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# Deuterium Depleted Water Inhibits the Proliferation of Human MCF7 Breast Cancer Cell Lines by Inducing Cell Cycle Arrest

Kamal Yavari[a] and Lida Kooshesh[b]

[a]Nuclear Science and Technology Research Institute, Tehran, Iran; [b]Department of Biology, Tehran North Branch, Islamic Azad University, Tehran, Iran

**ABSTRACT**

Recent studies have shown that the depletion of naturally occurring deuterium can result in tumor regression. The aim of the present study was to show the growth inhibitory effects of DDW discretely and in combination with 5-FU on MCF-7 breast cancer cells and to determine possible mechanisms underlying these changes. MCF7 cells were grown in RPMI medium with decreasing deuterium concentrations of DDW individually, 5-FU alone and both for 24, 48, and 72 h. Cell viability was determined with the MTT assay. The cell cycle and antioxidant enzymes status were measured using flow cytometry and quantitative luminescence methods, respectively. Our results showed that treatment with DDW especially in 30–100 ppm concentrations imposed the highest cell growth inhibitory effect. 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 MCF-7 cells. The activities of superoxide dismutase (SOD) and catalase (CAT) enzymes also increased in the same low concentrations of DDW. In conclusion, DDW can open new strategic approach in breast cancer therapy.

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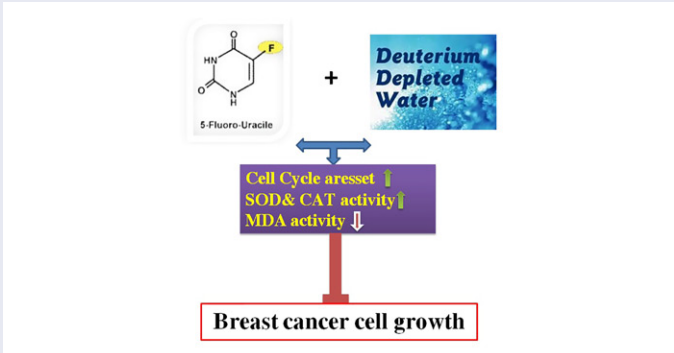
**HIGHLIGHTS**

- DDW cause lethality in cancer cells.
- DDW augmented 5-FU inhibitory effects on breast cancer cell lines.
- Cell inhibitory results lead to the discovery of synergic effects of DDW–drug combinations
- Synergistic anti cancer effects of DDW with 5-FU is enhanced by decreasing deuterium content of the DDW.
- DDW exerts effects on the cell cycle, changes in cell configuration and induces antioxidant enzymes *in vitro.*
- DDW can be considered as an adjuvant to conventional anticancer agents in future trials.

**GRAPHICAL ABSTRACT**




**CONTACT** Kamal Yavari kyavari@aeoi.org.ir Nuclear Science and Technology Research Institute, Tehran, P.O. Box: 14395-836, Iran.
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## Introduction

Breast cancer is one of the most prevalent female's cancers in the world and its incidence rates are increasing. In spite of advances in breast cancer diagnosis and therapy techniques, but death rates still remain high and so new therapeutic strategies are needed for breast cancer treatment (1–7). Surgery is the main modality treatment of primary breast cancer, but chemotherapy, hormone therapy and radiation therapy all have significant roles in the treatment of breast cancer. 5-Fluorouracil (5-FU) is a widely used as an active agent against human cancers. Since 1957, it is used for patients with breast and other cancers. In addition, its combination with other anti-cancer drugs has improved the response rates to 40–50% (8, 9). 5-FU is a heterocyclic aromatic compound with a structure similar to DNA and RNA that has a fluorine atom replacing the hydrogen atom at the C-5 position. 5-FU can be incorporated into RNA and DNA of a cell and leading to the cytotoxicity and apoptosis (8, 9).

Despite extensive use of this variety of therapies, chemotherapy, radiation therapy and hormonal therapy can contribute to the development side effects, including: fatigue, lymphedema, cognitive impairment (10–17), fatigue (18–20), sleep difficulty (21), pain (22–24), chemotherapy-induced peripheral neuropathy, cardiotoxicity (25, 26), and bone loss (27, 28). These side effects negatively influence quality of life and daily activities of breast cancer pateint, lead to noncompliance treatments, and ultimately affect survival outcomes (29, 30). Approximately 30–50% of patients have the recurrence of the tumor and the average survival rate for the patients with advanced breast cancer remains extremely poor. Thus, interventions are needed to enhance quality of life and functional capacity, and improve adherence to therapy and thus, there is a critical need to develop new therapeutic agents (7).

Deuterium is the heavier stable isotope of hydrogen. Since 1992, great importance has been attached to the deuterium content in water. It has been known for decades there exist strong mass differences between hydrogen and deuterium and their different masses cause these isotopes to behave differently in chemical reactions. The concentration of deuterium is about 150 ppm (over 16 mM/L) in regular water and 12–14 mM/L in living organisms. In order to reveal the possible role of naturally occurring D in living organisms, the replacement of regular water with deuterium-depleted water (DDW) in a range from 25 to 125 ppm was investigated in cell cultures. As a control of these experiments, regular water (150 ppm) was considered to compare the effects of decreasing the amount of dutrium in biological systems.

It has been suggested that DDW might play a role both in treatment and prevention of cancers. Some studies yielded promising results for using DDW as adjuvant therapy to cancer conventional regimen (31–36). However, the precise mechanisms underlying the cytotoxic properties and antitumor effects of DDW are still unclear. In this study, we have investigated the effect of DDW alone or combined with 5-Fu on MCF7 human breast cancer cell lines to analyze their in vitro effects on the tumor cellular growth, cell cycle and anti oxidant status.

## Materials and Methods

RPMI1640 (Gibco, USA), Fetal bovine serum, phosphate-buffered saline (PBS), trypsin–EDTA solution (0.25% trypsin, 1 mM EDTA), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. DDW was provided from Atomic Energy Organization of Iran (Tehran-Iran). The Human breast cancer cell line (MCF7) was purchased from Pasteur Institute of Iran.

### *Cell Culture*

The human breast cancer cell line (MCF7) was cultured in RPMI 1640 medium at 37 °C in 5% $CO_2$. For *in vitro* studies, the cells were seeded in T25 tissue culture flasks and then cells were grown in complete growth medium. After the cells reached the 80% confluence, 0.25% trypsin was used mainly to detach the cells from the flask. Next, MTT-based cytotoxicity assay was carried out according to the following protocol.

### *MTT-Based Cytotoxicity Assay*

MTT-based cytotoxicity assay was carried out as short-form and long-form assays. In the short time assay, the cells were seeded on a 96-well microtitration plates at a density of 5000 cells per well in 100 µL RPMI medium. The cells were incubated at 37 °C and 5% $CO_2$. After entering the cell to the logarithmic phase of growth, next day, exposure period was started and the cells was treated with RPMI1640 media containing different deuterium concentrations of DDW (30–150 ppm), alone or in combination with

various concentrations of 5-FU (2–32 μM). After 24 h, media containing DDW and 5-FU were removed from the wells and recovery period started in order to demonstrate retention of regenerative capacity of the exposed survived cells. Duration of recovery periods was considered 72 h and in this period, medium of the plates daily replaced with fresh growth media. Each treatment was run triplicate. The cells untreated with 5-FU were considered as controls. Of course, DDW150ppm was considered as controls for all experiments. In the long-form assay, the cells were seeded on a 96-well microtitration plates at a seeded of 1,000 cells per well. DDW and 5-FU were added three days later with the same concentrations mentioned for short-form assay. In this form of study, the exposure and recovery duration times were considered as 72 and 96 h, respectively.

In both types of assays, at the end of the recovery periods, 20 μL MTT (5 mg/mL) solutions were added to each well and then the plates were further incubated for 4 h. Next, the entire medium was removed and the formazan product dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader (Biotech, Korea) at 570 nm and viability was expressed as percentage of viability compared to nontreated controls (37). Each cell sample was measured three times and reported as the mean results.

### Flow Cytometry Analysis of the Cell Cycle

The cells were conveyed to 6-well microtitration plates at a density of 500,000 cells/well in 1000 μL. The cells were treated with DDW alone or combined with 5-FU for48h, then washed twice in ice-cold PBS and re-suspended in ice-cold PBS and fixed with ethanol. After removing the ethanol, cells were washed after centrifugation, and resuspended in 1 mL propidium iodide (PI)/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/mL RNase A and 10 μg/mL PI) for 30–45 min at room temperature. Stained cell suspensions were analyzed with the Flowmax software.

### Antioxidant Activity

#### Superoxide Dismutase (SOD)

For SOD activity (U/mL) assay, the cells were treated with various concentrations of 5-FU and DDW for 48 h and absorbance was recorded at 560 nm using spectrophotometer. The cell homogenate was prepared in ice-cold reaction solution containing 1.42% Triton X-100, 16 mM of pyrogallol solution, 0.98 mM of NBT and 0.5 mM of Tris–cacodylic buffer.

#### Catalase (CAT)

The activity of the enzyme catalase was examined in cells cultured for long 48 h with media containing DDW and 5-FU and the changes in absorbance were recorded at 240 nm against the reagent blank using spectrophotometer. The reaction solution for catalase activity (U/mL) composed of 1% Triton X-100, 1 mM of EDTA, 1 mM of PMSF and 100 mM of potassium phosphate buffer.

### Malonyl Dialdehyde (MDA)

Cell membrane damage of DDW and 5-FU treated cells determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532 nm)/fluorometric ($\lambda ex = 532/\lambda em = 553$ nm) product, proportional to the MDA concentration (μM MDA/gFW) using $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ formula.

## Statistical Analysis

Statistical analyses were performed with two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. For all tests, a $P$ value of 0.05 was considered statistically significant. The statistical analyses were carried out using Graph Pad Prism, version 5.01 (Graph Pad, San Diego, CA, USA).

## Results

Evaluation of the cell growth through short-term treatment by DDW using MTT test:

Figure 1 represents of survival fraction curve of MCF7 cells 24 h after treatment with various concentrations of DDW and 5-FU.

As shown in Figure 1, there was seen a significant differences in the cell survivals of the cells co-treated with increasing concentration of the 5-FU (with a $P$ value $< 0.0154$) and different concentrations of DDW ($P$ value $< 0.001$). There was a positive correlation between increasing concentrations of 5-FU and cell cytotoxicity rate. Different concentrations of DDW alone demonstrated differences in the surviving fractions of MCF7 cells. DDW with 30 ppm deuterium strikingly decreased the survival fraction of cell compared to control cells (DDW150ppm) assessed. Statistically significant effect on cell viability was identified in the co-treated 5-FU and DDW cells ($P$ value $< 0.001$).

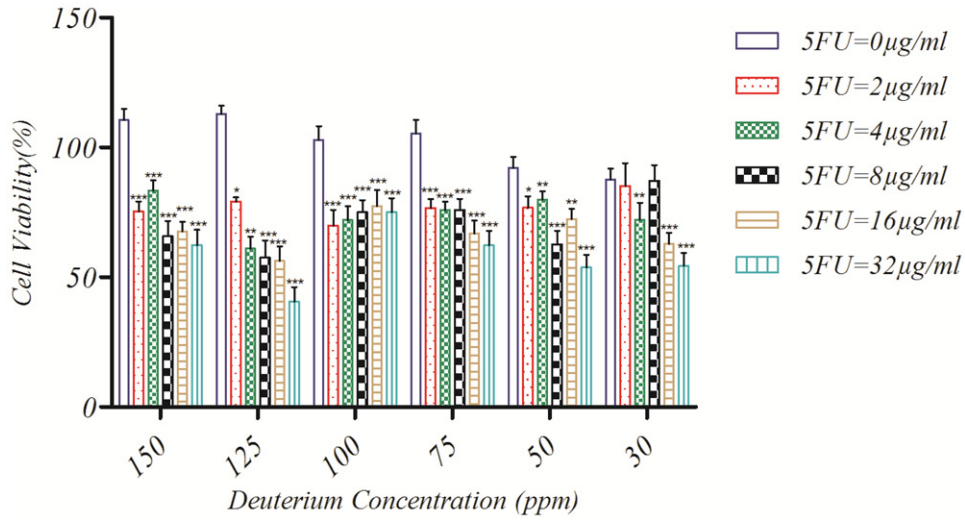


**Figure 1.** The effect of different concentrations of 5-FU and DDW on the survival rate of MCF7 cell line compared with control 24 h after treatment in short-form assay.

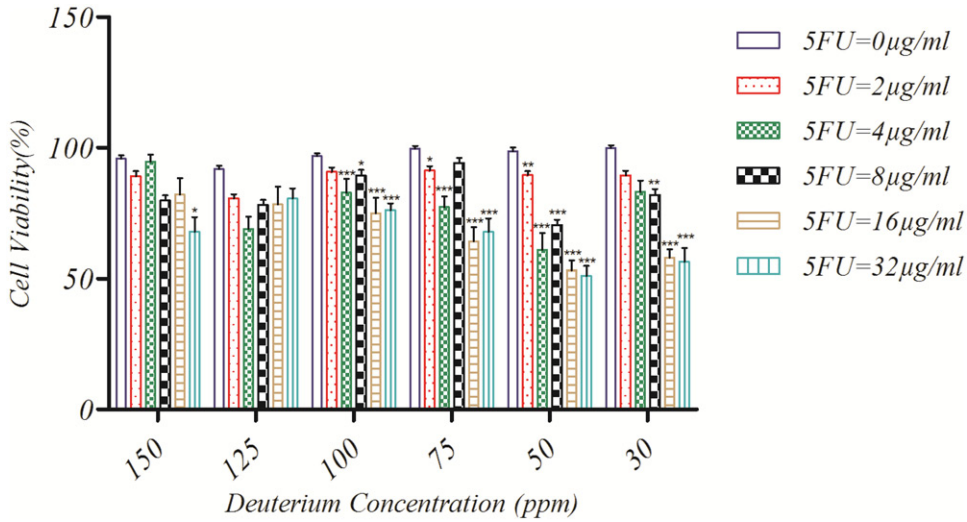


**Figure 2.** The effect of different concentrations of 5-FU and DDW on the survival rate of MCF7 cell line compared with control 48 h after treatment in short-form assay.

In the 48-h short MTT form assay, the combined effects of increasing concentration of 5-FU ($P$ value $< 0.244$) and decreasing of deuterium concentration of water ($P$ value $< 0.001$) showed a significant reduction in cell survival. After a 48 h treatment, synergistic antiproliferative effect of 5-FU and DDW was significant ($P$ value $< 0.0001$) (Figure 2).

Figure 3 shows survival percentage of the cells were treated with different concentrations of deuterium ($P$ value $< 0.001$) alone or with 5-FU ($P$ value $< 0.0425$) for 72 h. The highest and lowest inhibitions in cell growth were observed in cells treated with 30 and 150 ppm of DDW, respectively. The proliferation inhibition rates of cancer cells after treatment with DDW75ppm in combination with 0, 2, 8, 16 and 32 µM of 5-FU were 13.77%, 17.74%, 23.8%, 36.3% and 40.24%, respectively.

### *MTT Results in Long-Form Study*

Figure 4 shows the effect of increasing concentration of 5-FU ($P$ value $< 0.479$) and decreasing concentration of DDW ($P$ value $< 0.001$) on the viability of MCF7 cancer cell lines in the long-form MTT assay. Increasing the concentration of 5-FU from 2 to 32 µg/mL resulted in decreased cell viability. At all 5-FU concentrations, the highest

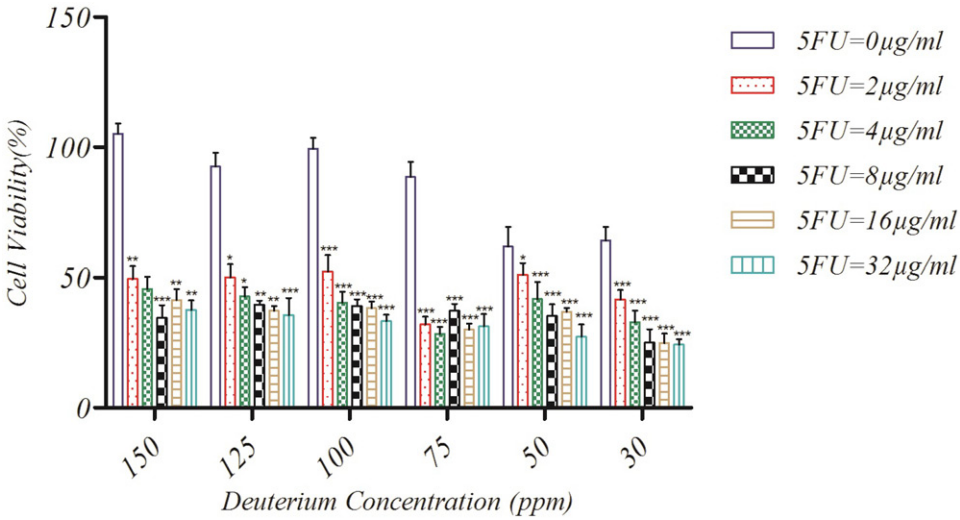


**Figure 3.** The effect of different concentrations of 5-FU and DDW on the survival rate of MCF7 cell line compared with control 72 h after treatment in short-form assay.

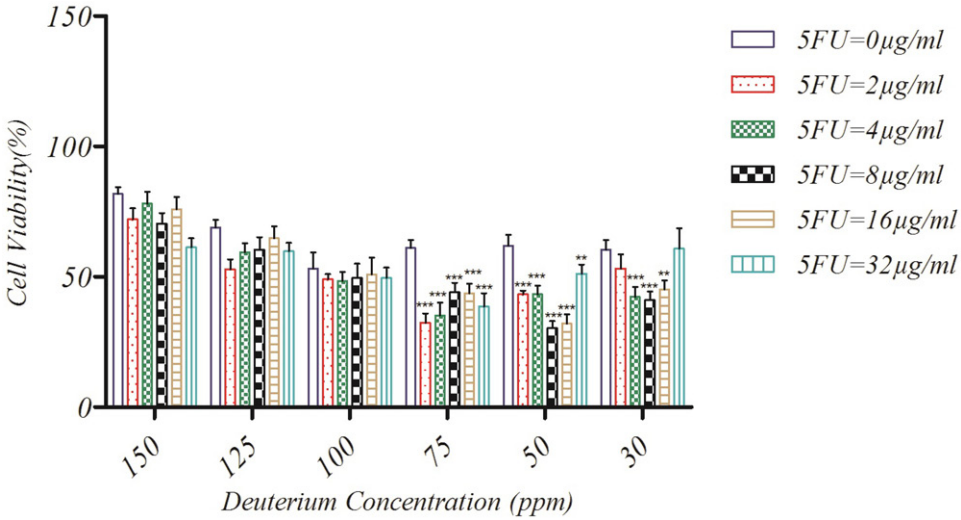


**Figure 4.** The effect of different concentrations of 5-FU and DDW on the survival rate of MCF7 cell line compared with control in long-form assay.

percentage of cell survival was related to DDW150ppm. DDW without 5-FU, also had a different effect on cell survival and the highest and lowest cell survival rates related to DDW 150 and 30 ppm, respectively (Figure 4).

### *Cell Cycle Changes*

In this study, the cell cycle alterations in the cells treated with DDW singly and with 8 and 32 μM concentrations of 5-FU were analyzed by flow cytometry. Flow cytometric study showed changes in the cell cycle outflow of treated cancer cells, involving reduced numbers of S and G2 to M phase's cells and increased numbers of G0 to G1 phase cells in the DDW/5-FU-treated cells compared to the corresponding controls.

### *Cell Cycle Changes in MCF7 at 0 M Concentration of 5-FU*

Cells in the S phase fraction decreased 2.25% at DD30ppm, 1.82% at DDW75ppm, 0.25% at DDW100ppm and were undetectable at DDW125ppm and DDW50ppm compared to the control group (DDW150ppm). In the M/G2 phase, the decrease in cell population of media containing deuterated water (30, 50, 75,100, 125 ppm) was 1.36, 6.54, 2.26, 4.48, and 5.87% compared to cells

**Table 1.** Cell cycle alterations in MCF7 cell lines after treatment with various concentrations of Deuterium-depleted water (30–150 ppm) alone and combined with various concentrations of 5-FU.

| Cell line | Concentration of DDW (ppm) | Concentration of (μM) 5FU | *G*1% | *S*% | *G*2M% |
|---|---|---|---|---|---|
| MCF7 | 30 | 0 | 53.317 ± 0.982(a) | 30.857 ± 0.600(*) | 13.450 ± 0.918(ac) |
| | 50 | 0 | 47.527 ± 1.151(b) | 32.783 ± 0.847(*) | 8.470 ± 0.892(be) |
| | 75 | 0 | 59.160 ± 0.785(c) | 31.207 ± 0.841(*) | 12.627 ± 0.988(c) |
| | 100 | 0 | 56.347 ± 1.175(de) | 32.647 ± 1.270(*) | 10.807 ± 0.704(d) |
| | 125 | 0 | 57.467 ± 1.067(cef) | 33.447 ± 1.155(*) | 8.980 ± 0.784(e) |
| | 150 | 0 | 55.907 ± 0.965(df) | 32.743 ± 1.109(*) | 15.087 ± 0.955(f) |
| | 30 | 8 | 70.593 ± 0.519(ad) | 15.260 ± 0.815(ade) | 4.530 ± 0.605(ab) |
| | 50 | 8 | 77.490 ± 0.943(b) | 11.470 ± 0.242(b) | 4.560 ± 0.907(b) |
| | 75 | 8 | 73.080 ± 1.005(c) | 17.543 ± 1.118(ch) | 8.503 ± 1.097(cef) |
| | 100 | 8 | 71.087 ± 0.942(d) | 15.567 ± 1.112(dcfg) | 8.467 ± 0.89(dcg) |
| | 125 | 8 | 63.527 ± 1.305(e) | 14.687 ± 1.076(eg) | 9.123 ± 0.853(edh) |
| | 150 | 8 | 66.213 ± 1.076(f) | 17.240 ± 0.896(fah) | 8.340 ± 1.115(fgh) |
| | 30 | 32 | 76.387 ± 0.940(a) | 10.970 ± 0.836(acg) | 5.390 ± 0.896(abghd) |
| | 50 | 32 | 70.547 ± 0.325(be) | 13.400 ± 1.173(bie) | 7.573 ± 0.776(bcjk) |
| | 75 | 32 | 88.990 ± 0.819(c) | 12.277 ± 0.977(cdbh) | 7.613 ± 1.165(cail) |
| | 100 | 32 | 72.467 ± 1.104(d) | 12.603 ± 0.506(daij) | 4.473 ± 0.983(de) |
| | 125 | 32 | 69.113 ± 0.902(e) | 12.343 ± 1.040(eghj) | 6.123 ± 0.993(egijf) |
| | 150 | 32 | 65.193 ± 1.046(f) | 18.187 ± 0.821(f) | 6.663 ± 0.405(fhkl) |

Data are presented as the means ± SD for three independent experiments. The similar letters show non-significant difference at $P \leq 0.05$.

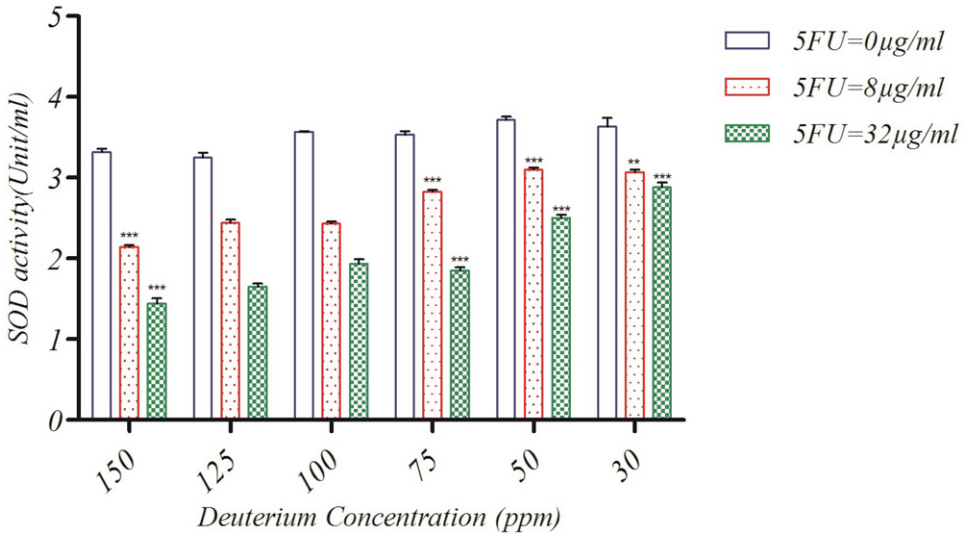


**Figure 5.** Activity profile of the superoxide dismutase enzyme in MCF7 cells after 48 h of treatment with DDW and 5-FU.

that were cultured in DDW150ppm medium (Table 1).

### Cell Cycle Changes at DDW + 5-FU (8 g/mL)

DDW and 5-FU (8 μg/mL) combination induced a more DDW concentration-dependent decrease in the S population (1.57, 5.91, 1.79, and 2.3% at DDW30ppm, DDW50ppm, DDW100ppm, and DDW125ppm, respectively, as compared to DDW150ppm). Cells in the M/G2 fraction decreased by 4.09% at DDW30ppm and 4.01% at DDW50ppm (Table 1).

### Cell Cycle Changes at DDW + 5-FU (32 g/mL)

The population of cells in the S phase was also decreased more by DDW+ 5-FU (32 μg/mL) in all the DDW concentrations tested. The decreasing population of cells in S phase of cell groups exposed to media containing low deuterium concentrations (30, 50, 75, 100 and 125 ppm) in combination with 5-FU (32 μg/mL) compared to the control group (DDW150ppm) were 7.22, 4.94, 5.9, 5.45, and 5.53%, respectively. At concentrations of 30, 100 and 125 ppm, the cell population decreased in the M/G2 phase by 1.33, 1.85 and 0.81%, respectively (Table 1). The synergistic effect of the DDW with 5-FU in a concentration of 32 μM has been reported with a significant $P$ value of $<0.05$.

### SOD Activity

We determined whether combining DDW with 5-Fu would affect antioxidant systems in breast cancer cells. As seen in Figure 5, SOD activity at low concentrations of deuterium, especially in concentrations

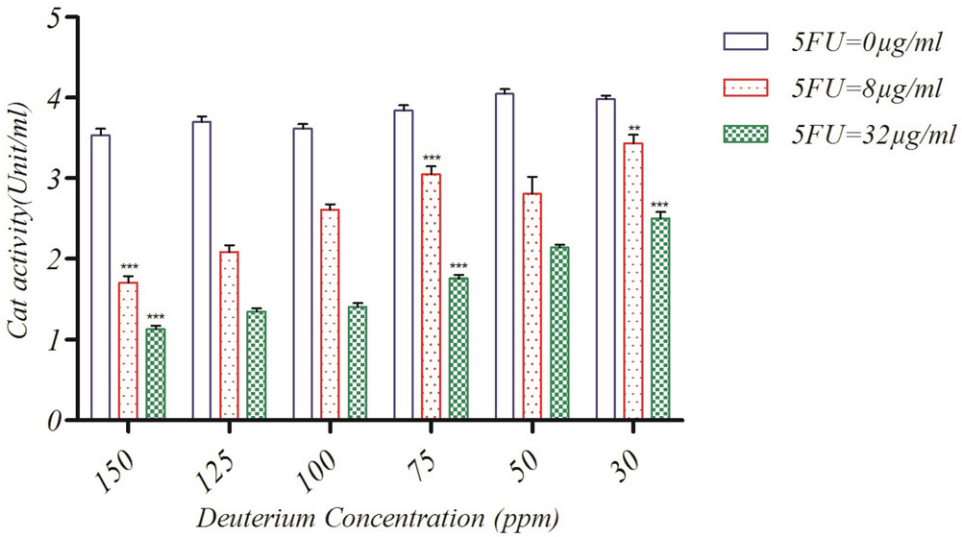


**Figure 6.** CAT activity after 48 h treatment with DDW different concentrations and 5-FU.

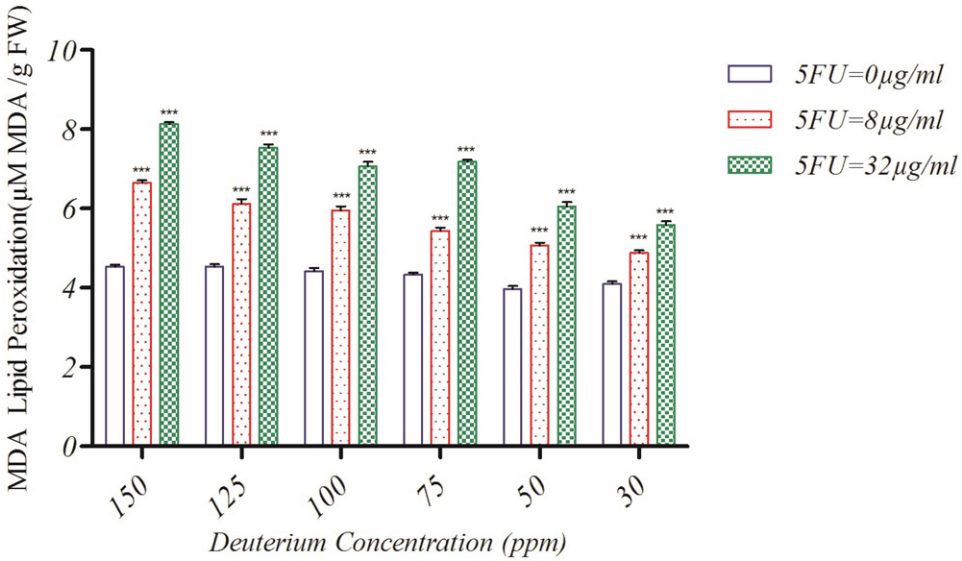


**Figure 7.** MDA concentration after 48 h treatment with DDW different concentrations and 5-FU.

of 30 and 50 ppm, was significantly increased ($P < 0.001$) compared to control group (DDW150ppm) (Figure 5).

### *Catalase Enzyme Activity*

An increasing of catalase at DDW50ppmas was registered. The most important decreasing ($P < 0.001$) was registered at DDW150ppm + 5-FU (32 μg/mL) groups (Figure 6).

### *Malondialdehyde Concentration*

DDW150ppm has a prooxidant effect and MDA values were increased ($P < 0.001$). MDA levels increased in the cells cotreated with DDW and 5-FU in comparison cells treated with solely DDW (Figure 7).

## Discussion and Conclusion

Breast cancer is the most diagnosed invasive malignancy and the second leading cause of cancer death worldwide (38). During the last decade, survival rates for breast cancer have increased as a result of the use of adjuvant therapy (39). In the last few decades, studies have begun on new anti-cancer compounds (40). The effects of deuterium in biological systems was first investigated in the early 1990s (33). 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 (41). Subsequently, the effect of drinking water with 30 ppm 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% (41). The other studies presented results of DDW inhibitory effect on PC-3 (cancerous cell line), MDA (breast cancer), and M14 (melanoma) cancer cells lines (42, 43). Further studies reported DDW inhibitory effect on the human lung carcinoma cell line (A-549) and human nasopharyngeal carcinoma cells established with MTT assay (43, 44).

This study aimed to investigate the cytotoxicity, cell cycle arrest, and antioxidant effects of DDW on human breast cancer cells. We found that the DDW significantly suppressed the proliferation of the MCF7 cells. Deuterium depletion increased the cytotoxicity of 5-FU on the MCF7 cancer cell line in a time and concentration dependent manner.

The study slowed lower levels of DDW alone, especially in concentration of 30–100 ppm, led to inhibition of cancer cell growth. Also, 5-FU and DDW combination significantly inhibited the growth of MCF7. DDW enhanced cell growth inhibitory effect of 5-FU in a concentration-dependent manner. Toxicity of the 5-FU increased in lower concentrations than 125 ppm from DDW. Concentrations of 150 ppm had the least effect on the cell growth inhibition. DDW30 and 50 ppm + 5-FU (32 μg/mL) had effective retardation of tumor growth. In the MTT study, DDW with 50 ppm deuterium strikingly decreased the survival fraction of 5-FU-treated group compared to control, whiles DDW at 150 ppm concentrations showed a trend towards the increase of survival measures of cells treated with 5-FU. Thus, these results confirm the synergistic effects of DDW and 5-FU in inhibiting the cancer cells growth.

In the previous studies, the growth inhibitory effect of DDW alone and in combination with the anti-cancer drugs including etoposide, taxol, doxorubicin and cisplatin showed that DDW synergistically enhances the inhibitory effect of anti cancer drugs in almost all cancers cultures tested. Also, synergistic inhibitory effect was 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 studies, theses preclinical finding was supported with the clinical studies. DDW as an adjuvant therapy came into clinical use recently and application of the it as an adjutant in conventional therapy regimens in patients with 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 (32, 34, 45–47).

In accordance with these previous investigations, our study also showed that DDW strengthens the cytotoxic effect of 5-FU as a chemotherapeutic agent on the MCF7 cells. The results of our study showed DDW have anti proliferative effects when used alone, but its anti-growth cellular effect is much higher in combination with 5-FU.

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, it is suggested that DDW playing a key role in the cell cycle regulation, apoptosis and tumor development (48, 49).

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 (50, 51).

In order to investigate the mechanisms of the cell growth inhibition under treatment with deuterium depleted water alone or in combination with 5-FU, the cell cycle changes of the treated and control cells were studied.

In the present study, we found that DDW50ppm 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 MCF-7 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. Also the cells in treated with DDW and 5-FU, the percentage of stopped cells in the G0/G1 stage was higher than that one's treated with DDW alone.

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 50 ppm of deuterium water compared with the control (150 ppm) (43). The results of Song and ea tal study showed The S phase (11.38%) increased whereas the G0 to G1 phase (8. 4%) and G2 to M phase (9.6%) were reduced in DDW-treated cells as compared to the control cells (43). 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 100 ppm of the DDW in five NPC cells, revealed that the cell cycle undergoes a changes in the *D*/*H* ratio can trigger cellular and molecular mechanisms having key role in cell cycle regulation. He hypothesized that increase in deuterium concentration is the real trigger for the cells to inter into S phase.

The results of o 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 (44). When the cells are cultured in a low concentration deuterium diet, the cell growth is inhibited due to the increased the time needed to reach the proper ratio of *D*/*H* (50, 51). *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 antioxidant enzymes and MDA concentration were studied. Superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) is active oxygen species 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 (52). Cancer cells show patterns of metabolism different from normal cells, that way cancer cells exhibit altered metabolism with a high glycolytic rate and a low level of respiration and these alterations in metabolism enhance oxidative stress (53–55). This high oxidative state in the cell stimulates tumor formation through cancer cells grow and divide in an uncontrolled manner and turning off tumor suppression functions (53–56). The effects of deuterium are highlighted in many studies (57, 58). Tumor growth could be inhibited in vitro by alteration of the environment around tumor cells to a more reducing agents. Then, development a cell redox state would also support increased apoptosis, which would inhibit tumor formation. Studies showed that entire antioxidant system was influenced by the deuterium depleted water. Alexander showed that DDW consumption before hypoxia modeling in rats improves antioxidant defense enzymes activity in the blood, increasing its antioxidant potential by 20% (31).

Our study showed that DDW improves antioxidant defense enzymes (catalase and superoxide dismutase of the cells, while level of proxidation factor (MDA) decreased. The elevated level of SOD and CAT activity related to DDW with low *D*-concentration, the same group of DDWs that have further inhibited cell growth and arrested the cell cycle. On the other hand, by increasing the amount of deuterium, the MDA level increased also. Therefore, it can be concluded that in addition to the ability of DDW to suppress MCF7 growth, we also observed that cell cycle arrest and antioxidant enzymes activity were significantly increased as the deuterium concentrations in MCF7 cells declined. This study is in aggrement with the study conducted by 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 (31, 59).

In summary, this *in vitro* study provided evidence that DDW alone suppresses cell growth, and augments the anti-proliferatory effect of 5-FU on the human neoplastic cell lines of the breast. These results demonstrated that DDW is a novel, nontoxic adjuvant therapeutic agent that suppresses MCF7 cell proliferation through the induction of antioxidant enzymes and cell cycle arrest. Collectively, our results suggest the potential for DDW as an antitumor adjutant with clinical application.

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## Disclosure Statement

The authors have no conflict of interest to declare.

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