RESEARCH ARTICLE

in vitro Assessment of Antineoplastic Effects of Deuterium Depleted Water

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Abstract

Background: *In vitro*, *in vivo* and clinical studies have demonstrated anti-cancer effects of deuterium depleted water (DDW). The nature of this agents action, cytotoxic or cytostatic, remains to be elucidated. We here aimed to address the point by examining effects on different cell lines. Materials and Methods: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) -based cytotoxicity analysis was conducted for human breast, stomach, colon, prostate cancer and glioblastoma multiforme cell lines as well as human dermal fibroblasts. The cell lines were treated with decreasing deuterium concentrations of DDW alone, paclitaxel alone and both. One way analysis of variance (ANOVA) was used for statistical analysis. Results: Treatment with different deuterium concentrations of DDW alone did not impose any significant inhibitory effects on growth of cell lines. Paclitaxel significantly decreased the survival fractions of all cell lines. DDW augmented paclitaxel inhibitory effects on breast, prostate, stomach cancer and glioblastoma cell lines, with influence being more pronounced in breast and prostate cases. Conclusions: DDW per se does not appear to have inhibitory effects on the assessed tumor cell lines as well as normal fibroblasts. As an adjuvant, however, DDW augmented inhibitory effects of paclitaxel and thus it could be considered as an adjuvant to conventional anticancer agents in future trials.

Keywords: Deuterium depleted water - cancer cell lines - *in vitro* - paclitaxel

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Introduction

Deuterium is the heavier stable isotope of hydrogen1998).

Following such a promising body of data, human clinical studies evaluated the effectiveness of DDW on the treatment of different cancers. A retrospective study showed that administration of DDW as adjuvant to, or as extension of conventional therapy increases the median survival time of patients with breast cancer Somlyai(2004). A recent cohort study among 129 lung cancer patients showed that DDW as adjuvant to the conventional chemoradiotherapy increased the median survival (Gyongyi et al., 2013). In addition, a controlled interventional study yielded promising results for using DDW as adjuvant therapy to prostate cancer conventional regimen (Kovacs et al., 2011).

Although no report of major side effect attributable to DDW consumption exists in the literature, the safety of this water on human remains to be methodologically confirmed. In addition, there exists no well-designed study to determine the appropriate dosage and regimen of DDW administration. Thereafter a phase I clinical trial seems to be mandatory to fill in these gaps.

According to international guidelines on conducting phase I clinical trials, (Korn et al., 2001; Eisenhauer et al., 2009) type of proposed anti-cancer agent, whether cytotoxic or cytostatic, influence the design of trial. However, it is yet to be clearly determined the cytotoxic or cytostatic behaviors of the DDW (Somlyai et al., 1993; 1997; 1998; 2010). We conducted an *in vitro* study on five neoplastic and one normal cell lines to determine the nature of DDW effect alone and in combination with a conventional cytotoxic drug.

Materials and Methods

Materials and cell lines

Dulbecco's modified eagle medium (DMEM),fetal bovine serum, phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA), amphotericin B and penicillin-streptomycin solutions were obtained from Invitrogen (Carlsbad, CA, USA). All cell culture vessels were purchased from BD Biosciences (Franklin Lake, NJ, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) glycine and paclitaxel were obtained from Sigma-Aldrich (Steinheim, Germany).MDA-MB-231(human breast adenocarcinoma), HCT-116 (human colon cancer), PC-3 (human prostate adenocarcinoma)

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and U-87MG (glioblastoma multiforme) cell lines were purchased from National Cell Bank of Iran (Tehran, Iran). AGS (human gastric adenocarcinoma) and human normal dermal fibroblast cell line (HDF-1) were obtained from Iranian Biological Resource Center (Tehran, Iran). DDW was gifted by Atomic Energy Organization of Iran (Tehran, Iran).

MTT-based cytotoxicity assay

MTT-based cytotoxicity assay was carried out in accordance with the protocol previously described by Plumb and her colleagues (Plumb et al., 1989). Based on population doubling time of cell lines, cytotoxicity test was designed as short-form and long-form assays. Since the doubling time of HCT-116 and AGS is shorter than 24 hours, these cell lines were included in the short-form assay. But the cytotoxicity of DDW on MDA-MB-231, PC-3, U-87MG and HDF-1 cell lines with the doubling times longer than 24 hours was examined with the long-form assay.

In the short-form assay, AGS and HCT-116 cells were conveyed to 96-well microtitration plates with a seeding density of 5,000 cells per well in 200 μL DMEM medium containing 10% FBS and 2mM L-glutamine. The plates were incubated in humidified air containing 5% CO_2 at 37°C. The next day, when the cells were entered to the logarithmic phase of growth, exposure period was started by adding water with deuterium concentrations of 40 ppm, 62 ppm, 84 ppm, 106 ppm, 128 ppm and 150 ppm, alone or in combination with 0.5 μM paclitaxel. Each treatment was run triplicate. 24 hours later, the drugs were removed from the wells. In order to demonstrate retention of regenerative capacity of the exposed survived cells, a 48 hours recovery period were considered.

In the long-form assay, cells were seeded in microtitration plates with the density of 1,000 cells per well. Drugs were added three days later with the same concentrations mentioned for short-form assay. In the long-form assay, the durations of exposure and recovery periods were considered as 72 and 96 hours, respectively.

During the recovery period, the plates were fed daily with fresh medium. At the end of the recovery period, 50 μL of MTT (5mg/mL) solution was added to each well and then the plates were further incubated for four hours. All remaining supernatant were removed and 200 μL of DMSO was added to dissolve the formed insoluble formazan crystals. 25 μL of glycine buffer was added to each well to adjust the final pH. Then, absorbance was immediately recorded at 570 nm using microtitration plate reader (BioTek®, USA). The absolute values of the absorbance were converted to surviving fraction data as the percentage of living cells of the control.

Statistical analysis

Data were represented as mean±standard error of the mean (SE). Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Bonferroni test to adjust for multiple testing. Linear regression analysis was used to find out the concentration-response relationship. Level of significance was set at $p<0.05$. The statistical analyses were carried out using BioStat 2008 software.

Results

Treatment with different deuterium concentrations of DDW alone, did not illustrate statistically significant differences in the surviving fractions of all neoplastic and normal cell lines assessed.

Paclitaxel significantly decreased the surviving fractions of all cell lines tested in this study compared to the control ($p<0.001$).

There was a statistically significant difference in the survival of MDA-MB-231 cells when co-treated with paclitaxel and different concentrations of DDW ($p=0.002$). In this cell line, the surviving fractions of paclitaxel-treated groups in combination with DDW at 40 ppm (22.37±5.23%) and 62 ppm (30.33±1.66%) were significantly decreased compared to 150 ppm as control (50.14±3.87%) ($p=0.002$; $p=0.026$; respectively) (Figure 1A). As illustrated in Figure 2, there was a significant positive correlation between deuterium depletion and paclitaxel cytotoxicity in MDA-MB-231 cell line, when used together ($p<0.001$, $r=0.993$). Other concentrations of DDW had no significant effects on the growth inhibitory properties of paclitaxel.

There was illustrated a statistically significant difference in the survival measures of PC-3 cells treated with different concentrations of DDW in combination with paclitaxel ($p=0.014$). In this cell line, DDW with 40 ppm deuterium significantly decreased the surviving fraction of paclitaxel-treated group compared to control (35.16±3.98% vs 59.56±4.68%; $p=0.007$). Deuterium in other concentrations did not show any significant effect on the survival of paclitaxel-treated group (Figure 1B).

We observed statistically significant difference between the survival measures of AGS cells co-treated with paclitaxel and different concentrations of DDW ($p=0.011$). In this cell line, DDW with 84 ppm deuterium strikingly decreased the survival fraction of paclitaxel-treated group compared to control (26.71±1.98% vs 46.96±0.94%; $p=0.001$). Although DDW at 40 and 62 ppm concentrations showed a trend towards the increase of survival measures of cells treated with paclitaxel, these values were not statistically significant compared to 84 ppm concentration. Other concentrations of deuterium didn't demonstrate any significant effect on the survival fraction of paclitaxel-treated group in this cell line (Figure 1C).

We observed a statistically significant difference in the survival measures of U-87MG cells treated with different concentrations of DDW in combination with paclitaxel, as determined by one-way ANOVA ($p=0.011$). The surviving fraction of paclitaxel-treated group in combination with 62 ppm DDW (36.61±0.64%) was significantly lower than those observed in co-treatment with 106 ppm (51.83±0.07%, $p<0.001$), 128 ppm (44.21±0.91%, $p=0.002$), and 150 ppm DDW (52.47±1.58%, $p=0.001$). Although in paclitaxel-treated group the survival measure of GBM cells co-treated with 40 ppm DDW was higher than that seen in 62 ppm, but this difference was not statistically significant. Other concentrations of DDW

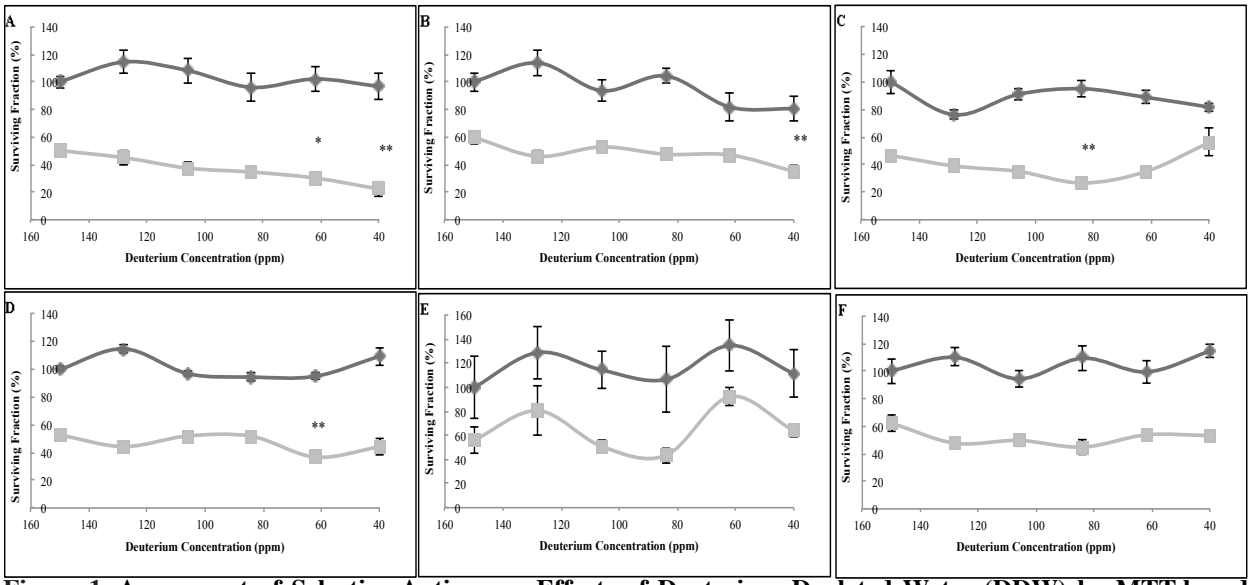

Figure 1. Assessment of Selective Anticancer Effects of Deuterium Depleted Water (DDW) by MTT-based Cytotoxicity Assay. Surviving fractions plotted in Y axis, are calculated as percentage of living cells of the control. Black curves represent survival of cells treated with water containing 150 ppm, 128 ppm, 106 ppm, 84 ppm, 62 ppm and 40 ppm of deuterium. Grey curves indicate to the survival of cells co-treated with paclitaxel (50 uM) and serial concentrations of DDW. As illustrated in this figures, deuterium depletion didn't show cytotoxic or cytostatic effect on neoplastic and normal cell lines in itself, but it could increase the cytotoxicity of paclitaxel on some neoplastic cells. A) MDA-MB-231 (human gastric adenocarcinoma cell line); B) PC-3 (human prostate adenocarcinoma cell line); C) AGS (human gastric adenocarcinoma cell line); D); U-87MG (human glioblastoma multiforme); E) HCT-116 (human colon adenocarcinoma); F) HDF-1 (normal human dermal fibroblast cell line). Error bars represent SE. *p<0.005; **p<0.01 (Bonferroni t-test; compared to paclitaxel with 150 ppm DDW)

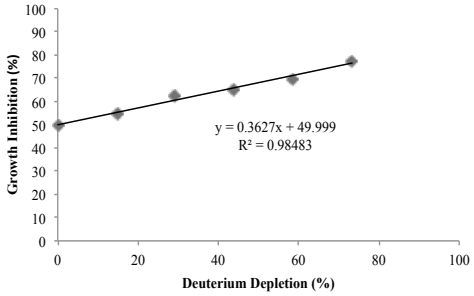

Figure 2. Correlation of Deuterium Depletion from Cultrue Medium and Cytotoxicity of Paclitaxel in Breast Adenocarcinoma Cell Line (MDA-MB-231). Cells were treated with paclitaxal (0.5 μM) in media composed of water containing 150 ppm, 128 ppm, 106 ppm, 84 ppm, and 40 ppm deuterium. Then, MTT-based cytotoxcity assay was performed. The X-axis demonstrates the percentage of deuterium depleted from 150 ppm water. As seen in this figure, deuterium depletion significantly increased the cytotoxicity of paclitaxel in a concentration-dependant manner (p<0.001, r=0.993)

didn't cause any effects on the cytotoxicity of paclitaxel in U-87MG cell line (Figure 1D).

DDW neither alone nor in combination with paclitaxel demonstrated any effect on the survival measure of HCT-116 and HDF-1 cell lines (Figures 1E and 1F), (Preferred position for Figures 1 and 2).

Discussion

The present study aimed to investigate the selective anticancer properties of DDW in a cell line panel including neoplastic (MDA-MB-231, PC-3, AGS, U-87MG, HCT-116) and normal (HDF-1) cell lines. DDW didn't show cytotoxic or cytostatic effects on this panel of cell lines when used alone. However, deuterium depletion increased the cytotoxicity of paclitaxel on the breast cancer cell line in a concentration-dependent manner. Also DDW enhanced the growth inhibitory properties of paclitaxel on PC-3, AGS and U-87MG cell lines at lower levels of deuterium. DDW didn't show significant effects on the paclitaxel cytotoxicity on HCT-116 and HDF-1 cell lines.

Previous *in vitro* studies, almost in consensus, reveal findings that DDW per se could be of inhibitory effect on tumorous cell lines. One of these studies (Somlyai et al., 2010) investigating the DDW effect on PC-3 (human prostate), MDA (human breast), HT-29(human colon) and M14 (human melanoma) tumorous cell lines, showed that DDW imposes inhibitory effect on cell lines growth by delaying the cell proliferation cycle. Another study using real time cell analyzer technique, presented results of DDW inhibitory effect on MCF-7 (human breast), HT-199 (human melanoma) and A-549 (human lung) tumorous cell lines (Nagy et al., 2012). Two other studies reported DDW inhibitory effect on human lung carcinoma cell line (A-549) and human nasopharyngeal carcinoma cells established with MTT-assay; the former also showed that DDW induces apoptosis in lung cancer cells accounting in part for the growth inhibitory effect (Cong et al., 2010; Wang et al., 2012). FAC-Scancytofluorimeter analysis indicated inhibitory effect of DDW on acute leukemic (both myeloid and lymphoid) cells in another study (Roumyantsev et al., 2012). Surprisingly contracting these findings, a remote study showed DDW to be a stimulant for neoplastic and normal cell lines growth, by means of MTT assay (Bild et al., 2004). Limited number of tumorous cell lines assessed in most of these studies, decreases their weight of evidence. In addition, diversity of techniques employed to analyze cell growth makes the results somehow heterogeneous to sum up.

Unlike the bulk of existing body of evidence, our study indicates that DDW per se imposes neither inhibitory nor stimulatory effect on assessed normal or tumorous cell lines growth. High number of cell lines analyzed, augments the strength of this result. Such a finding would call in to question the former seeming consensus that DDW alone could have growth inhibitory effects. This discrepancy would be of notable importance when the previous data has led to conduction of higher levels of studies as far as a clinical trial on human (Kovacs et al., 2011).

The *in vitro* study investigating the growth inhibitory effect of DDW alone and in combination with known antineoplastic drugs etoposide, taxol, doxorubicin and cisplatin, indicated that DDW synergistically enhances the inhibitory effect of doxorubicin in almost all tumorous cultures tested; such a synergistic inhibitory effect was also reported with cisplatin in A-549 (human lung carcinoma) and HT-199 (human melanoma) cultures (Nagy et al., 2012). Later on, this preclinical finding was supported with clinical studies. Use of DDW as adjuvant to conventional chemotherapy regimens in patients with prostate, lung and breast malignancies, yielded clinical benefit in terms of prolonged median survival time, decreased tumor size, attenuated subjective symptoms and molecular responses (Somlyai, 2004; Krempels et al., 2008; Kovacs et al., 2011; Gyongyi et al., 2013). In accordance, our study showed that DDW strengthens the cytotoxic effect of paclitaxel as a chemotherapeutic agent on tumorous cell lines of breast, prostate, stomach and glioblastoma. This effect was most prominent in MDA-MB-231 (human breast adenocarcinoma) cell line. The routine use of paclitaxel in breast adenocarcinoma chemotherapy in clinic, might justify this difference. Assessing further combinations of DDW with other conventional anti-cancer agents could have far strengthened our results to better deduce DDW efficacy as an adjuvant therapy.

The synergistic effect of DDW and paclitaxel could be explained upon their already reported common properties of apoptosis induction and cell-cycle halting. As reported (Jordan and Wilson, 2004), paclitaxel stabilizes the microtubules polymers and protects them against disassembly in a mitotic cell cytoskeleton; this inhibits chromosomal metaphase spindle configuration with consequent blocking of mitosis which results in apoptosis or regression of the cell back to G phases. Similarly, apoptotic and cell cycle halting properties have been described for DDW as well (Cong et al., 2010). Having this already published data, we thought of mechanistic assessments to be of no new benefit in this study.

Upon findings of this study, it does not seem rational to consider DDW as a monotherapy (neither cytotoxic nor cytostatic) agent in design of a clinical trial; rather appropriate combinations of this agent with other routine cytotoxic drugs could be tailored and tested. Since having the efficacy of such combinations proven with trials, the DDW would be a good candidate to enter routine cancer therapy regimens as an adjuvant agent.

In conclusion, according to this *in vitro* study, deuterium depleted water alone does not impose any significant growth inhibitory effect on human breast, prostate, stomach, colon and glioblastoma tumorous cell lines; however, it can augment the anti-proliferatory effect of the known chemotherapeutic agent, paclitaxel on human neoplastic cell lines of breast, prostate, stomach and glioblastoma. This synergistic effect was more pronounced on human breast adenocarcinoma (MDA-MB 231) cell line. Further *in vitro* studies on different well-tailored combinations could prepare enough data to select good choices for subsequent clinical trials.

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