Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2019; 26 (5): 357-367

The Effect of Deuterium Depleted/Enriched Water on the Growth of A549 and HepG2 Cell Lines

Ali Mandegary, Ph.D.[1], Elham Sharif, Pharm D.[2], Rokhsana Rasooli, D.V.M.[3], Abdolreza Hassanzadeh, Ph.D. [4]

1- Associate Professor, Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

2- Pharmacist, Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

3- Veterinarian, Department of Veterinary, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

4- Assistant Professor, Department of Medicinal Chemistry, Faculty of Pharmacy & Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran (Corresponding author; E-mail: a_hassanzadeh@kmu.ac.ir)

Received: 7 July, 2019 Accepted: 11 October, 2019

ARTICLE INFO

Article type:
Original Article

Keywords:
Deuterium enriched water
Deuterium depleted water
Cell growth
Doubling time

Abstract

Background: Although advances in cancer therapy continue to develop, the overall survival rate is poor for some cancer cases. The search for a new adjuvant strategy is the focus of cancer treatment. There is some evidence suggesting a change in the mechanism of cell function by a change in the content of deuterium in the medium. So, the aim of this study was to investigate the effect of deuterium depleted water (DDW) and deuterium enriched water (DEW) on the cell growth and cytotoxicity of two A549 and HepG2 cell lines.

Methods: The A549 and HepG2 cell lines were cultured in media at different concentrations of deuterium and different exposure times. Cell proliferation was carried out by trypan blue dye exclusion technique. The cytotoxicity effects of DEW and DDW were determined by MTT assay on different exposure times (24, 48 and 72 h).

Results: In this study, DEW acted as a dose- response growth inhibitor at deuterium concentration of 50×10^3 ppm and greater, while no considerable effect was seen upon short and long term exposures to DDW (31 ppm and 127 ppm deuterium). Both DEW and DDW at used deuterium concentrations were found not to be toxic on the studied cell lines.

Conclusion: In conclusion, long term treatment with DEW could inhibit the proliferation of A549/HepG2 cell lines. Therefore, it can be considered as an adjuvant in cancer therapy.

Citation: Mandegary A, Sharif E, Rasooli R, Hassanzadeh A. The Effect of Deuterium Depleted/Enriched Water on the Growth of A549 and HepG2 Cell Lines. *Journal of Kerman University of Medical Sciences*, 2019; 26 (5): 357-367.

Introduction

Cancer is the major public health problem worldwide (1). Although our knowledge about the biology of cancer and its treatment has advanced much, cancer has remained the second most common cause of death in the world. Because of the number of sufferers and high mortality rate, the search for new therapies is still ongoing. It has been observed that cancer survival rate depends strongly on the available adjuvant therapy (2). Therefore, introduction of new adjuvant protocols could be a footstone for future investigations.

Water is a fundamental factor of all the biological systems and is an essential requirement for life. The replacement of hydrogen (^1H) by deuterium (^2H) at H_2O results in deuterium oxide (D_2O) known as heavy water. It has been known for decades and well documented that the behavior of the molecules in solutions containing different deuterium concentrations is altered in chemical reactions and this alteration manifests in biological systems (3). The ratio of deuterium concentration is about 150 ppm in nature (4); therefore, the higher and less amounts of deuterium are considered as deuterium enriched water (DEW) and deuterium depleted water (DDW), respectively. Normal concentration of deuterium is essential to initiate and maintain normal cellular growth (5) and any deviation from normal values may affect cell growth and survival. There are many reports which have demonstrated the toxic biological effects of DEW and DDW on both benign and malignant cells (6, 7). It is shown that incubation of tumor cells with various contents of deuterium leads to inhibition of tumor cell proliferation (8-10). As a result, DEW and DDW might help in the chemotherapeutic attempts in human tumors (4, 8).

To our knowledge, there are no studies about long term effects of DDW and DEW on the growth and viability of cancerous cell lines; therefore, the aim of this study was to investigate the effects of DDW and DEW on growth of A549 and HepG2 cell lines.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) (high glucose), Fetal Bovine Serum (FBS), and penicillin/streptomycin were purchased from PAA (Australia). Culture flask (25 and 75 cm^2) was purchased from SPL Company (Korea). Centrifuge tube (15 and 50 ml), micro-centrifuge tube and multi-well plates (6-well, 24-well, and 96-well) were obtained from Nest Company (China). Trypsin-EDTA was bought from Biosera (England) and MTT assay kit was purchased from Sigma Chemical Company (UK). The waters containing various concentrations of deuterium were taken from Atomic Energy Organization of Iran (AEOI).

Cell culture

Two human cancer cell lines including A549 (lung cancer) and HepG2 (hepatocellular carcinoma) were purchased from Iranian Biological Resource Center (IBRC) Tehran, Iran. The cells were grown in DMEM supplemented with 10% FBS and 100 units/ml of penicillin/streptomycin and incubated at standard condition (5% CO_2 at 37 ˚C). The cells were not allowed to reach conflux by sub-culturing, in order to prevent any reduction of cell responses to stimulators and/or inhibitors (11).

Preparation of media containing different concentrations of deuterium

The types of water with reduced concentrations of deuterium (i.e. 31, 69, 91, 109 and 127 ppm deuterium) were used as supplied by the AEOI. For the deuterium enrichment purposes, the 5, 10, 20 and 30 % D_2O were used.

The cell culture media were prepared by dissolving components of the culture medium in water with different

concentrations of deuterium and then they were sterilized using 0.2 μ filters.

Cell treatment

The effect of short-term treatment with DDW and DEW on the growth curve

In the short-term exposure, a cell suspension containing 5×10^4 cells of A549 or HepG2 were seeded into each well of a flat bottomed 24-well tissue culture plate containing normal water (for control group) or water with various deuterium content (31, 127, 50000, 100000, 200000 and 300000 ppm) and incubated for 24 h. Each treatment was run in triplicate. Cell numbers were determined with hemocytometer and trypan blue dye exclusion at 0, 1, 2, 3, 4, 5, 6 and 7 days of cell seeding. Doubling time of cell line was calculated by the following formula (11):

DT = T.ln2/ln (Xe/Xb)

Where: DT is doubling time, T is the time that is considered between two cell counts, Xb is the cell number at the beginning of T time and Xe is the cell number at the end of T time.

The effect of long-term treatment with DDW and DEW on the growth curve

In the long-term assay, logarithmically-growing A549 or HepG2 cells were incubated in various concentrations of deuterium (31, 127, 50000, 100000, 200000 and 300000 ppm of deuterium) for 21 days. Hence a cell suspension containing 5×10^4 cells was seeded into each well of a 24-well tissue culture plate. For the control group, cells were cultured in DMEM prepared by normal water, and the experimental groups were cultured in DMEM prepared using different concentrations of deuterium (31, 127, 50000, 100000, 200000 and 300000 ppm). Cell numbers were determined as described above.

Cytotoxicity assay

A suspension containing 7×10^3 cells of A549 or HepG2 cells in the logarithmic phase were cultured in 96-flat bottomed plates. After 24 h, the cells' medium were replaced with medium containing different concentrations of deuterium (31, 69, 91, 109, 127, 50000, 100000, 200000 and 300000 ppm) and incubated for 24, 48 and 72 h at standard condition. After the treatment, the viability of cells was determined by using MTT assay. Each concentration was tested in triplicate in a single experiment, which was repeated three times. The growth inhibition percentage was calculated by using Excel Software Version 14.0.7224.5000(32-bit).

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Data were analyzed using SPSS for Windows (SPSS Inc. Chicago II, version 18). Statistical differences between the treatment and control groups were evaluated by one-way ANOVA, followed by Newman-Keuls post hoc test and $p\leq 0.05$ was considered as significant.

Results

The effect of short- and long-term exposure to DDW and DEW on A549 cell line

The effects of short and long term exposure to DDW and DEW on the growth curves and doubling time of A549 cells have been shown in Figure 1 and Table 1. In comparison to the control group, DDW- treated cells showed a decrease in doubling time of A549 cells in short- and long-term exposures, while DEW could increase the doubling time.

Table 1. The effects of water with different concentrations of deuterium on A549 cell doubling time (D. T.)

Exposure time		Deuterium concentration (ppm)						
		150 (control)	31	127	50000	100000	200000	300000
Short	**D. T. (h)**	26.8	25.5	24.8	26.9	26.3	27.0	27.8
	(% change)		(- 4.8)	(- 7.5)	(+ 0.4)	(- 1.9)	(+ 0.7)	(+ 3.7)
Long	**D. T. (h)**	26.8	25.4	25.6	27.9	28.7	31.0	34.0
	(% change)		(- 5.2)	(- 4.5)	(+ 4.1)	(+ 7.1)	(+ 15.7)	(+ 26.9)

The effect of short- and long-term exposure to DDW and DEW on HepG2 cell line

The effects of short and long term exposure to DDW and DEW on the growth curves and doubling time of colony formation of $HepG_2$ cells have been shown in Figure 2 and Table 2. There was a little decrease in the proliferation of HepG2 cells in short-time exposure to DDW only for the sample of 127 ppm of deuterium. In long-term exposure, DDW reduced HepG2 cells doubling time in comparison to the control group. DDW- treated cells show a decrease in doubling time in dose- and exposure time- dependent manners.

Table 2. The effects of water with different concentrations of deuterium on Hep-G2 cell doubling time (D. T.)

Exposure time		Deuterium concentration (ppm)						
		150 (control)	31	127	50000	100000	200000	300000
Short	**D. T. (h)**	23.8	23.9	23.4	23.9	23.9	24.3	24.9
	(% change)		(+ 0.4)	(- 1.7)	(+ 0.4)	(+ 0.4)	(+ 2.1)	(+ 4.6)
Long	**D. T. (h)**	23.8	23.4	23.4	24.9	26.7	28.6	30.8
	(% change)		(- 1.7)	(- 1.7)	(+ 4.6)	(+ 12.2)	(+ 20.2)	(+ 29.4)

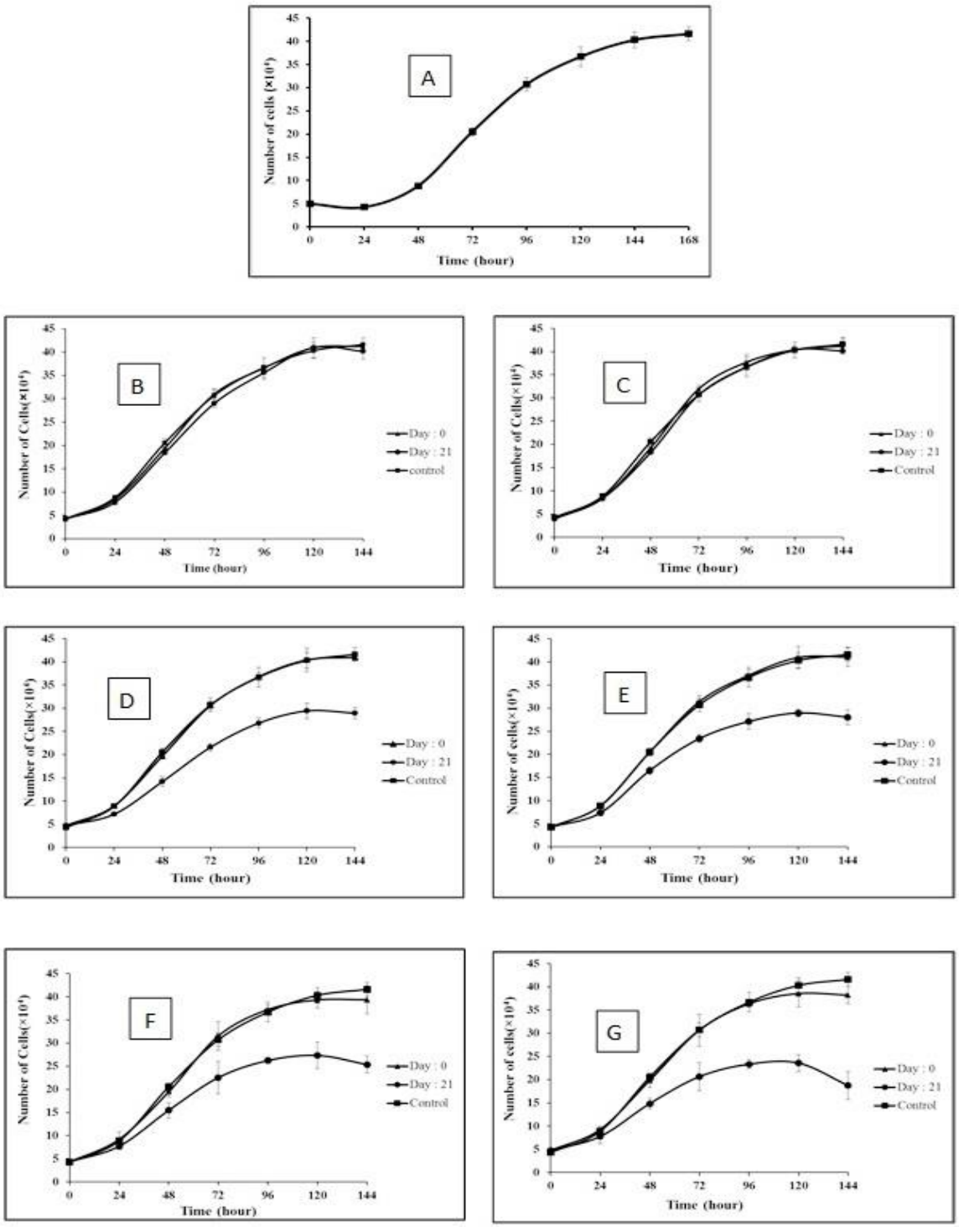

Figure 1. The effect of water with different concentrations of deuterium on A549 growth (A: control, B: 31, C: 127, D: 50000, E: 100000, F: 200000, G: 300000 ppm deuterium)

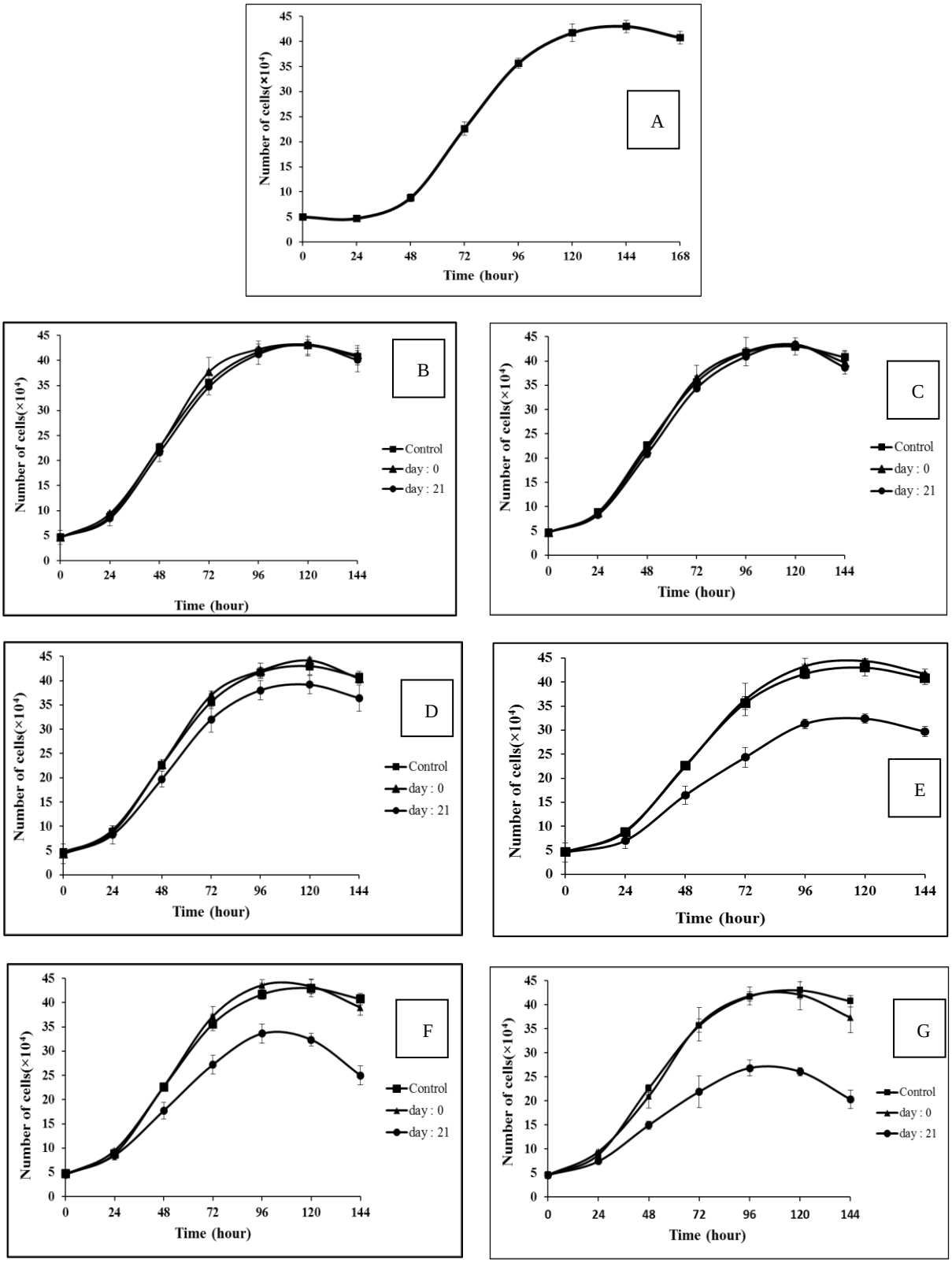

Figure 2. The effect of water with different concentrations of deuterium on HepG2 growth (A: control, B: 31, C: 127, D: 50000, E: 100000, F: 200000, G: 300000 ppm deuterium**)**

Determination of viability by MTT assay

Treatment of both cell lines with different concentrations of deuterium did not affect cell viability statistically during 24, 48 and 72 h. The cytotoxicity effect of water at various deuterium concentration is depicted separately in Figures 3 and 4 for respectively A549 and HepG2 cell lines,.

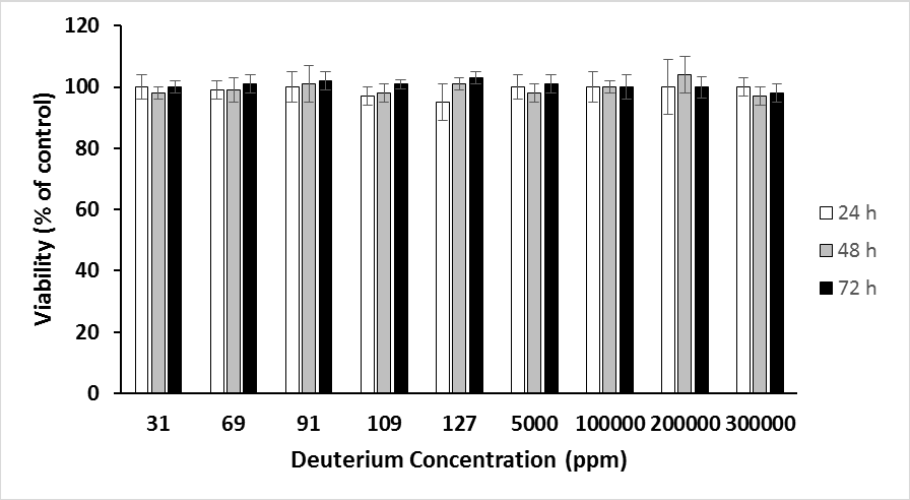

Figure 3. Cytotoxicity of DEW and DDW on A549 cell line

The A549 cells treated with different concentrations of deuterium for 24, 48 and 72 h. Data are shown as Mean ± SD.

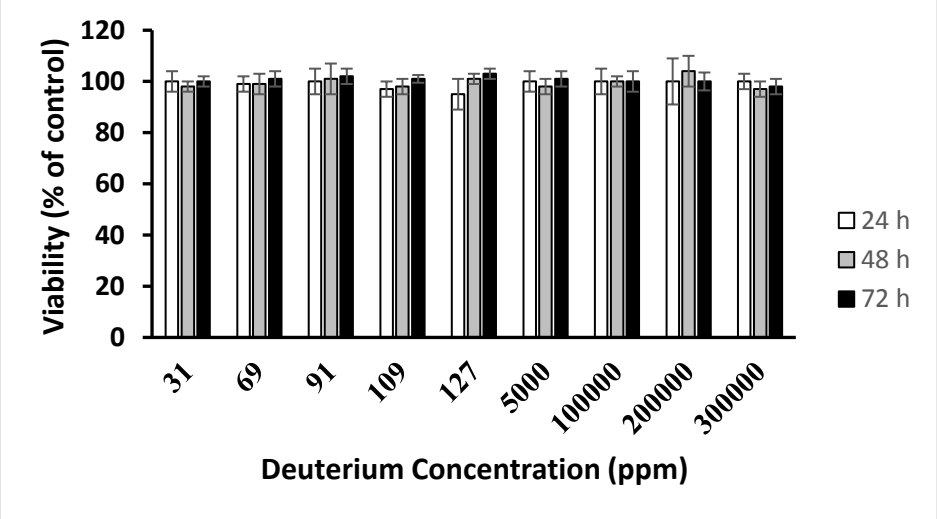

Figure 4. Cytotoxicity of DEW and DDW on the Hep-G2 cell line

The HepG2 cells treated with different concentrations of deuterium for 24, 48 and 72 h. Data are shown as Mean ± SD.

Discussion

Hepatocellular carcinoma (HCC) and Non-small cell lung cancer (NSCLC) are the leading cause of tumor-related death worldwide, highlighting the need for more effective treatment strategies. Despite improved outcomes with various chemotherapeutic agents and regimens in patients with these cancers, most treatments ultimately may fail due to the resistance or intolerable toxicity (12, 13). Therefore, recent efforts have focused on identifying new anticancer components to obtain enhanced anticancer efficacy and reduced toxicity. Deuterium depleted water and deuterium enriched water are new anticancer compounds declared to have no adverse effects at appropriate doses.

We have already shown that cyclooxygenase inhibitors combined with deuterium-enriched water augment cytotoxicity in A549 lung cancer cell line *via* activation of apoptosis and MAPK pathways (14). Although high deuterium contents may be seen toxic, it has been found that mammalian cells can tolerate D_2O in a concentration of at most 35%; a higher concentration of D_2O may show a lethal effect in them (15). The low toxicity of D_2O in mammals is reflected in its widespread use for measuring water spaces in humans and other animals. Higher concentration (usually >20% of body weight) can be toxic to animals and animal cells (16). Acute exposures of up to 23% D replacement in plasma have been tolerated without reported adverse effects (17). Replacement up to 25% of brain water by heavy water has been reported by Blagojevic and co-workers for the purpose of minimizing (n,γ) reactions on hydrogen in Boron Neutron Capture Therapy applications to glioblastomas (17). No adverse events have been reported in metabolic labeling studies in which healthy subjects consumed daily doses of up to 9.8 g D in the form of D_2O for up to 4-9 weeks and treatments have been sufficient to maintain1.0-2.0 % D enrichment in body water (18). Furthermore, it has been shown that D_2O is much more effective in killing malignant melanoma and carcinoma cells (colon carcinoma, glioblastoma, and small lung cell cancer cells) than PHA stimulated lymphocytes and normal glial cells; for example, 90% D_2O is considered deadly for 70% of the former but only for 5% of the latter group (19). At a concentration of 80%, it has been reported that D_2O acts as an inhibitor of mitosis on PtK1 cells (20).

In the present study, we found the inhibitory effects of DEW on both Hep-G2 and A549 cells growth. DEW could inhibit cell growth in both A549 and Hep-G2 cell lines in a dose dependent manner. The anti-tumor effects of DEW on various cell types have been shown in several studies. Katz and coworkers (21) examined the effects of deuteration on the growth rate of Krebs-2 ascites tumor and P-1534 leukemia and when deuterium was administered to mice prior to the whole-body X-ray radiation, the LD_{50} for the deuterated animals was slightly higher than the LD_{50} for control animals. The protection from the lethal effects of x-rays in cells pretreated with D_2O prior to radiation exposure was found slight but appeared significant (22, 23). In Gross and Spindel study, high concentrations of deuterium in water induced stagnation mitosis (24). Deuterium oxide has been reported to have *in vitro* anticancer effect on bladder cancer cells (7) and human pancreatic carcinoma cells (10). It has been found that DEW has anti-proliferative, anti-adhesive and anti-invasive effects, and it has also been shown that an increased concentration and exposure time may lead to genetic changes which indicate the possibility of a favorable prognosis (7). The *in vitro* antimitotic activity of deuterium alone has also been well established in cultures of fertilized sea

urchin eggs exposed to deuterated seawater. Upon transfer of the eggs into normal sea water, the mitotic activity resumed, and large numbers of normal-looking embryos developed from these eggs, thus indicating a certain reversibility of the blocking effect (25). Our work also showed an increase in the doubling time of A549 and Hep-G2 cells in long-term exposure which is in agreement with Leonard and his co-worker's experiment on PtK1 cells (20). They reported that PtK1 cells exposed to culture medium containing up to 50 % D_2O were able to enter and complete mitosis, but the duration of mitosis increased proportionally to the concentration of applied D_2O (20).

Here, we report that neither short time nor long time exposure to DDW (31 and 127 ppm) had effects on the cell growth and viability of the cell lines. This finding is in agreement with Soleyman-Jahi and his coworkers' findings (26) and is in contrary to some other studies. Some evidences have demonstrated that DDW suppresses the proliferation of tumor cells (5, 9), while others believe that DDW has the potential to be a stimulant for neoplastic and normal cell growth (27). It seems that the cell cycle regulatory system perceives the D/H ratio, and at the threshold level it triggers cell division (9). Therefore, one reason for various responses to deuterium would be differences in the threshold level of different cell lines.

In conclusion, due to the obtained results of DDW and DEW effect on the cell growth, the long-term exposure to deuterium enriched water has the potential to inhibit or slow down the proliferation of A549 and HepG2 cell lines. Therefore, it can be considered as a promising anticancer agent for future clinical applications.

Declarations of interest

There are no declarations of interest.

Acknowledgements

The authors express their gratitude to Mesbah Energy Company of Iran for funding the project and providing water samples with various concentrations of deuterium.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64(1):9-29.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009; 59(4):225-49.
3. Rundel PW, Ehleringer JR, Nagy KA. Stable Isotopes in Ecological Research. New York: Springer; 1989.
4. Somlyai G, Molnar M, Laskay G, Szabo M, Berkenyi T, Guller I, et al. Biological significance of naturally occurring deuterium: the antitumor effect of deuterium depletion. *Orv Hetil* 2010; 151(36):1455-60. [In Hungarian].
5. Somlyai G, Jancso G, Jakli G, Vass K, Barna B, Lakics V, et al. Naturally occurring deuterium is essential for the normal growth rate of cells. FEBS Lett 1993; 317(1-2):1-4.
6. Uemura T, Moritake K, Akiyama Y, Kimura Y, Shingu T, Yamasaki T. Experimental validation of deuterium oxide-mediated antitumoral activity as it relates to apoptosis in murine malignant astrocytoma cells. J Neurosurg 2002; 96(5):900-8.
7. Bahk JY, Lee JH, Chung HS, Lee HY, Chung BC, Park MS, et al. Anticancer effect of deuterium oxide on a bladder cancer cell related to Bcl-2 and Bax. J Ind Eng Chem 2007; 13(4):501-7.

8. Bader Y, Hartmann J, Horvath Z, Saiko P, Grusch M, Madlener S, et al. Synergistic effects of deuterium oxide and gemcitabine in human pancreatic cancer cell lines. Cancer Lett 2008; 259(2):231-9.

9. Cong FS, Zhang YR, Sheng HC, Ao ZH, Zhang SY, Wang JY. Deuterium-depleted water inhibits human lung carcinoma cell growth by apoptosis. Exp Ther Med 2010; 1(2):277-83.

10. Hartmann J, Bader Y, Horvath Z, Saiko P, Grusch M, Illmer C, et al. Effects of heavy water (D_2O) on human pancreatic tumor cells. Anticancer Res 2005; 25(5):3407-11.

11. Peterson J. Animal cell culture: a practical approach. Yale J Biol Med 1993; 66(4):336-7.

12. Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004; 5(3):215-9.

13. Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008; 48(6):2047-63.

14. Hassanzade A, Mandegary A, Sharif E, Rasooli R, Mohammadnejad R, Masomi-Ardekani Y. Cyclooxygenase inhibitors combined with deuterium Enriched water augment cytotoxicity in A549 lung cancer cell line via activation of apoptosis and MAPK pathways. Iran J Basic Med Sci 2018; 21(5):508-16.

15. Somlyai G. Pharmaceutical products for curing tumorous diseases and process for preparing same. [cited October 30, 2019]. Available from: http://www.google.com/patents/US5855921.

16. Kushner DJ, Baker A, Dunstall TG. Pharmacological uses and perspectives of heavy water and deuterated compounds. Can J Physiol Pharmacol 1999; 77(2):79-88.

17. Blagojevic N, Storr G, Allen BJ, Hatanaka H, Nakagawa H. Role of heavy water in boron neutron capture therapy. In: Zamenhof RG, Solares GR, Harling OK. Topics in Dosimetry and Treatment Planning for Neutron Capture Therapy. USA: Advanced Medical; 1994.

18. Collins ML, Eng S, Hoh R, Hellerstein MK. Measurement of mitochondrial DNA synthesis in vivo using a stable isotope-mass spectrometric technique. J Appl Physiol (1985) 2003; 94(6):2203-11.

19. Bogdahn U, Bauer A, Haase A. Deuterium-containing pharmaceutical compositions for destroying tumors. [cited October 30, 2019]. Available from: https://patents.google.com/patent/WO1996003996A1/en.

20. Leonard PJ, Mullins JM. D_2O induced alterations of mitosis in PtK_1 cells. Exp Cell Res 1987; 172(1):204-211.

21. Katz JJ, Crespi HL, Hasterlik RJ, Thomson JF, Finkel AJ. Some observations on biological effects of deuterium, with special reference to effects on neoplastic processes. J Natl Cancer Inst1957; 18(5):641-59.

22. Biggs MW, Eiselein JE, Wilcox G. Observations on murine leukemia treated with deuterium and X-ray. Cancer Res 1963; 23:1059-62.

23. Laeng RH, Mini RL, Laissue JA, Schindler R. Radioprotection of cultured cells by preincubation in medium containing deuterium oxide. International Journal of Radiation Biology 1991; 59(1):165-739.

24. Gross PR, Spindel W. Heavy water inhibition of cell division: an approach to mechanism. Ann N Y Acad Sci 1960; 90:500-22.

25. Takahashi TC, Sato H. Effects of heavy water (D_2O) on the length of the mitotic period in developing sea urchin eggs. Cell Struct Funct 1983; 8(4):357-65.

26. Soleyman-Jahi S, Zendehdel K, Akbarzadeh K, Haddadi M, Amanpour S, Muhammadnejad S. In vitro assessment of antineoplastic effects of deuterium depleted water. Asian Pac J Cancer Prev 2014; 15(5):2179-83.

27. Bild W, Nastasa V, Haulica I. In vivo and in vitro research on the biological effects of deuterium-depleted water: 1. Influence of deuterium-depleted water on cultured cell growth. Rom J Physiol 2004; 41(1-2):53-67.