU on the growth of the HT29 cells after 48 treatment are shown in Fig. 2. After 48h treatment of the cells, the effect of the drug concentration, deuterium concentration and as well as synergistic effect of these two agents (P value < 0.0001) was statistically significant. Except for Fig. 2 (A), where the lowest percentage of the cell survival is related to the concentration of DDW30ppm, in the cells groups of Fig. 2B, 2C and 2D, the lowest survival rate is related to the concentration of 100ppm. In Fig. 2E the cell line group, DDW with 75ppm deuterium strikingly decreases the survival fraction of 5-FU treated group compared with the control. The ratio of survival the percentage difference between the cells treated with high concentrations of the 5-Fu (32μM), and the control samples reached its highest value.

In 72h, the cell viability of the treated cells (25, 50, 75,100 and 125ppm of DDW alone or combined with 5-FU) decreased significantly compared with the controls (150ppm DDW). Figure 3 shows the cell survival percentage of the DDW cultured cells with different concentrations of deuterium (, 125, 100, 75, 30 ppm) and 150ppm as the control with or without 5-FU. At a concentration of DDW75ppm, reductions in the survival of the cells treated with 1, 8, 16, 32 and 64μM of 5-FU were 13.77%, 17.74%, 23.8%, 36.3% and 40.24% respectively. In 72-h treatment, the effect of changes in deuterium and drug concentrations and synergistic effect of these two factors with P < 0.0001 was reported statistically.

# Investigating changes in the cell cycle

In order to investigate the effect of the reduction of deuterium alone and with 48 hours of treatment with 5-FU, we studied the cells of HT-29 with PI. Then used flow cytometry to assess the sub-G1 population. In this study, DDW with concentrations of 30, 75, 100 and 150ppm singly and with and 8μM concentrations of 5-FU were evaluated. The cell cycle alterations in the DDW-treated cells were analyzed by flow cytometry. The S and G2 to M phases reduced, whereas the G0 to G1 phases were increased in the DDW/5-FU-treated cells in compared to the control cells. In the cells treated with DDW alone(Table 1), in

concentrations of 75(Fig. 4C) and 100ppm(Fig. 4B), the reductions in the proportion of the cells in the S and G2 phases were observed in the cells treated for 72h as 8, 15 and 5, 63 respectively. Interestingly, the significantly decreased percentages of cells in S phase, along with the dramatically decreased cell population of G2/M phase observed. In my treated cells, there is 7.2 and 0.99 decrease in G2M phase compared with the control (DDW150ppm, Fig. 4A).

Table 1
Cell cycle population in HT29 cells (mean ± SD).

| Cell | DDW (ppm) | 5FU (µM) | G1% | S% | G2M% |
|---|---|---|---|---|---|
| HT-29 | 150 | 0 | 50.35 | 37.19 | 12.46 |
| | 100 | 0 | 56.96 | 31.56 | 11.47 |
| | 75 | 0 | 65.77 | 29.04 | 5.19 |

In the cells treated with DDW and 5-FU (8µM), also, a decrease in cell population in S and G2/M phases and an increase in the G1 phase were observed in comparison with control. The reductions in the S phase compared to the control group for concentrations of 100 and 75ppm from the DDW are 10% and 14.18%, respectively. The synergistic effect of the DDW with 5-FU in a concentration of 8µM has been reported with a significant P value of $< 0.05$(Table 2).

Table 2
Cell cycle population in HT29 cells (mean ± SD).

| Cell | DDW (ppm) | (µM) 5-FU | G1% | S% | G2M% |
|---|---|---|---|---|---|
| HT-29 | 150 | 8 | 53.4 | 30.5 | 16.1 |
| | 100 | 8 | 63.63 | 20.5 | 15.87 |
| | 75 | 8 | 72.01 | 16.32 | 11.67 |

Results of measurement of antioxidant enzymes activity

We determined whether combining DDW with 5-Fu would affect apoptosis in colon cancer cells. As seen in the figure, SOD activity at low concentrations of deuterium, especially in concentrations of 30 and 50 ppm, is significantly different ($P < 0.001$) than other groups in specific concentration of 150ppm (control). SOD activity in the cell treated with the DDW in the combination of 5-FU is higher than the activity of the cells treated with control (150ppm of DDW). An increase in blood SOD with 50.3% at DDW100ppm/5-FU (32 µM) as compared with the control group, was registered (Fig. 5).

Antioxidant enzymes assay also indicated that an increasing of blood catalase with 22.1% at DDW100ppm/5-FU (8 µM) as compared with the control group, was registered. The catalase activity decreased at DDW150ppm with 21%, $p < 0.001$(Fig. 6).

# DDW treatment alters HT29 cell morphology and structure

Under SEM, the control cells (Fig. 7A) showed a more uniform, regular, more extracellular matrix and smoother surface than those exposed to the DDW/5-FU In contrast, HT29 cells exposed to DDW/5-FU had a rough profile and numerous microvilli on the cell surface (Fig. 7B).

# Discussion

The present study aimed to investigate the in vitro effects of the DDW on growth rate, morphology and structure of the HT29 cells, cell cycle distribution and antioxidant enzymes. We found that the DDW significantly suppressed the proliferation of the HT29 cells in 72h. Deuterium depletion increased the cytotoxicity of 5-FU on the colon cancer cell line in a time and concentration dependent manner.

The previous in vitro studies demonstrated that the DDW could be of inhibitory effect on cancers cell lines. In the first study in this regard, Somlyai et al showed that 30 ppm DDW significantly decreased the growth rate of L929 fibroblast cells and also inhibited tumor growth in xenotransplanted mice [20]. Subsequently, the effect of drinking water with DDW with 30ppm deuterium concentration on mice transfected with MCF-7 and MDA-MB-231 cancer cells was investigated. The results of this study indicate an increase in tumor survival time and tumor disappearance in 59% [20]. The other studies presented results of DDW inhibitory effect on PC-3 (cancerous cell line), MDA (breast cancer), and M14 (melanoma) cancer cells lines [21, 22]. Further studies reported DDW inhibitory effect on the human lung carcinoma cell line (A-549) and human nasopharyngeal carcinoma cells established with MTT assay [22, 23].

The results in the present study are remarkably similar to those of the previous studies.

In the previous studies, the growth inhibitory effect of DDW alone and in combination with the known anti-cancer drugs: etoposide, taxol, doxorubicin and cisplatin, showed that DDW synergistically enhances the inhibitory effect of doxorubicin in almost all cancers cultures tested; such a synergistic inhibitory effect was also reported with cisplatin in the breast cancer cell lines (MDA-MB- 231), prostate (PC-3), intestine (HCT-116) and glioblastoma (U-87MG) cell cultures. In the next step, preclinical finding was supported with the clinical studies. DDW as an adjuvant therapy came into use recently. Application of the DDW as an adjutant in conventional chemotherapy in patients with some of malignancy including the prostate, lung and breast malignancies, resulted in clinical benefit in terms of noticeably prolonged median survival time (MST) in different populations under study, decreased the tumor size, the attenuated subjective symptoms and molecular responses [14, 16, 23–26].

In accordance with these previous investigations, our study showed that DDW strengthens the cytotoxic effect of 5-FU as a chemotherapeutic agent on the cancer cell lines of colon cancer. The results of the present study show that the toxic effects of less than 100 ppm concentrations of deuterium depleted water alone reflect the growth of both SW480 and HT-29 cell lines. In addition, the effect of concentrations of less than 100 ppm on DDW increased the toxicity of 5FU.

The synergistic effects of DDW and 5-FU can be explained based on their common characteristics in terms of induction of apoptosis and cell cycle arresting. As reported, 5-FU impair in the synthesis of

biocompatible macromolecules and inhibitory effects on the cell cycle by inhibiting the enzyme thymidilate synthase (TS) and incorrect placement within the RNA building. Similarly, the features of stopping apoptosis and cell cycle are also described for DDW [27, 28].

Our results support the hypothesis of Laskey et al, who hypothesized that mechanisms exist in the cells that detect changes in deuterium concentration. The possible cause of the cellular growth inhibition may be due to the changes in the isotope ratio of deuterium to hydrogen, because a number of studies have shown that high concentrations of deuterium are needed for the cell growth and cellular division and thus by decreasing the amount of deuterium the growth rate of the cancer cells also declines and the time required to reach the appropriate d/h ratio is high. [29, 30].

In order to investigate the mechanisms of the cell growth inhibition under treatment with deuterium depleted water alone or in combination with 5-0FU, the

cell cycle changes of the treated and control cells were studied.

In the present study, we found that DDW 75ppm alone led to stop in cellular proliferation at the G0 / G1 stage and decreased the S phase cell population. A greater proportion of the DDW-treated HT29 cells were arrested at S phase in the cells treated with the DDW and 5-FU combinations rather than at DDW treated alone, as observed by flow cytometry. Lowe D/H ratio triggered the molecular mechanism that finally prevented the cell to enter into the S phase.

A study was conducted in 2009 on the A549 cells and the cellular changes of the treated cells at a concentration of 50ppm of deuterium water compared with the control (150 ppm)[22]. The results of this study showed that the cell population in cell cycle S of DDW treatment groups increased by 11.38% and the control group in G0/G1 and the G2/M, respectively, decreased by 88%/4% and 49/6% [22]. However, the results presented in the study by H Wang et al in 2013[24] contradicted the previous research results. The study of H Wang et al on the three concentrations of 50, 75 and 100ppm of the DDW in five NPC cells, revealed that the cell cycle undergoes a change due to the decrease in deuterium concentration so that the decrease in the number of cells in the S phase and the increase in the cells in the G1 phase were significantly more than those of the control group.

The results of this study were consistent with the results of the H Wang study. As an interpretation of the cause of the changes in the cell cycle, it can be concluded that the cell division is sensitive to the changes in the concentration of intracellular deuterium and the natural concentration of deuterium is essential for the onset and progression of the cell growth. To enter the cells to stage S and start dividing the cell, the D/H ratio threshold is required (23). When the cells are cultured in a low concentration deuterium diet, the growth of the cells is inhibited by increasing the time needed to reach the proper ratio of D/H [29, 30]. D/H relative to the normal cells is faster than that of the cancer cells.

Subsequently, the changes in the activity of superoxide dismutase and catalase enzymes, which are important antioxidant enzymes, were studied. Among the active oxygen species, reference may be made

to superoxide and hydrogen peroxide radicals which are produced as a product of the natural metabolism of oxygen in the cells and tissues. Due to the intrinsic activity of ROS, these compounds can easily enter into reactions that ultimately damage the cells. Most of the cancer cells exacerbate and deteriorate due to the oxidative stress induced by oncogenic stimuli, increased metabolic activity, and mitochondrial malformations [31]. In cancer cells, the levels of the glycoltic and cellular respiration reactions are higher and lower than normal cell levels respectivly, and so this difference leads to an increased oxidative stress [32, 33]. This high oxidative state in the cell causes the tumor to grow through the cellular growth increment, and the suppression of antitumor activity can damage the DNA structure [34]. In our study on the antioxidant system at the cell surface, the level of enzyme activity of superoxide dismutase and catalase increased in the HT-29 cells at the same concentration of DDW, which coursed the highest growth-inhibitory and cell cycle-arresting properties. Therefore, it can be concluded that the cellular degradation or cellular growth inhibition, cell cycle arresting and antioxidant enzymes activity increases ultimately prevent the growth of the cancer cells by DDW alone or together with 5-FU, with a high degree of inhibition of cell growth. This study is in accordance with the study conducted by Lucia Olarium et al. Lucia Olarium et al. In 2007, measured the changes in the activity of red blood cell antioxidant enzymes in the DDW-treated rats and showed that the DDW has the antioxidant effects [35].

To investigate the morphological changes induced in DDW-treated cells alone or in combination with the 5-FU, we observed the morphology and structure of HT29 cells by two optical microscopes and scanning electron microscopy. According to the optical microscope observations, HT29 control cells were spindly smooth with a flat surface, while DDW-treated cells and the 5-FU were spindle-shaped and showed irregularities. In the observations made by the scanning electron microscope, the treated cells showed a more roughness than control. The extracted microscopic cells of the treated cells confirm the membrane degradation.

# Conclustion

In conclusion, according to our in vitro study, deuterium depleted water alone at concentrations lower than 125 ppm, especially at concentrations of 75 and 50ppm imposes growth inhibitory effect on the human colon cancer cells and it augments the anti-proliferatory effect of the known chemotherapeutic agent, 5-Fu on the human neoplastic cell lines of the colon. To elucidate the mechanism of DDW-induced cancer cell growth inhibition, we found that DDW exerts effects on the cell cycle and changes in the cell configuration and induces anti-oxidant enzymes in vitro. Collectively, our results suggest the potential for DDW as an anti-tumor adjutant with clinical application.

# Declarations

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**Author Contribution:**

Conceptualization: [Kamal Yavari], Methodology: [Kamal Yavari], investigation: [Kamal Yavari, Zahra Zarei Mahmoudabadi], Original draft preparation: [Kamal Yavari], Writing – review & editing: [Kamal Yavari], Data curation: [Kamal Yavari], Resources: : [Kamal Yavari], Supervision: [Kamal Yavari], Validation : [Kamal Yavari], Visualization[Kamal Yavari, Zahra Zarei Mahmoudabadi].

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# Figures

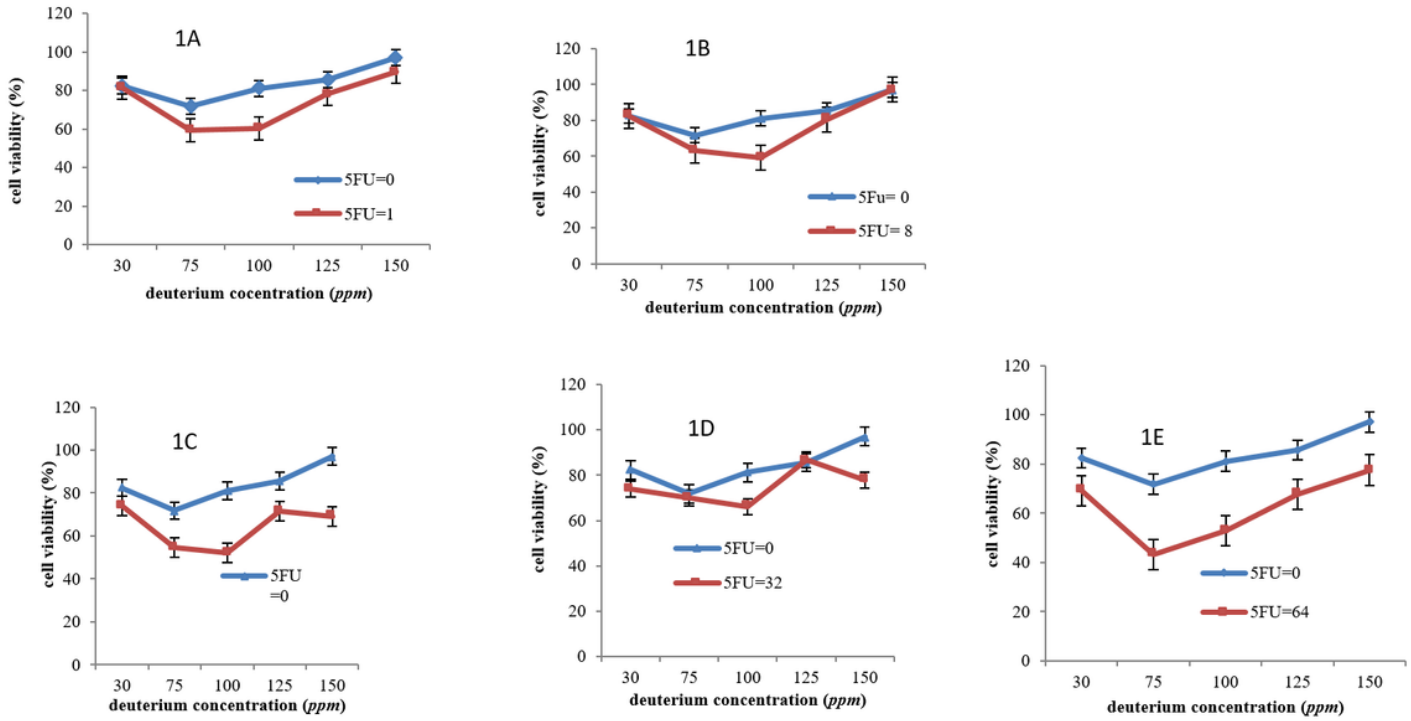
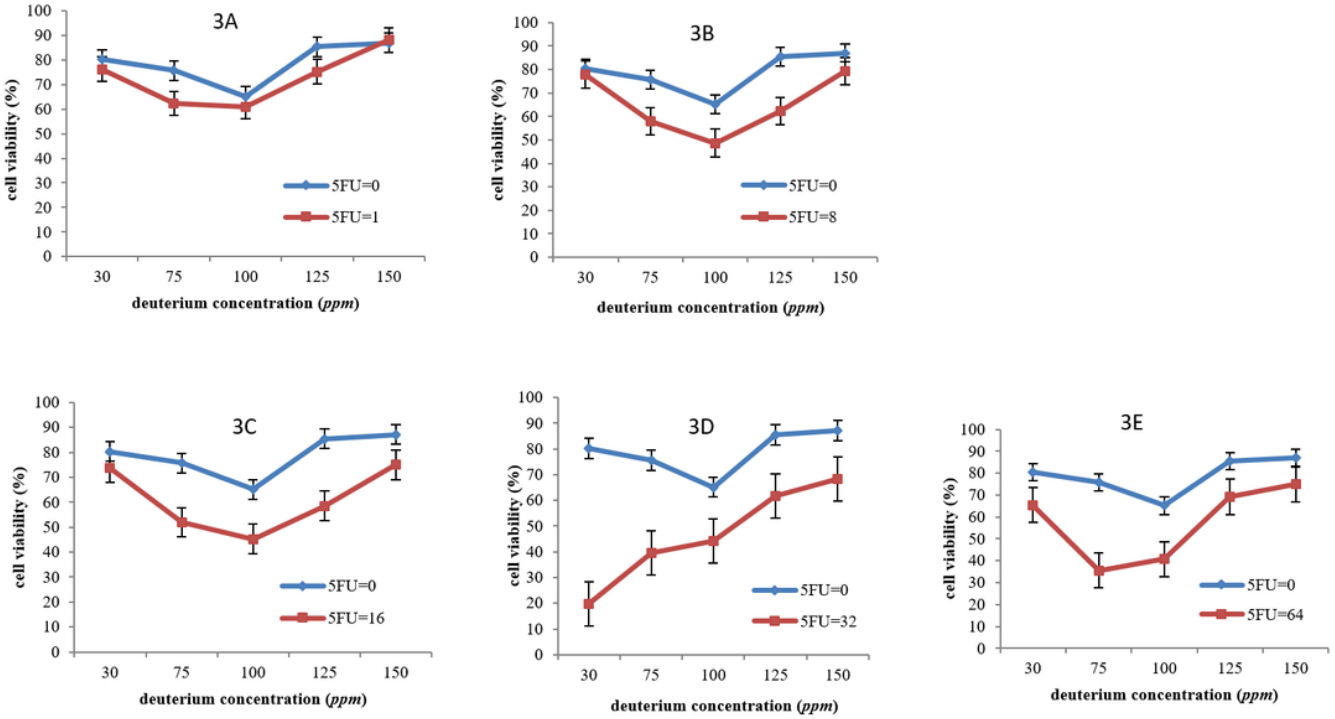


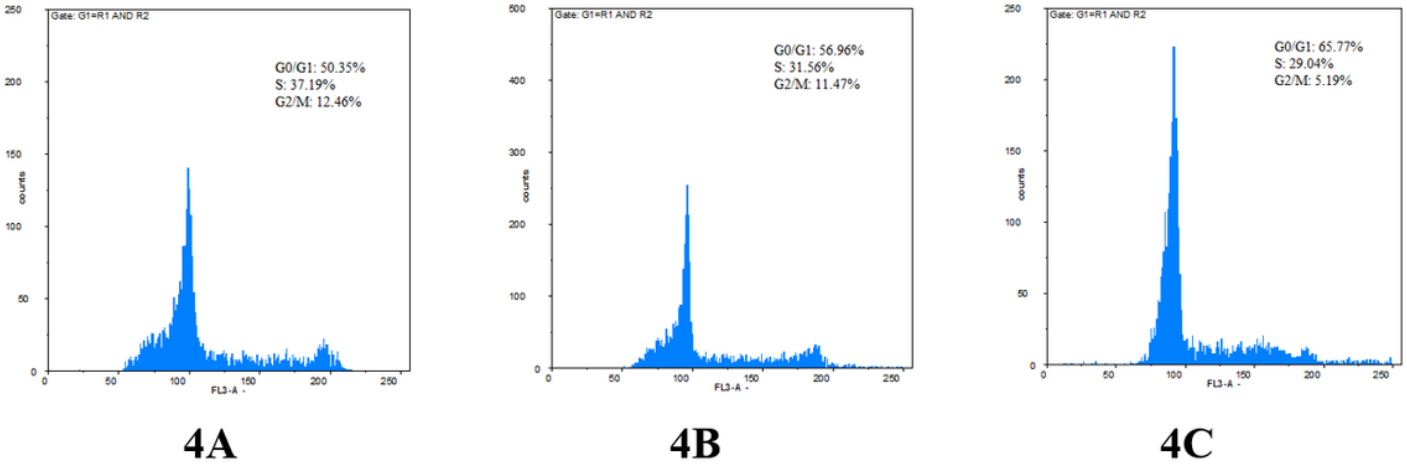

**Figure 1**

**The effect of different concentrations of 5FU and DDW on the survival rate of HT-29 cell line compared with control 24h after treatment**. (1A) 1μM concentration of the 5-FU, (1B) 8μM (1C) μM16 (1D) 32μM and (1E) 64μM concentration of 5-FU. The displayed data are± S.D mean ± 3 times the data of the experiments




## Figure 3

**Effect of different concentrations of 5FU and DDW on survival rate of HT-29 cell line compared with control 72h after treatment.** (3A) 1μM, (3B) μM8 (3C) 16μM, (3D) 32μM and (3E) 64μM concentrations of 5-FU. The displayed data are ± S.D mean ± 4 times the data of the experiments




## Figure 4

**Histogram of the DNA of the HT29 cell line treated with DDW alone**. (4A) 150 ppm as control; (4B) 100 ppm; and (4C) 75 ppm

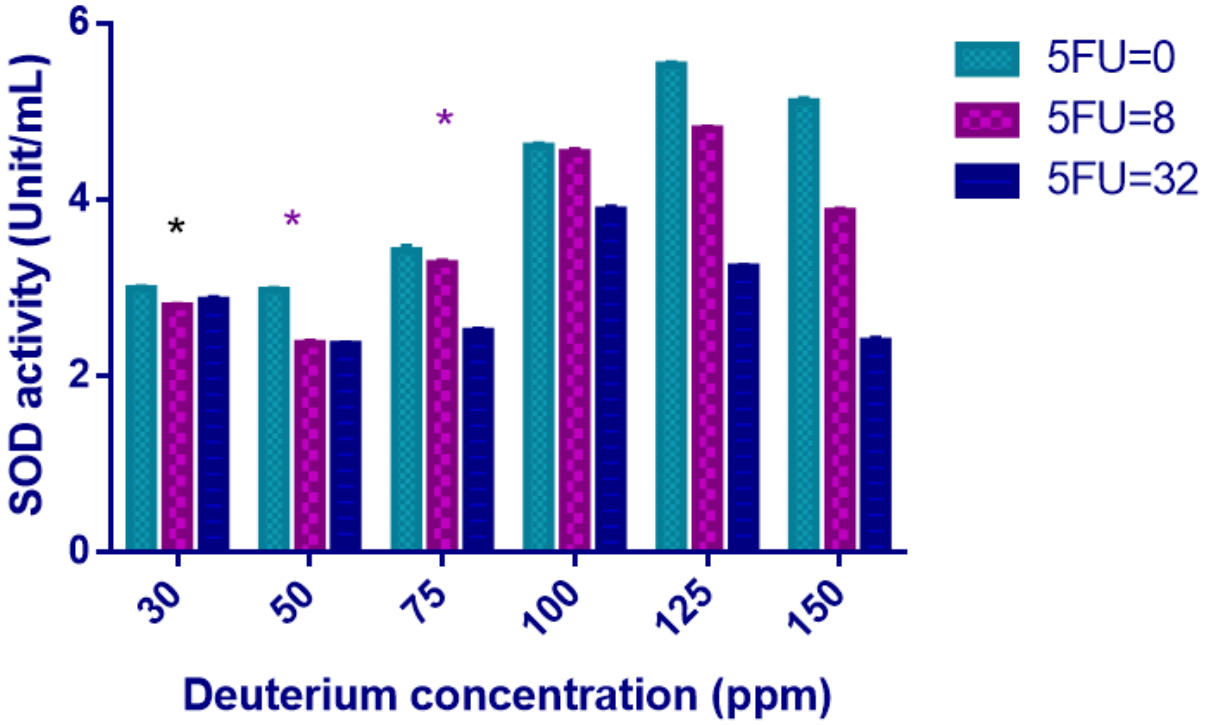



Figure 5

Activity profile of the superoxide dismutase enzyme in HT-29 cells after 48 hours of treatment with DDW and 5-FU

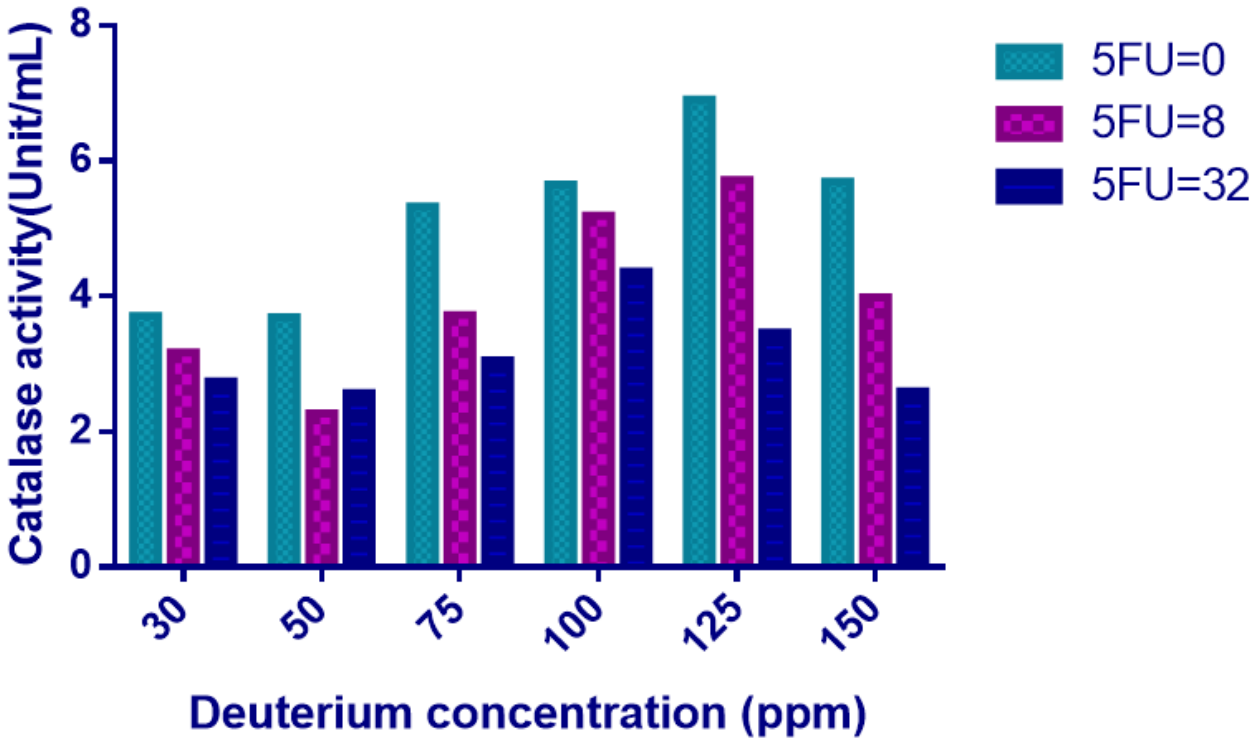



**Figure 6**

**Catalase activity in HT-29 cells after 48 hours of treatment with DDW and 32µM concentrations of 5-FU**. Each column of the mean of two tests was independent and the difference between the groups was calculated using graph pad software, two ways ANOVA

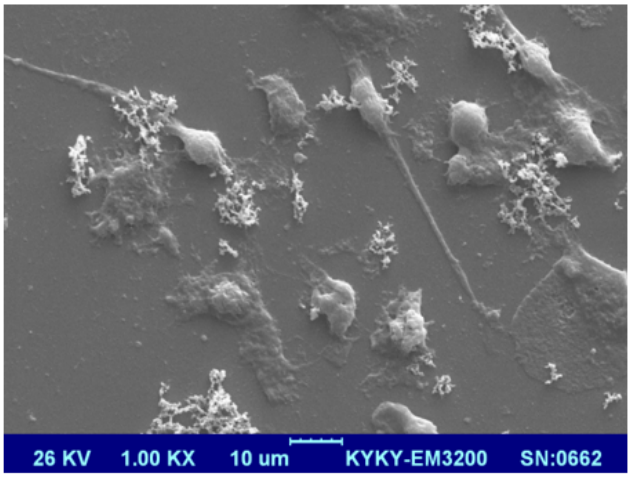



**Fig.7A**

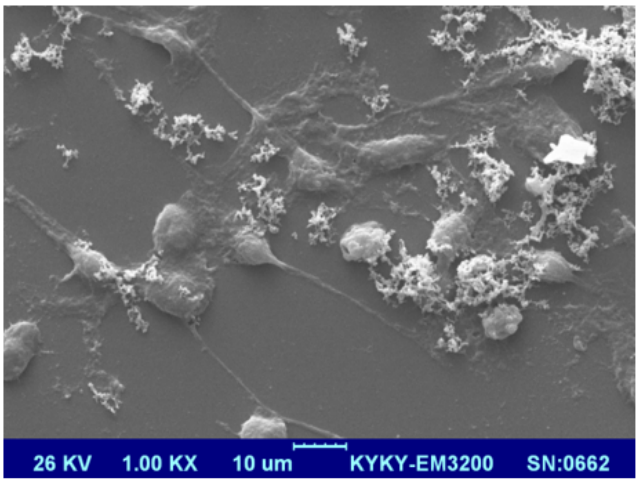



**Fig.7B**

**Figure 7**

**Scanning electron microscopic imaging of HT29 cells** (A) grown in DDW with a concentration of 150ppm and (B) with a DDW75ppm combined with 16μM of 5-FU

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graphicalabstract.jpg
- hilight.docx