Biomedicine & Pharmacotherapy 67 (2013) 489–496

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Original article

Deuterium-depleted water (DDW) inhibits the proliferation and migration of nasopharyngeal carcinoma cells in vitro

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ARTICLE INFO

Article history:
Received 20 January 2013
Accepted 4 February 2013

Keywords:
Deuterium-depleted water (DDW)
Nasopharyngeal carcinoma cell (NPC)
Preosteoblast cell
Cell proliferation
NADPH:quinone oxidoreductase-1 (NQO1)

ABSTRACT

Recent studies have demonstrated that natural water that has 65% of the deuterium concentration depleted, can exhibit anti-tumor properties. However, the anti-tumor effects of DDW on various nasopharyngeal carcinoma (NPC) cells have not previously been reported. In the present study, NPC cell lines and normal preosteoblast MC3T3-E1 cells were grown in RPMI1640 media containing different deuterium concentrations (50–150 ppm). The effects of DDW on the proliferation and migration of NPC and MC3T3-E1 cells were investigated using the MTT, plate colony formation, and Transwell assays, as well as Boyden chamber arrays, flow cytometry (FCM), western blot and immunofluorescence. We found that DDW was an effective inhibitor of NPC cell proliferation, plated colony formation, migration and invasion. In contrast, the growth of normal preosteoblast MC3T3-E1 cells was promoted when they were cultured in the presence of DDW. Cell cycle analysis revealed that DDW caused cell cycle arrest in the G1/S transition, reduced the number of cells in the S phase and significantly increased the population of cells in the G1 phase in NPC cells. Western blot analysis revealed that treatment with DDW significantly increased the expression of NADPH:quinone oxidoreductase-1 (NQO1), while immunofluorescence assay analysis revealed that treatment with DDW decreased the expression of PCNA and matrix metalloproteinase 9 (MMP9) in NPC cells. These results demonstrated that DDW is a novel, non-toxic adjuvant therapeutic agent that suppresses NPC cell proliferation, migration, and invasion by inducing the expression of NQO1 and causing cell cycle arrest, as well as decreasing PCNA and MMP9 expression.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck carcinoma that is common in the southern provinces of China and Southeast Asia [1,2]. The yearly incidence of NPC among Chinese males ranges from 9 to 30 per 100,000 [3,4]. NPC is difficult to detect clinically because the symptoms are not specific to the disease. Thus, NPC is detected in more than 60% of patients during the late stages of the disease (stages III and IV). According to statistics collected by the Tumor Hospital of Zhongshan Medical University, the number of new nasopharyngeal cancer cases detected annually accounts for 25–30% of all malignant tumors diagnosed. Patients suffering from inoperable tumors receive palliative therapy, including chemotherapy and radiation therapy. However, treatment options are very limited, and most individuals with recurrent or metastatic disease die within one year of diagnosis.

Water covers 70% of the surface of the earth, and organisms are composed of up to 70% water. Water is the origin of life and is composed of oxygen and hydrogen atoms. Hydrogen and deuterium (D) are two different isotopes, of which the non-radioactive isotope is stable. The compound formed by deuterium and oxygen atoms is referred to as heavy water. The ratio of deuterium to protium (D/H) in natural water is approximately 1:6600, and the natural fraction of deuterium is approximately 0.0139–0.0151% [5–9]. When the deuterium concentration is lower than 0.015% (150 ppm), the fraction is known as deuterium-poor water, low deuterium water, super light water, or deuterium-depleted water (DDW). Naturally occurring deuterium concentrations in living organisms are approximately 12 mM. Light water has a variety of different physicochemical properties and biological activities that are similar to those of deuterium isotopes. DDW has been shown to speed up exchange processes (both physicochemical and biological), resulting in the following physiological effects: immunostimulation,

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http://dx.doi.org/10.1016/j.biopha.2013.02.001

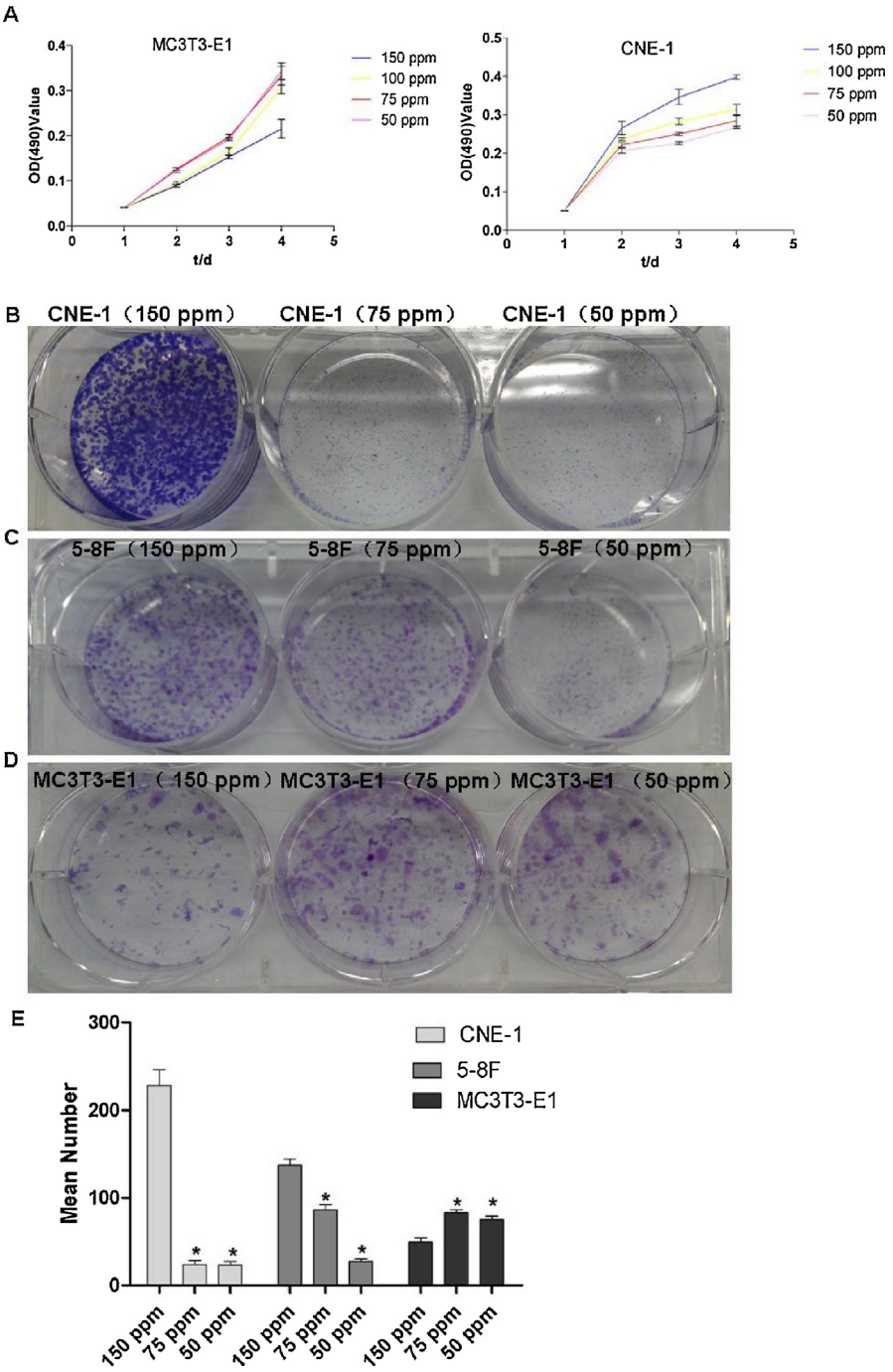

Fig. 1. Effects of deuterium-depleted water (DDW) on nasopharyngeal carcinoma (NPC) cell growth. A. NPC and MC3T3-E1 cell growth were examined using the MTT assay over a four-day period. Data are presented as the means ± SD from three independent experiments. $^{*}P < 0.05$ compared to 150, 100, 75, and 50 ppm of DDW in 1640 medium. CNE-1 (B) 5-8F and (C) MC3T3-E1 (D) Cell colony formation was examined using the plate colony formation assay. A decrease in the deuterium concentration in DDW markedly inhibited colony formation in NPC cells and increased colony formation in MC3T3-E1 cells. (E). Data are presented as the means ± SD for three independent experiments.$^{*}P < 0.05$ compared to 150, 75, and 50 ppm of DDW in 1640 medium.

reproductive effects, reversal of the Mn-induced decrease in the *Caenorhabditis elegans* life span, and interference with signal transduction pathways [10,11]. Previous studies have demonstrated that incorporating low D concentrations into conventional cancer therapy can significantly decrease the growth rate of various tumor cells and block metastasis and recurrence both in vitro or in vivo [12–18]. However, the underlying mechanisms remain unclear.

Because DDW has been shown to be active against various cancer cell lines in vitro or in vivo, the effects of different concentrations of deuterium on the growth of normal cells and NPC cell lines were examined in the present study. Due to the undesirable complications of chemoradiotherapy for NPC, which D/H ratios can actively block in this malignancy, a new approach, such as the use of DDW, may offer new options for the treatment of NPC.

2. Materials and methods

2.1. Reagents and materials

DDW (concentrations less than 0.0050% or 50 ppm) was obtained from Prof. Detao Xiao at the Institute of Nuclear Science and Technology, University of South China. Newborn calf serum

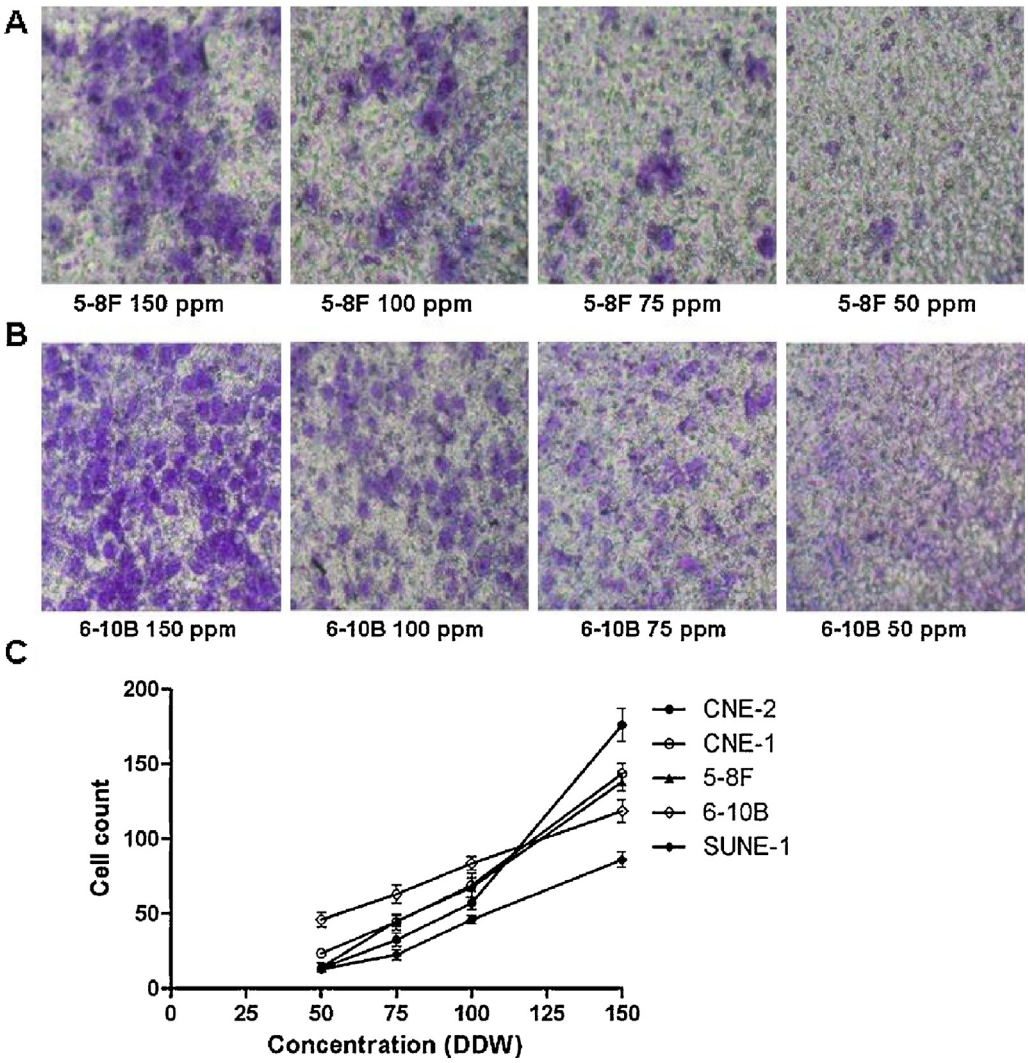

Fig. 2. Deuterium-depleted water (DDW) inhibited nasopharyngeal carcinoma (NPC) cell migration. A. The migrating capability of 5-8F cells treated with 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h was examined using Transwell and Boyden chamber assays. B. The migrating capability of 6-10B cells treated with 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h was examined using Transwell and Boyden chamber assays. C. The total numbers of cells in five NPC cell lines were counted under a microscope in five predetermined fields (×100). Data are presented as the means ± SD for three independent experiments. $^{*}P < 0.05$ compared to 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h.

was purchased from the Sijiqing Company, Hangzhou, China. An MTT kit was purchased from Boster, WuHan, China. RPMI 1640 medium was purchased from GIBCO. CNE-1, CNE-2, 5-8F, 6-10B, and Sune-1 NPC cells were obtained from the Cancer Research Institute of Southern Medical University. Normal preosteoblast MC3T3-E1 cells were obtained from the Sino-American Cancer Research Institute at Guangdong Medical College. Transwell apparatuses (8 μm pores in a polycarbonate membrane insert) were purchased from BD Biocoat. Anti-NQO1 (A180) (Genetic locus: 16q22.1) mouse monoclonal antibody was purchased from Santa Cruz Biotechnology. Goat anti-mouse IgG (H + L)/TRITC antibody was purchased from Invitrogen and was used as the secondary antibody. Anti-PCNA (PC10) mouse monoclonal antibody was purchased from Cell Signaling. Anti-matrix metalloproteinase 9 (MMP9) (ab3159) mouse monoclonal antibody was purchased from Abcam. Alexa Fluor® 594 goat anti-mouse IgG (H + L) was purchased from Invitrogen.

2.2. Cell culture

Five NPC cell lines, including CNE-1, CNE-2, 5-8F, 6-10B, and Sune-1, and normal preosteoblast MC3T3-E1 cells were grown in RPMI1640 dry powder culture medium (GIBCO) that was dissolved in water containing various concentrations of D (50, 75, 100, and 150 ppm) and supplemented with 10% heat-inactivated newborn calf serum, 1% sodium carbonate and 1% penicillin–streptomycin. The cells were maintained at 37 °C in a humidified atmosphere, containing 5% CO_2 and were deprived of serum for 12 h prior to experimentation.

2.3. MTT assay

Cell proliferation was analyzed using the MTT assay (Sigma, St. Louis, USA) by solubilizing formazan with dimethyl sulfoxide (DMSO). Briefly, logarithmically growing cells (2.0×10^3) were seeded in 96-well plates in triplicate for each condition. NPC cell lines and normal preosteoblast MC3T3-E1 cells were grown in RPMI1640 media, containing different deuterium concentrations (50–150 ppm). Cell viability was measured using the MTT assay after 24, 48 and 72 h of culture. The quantity of formazan product was measured using a spectrophotometric microtiter plate reader (Dynatech Laboratories, Alexandria, VA) at 490 nm. Each experiment was performed in triplicate.

Table 1
Cell cycle alterations in six cell lines after treatment with various concentrations of Deuterium-depleted water (50–100 ppm). Data are presented as the means ± SD for three independent experiments.

Cell	Medium (ppm)	G1	G2	S
6-10B	150	13.43 ± 0.65	15.20 ± 0.78	71.37 ± 2.36
	100	30.90 ± 1.50	0.24 ± 0.01	68.86 ± 1.75
	75	38.81 ± 1.64	0.31 ± 0.01	60.88 ± 1.56*
	50	44.15 ± 1.83	15.05 ± 0.75	40.80 ± 1.32*
CNE-2	150	38.65 ± 1.66	22.70 ± 0.97	38.65 ± 1.62
	100	48.10 ± 1.88	22.90 ± 0.98	28.99 ± 1.15*
	75	46.70 ± 1.67	26.58 ± 0.85	26.71 ± 1.07*
	50	53.90 ± 1.95	27.35 ± 0.91	18.85 ± 1.01*
CNE-1	150	55.21 ± 1.97	0.12 ± 0.01	44.65 ± 1.78
	100	47.78±1.50	13.05 ± 0.81	39.17 ± 1.54*
	75	56.81 ± 1.99	7.35 ± 0.71	35.84 ± 1.43*
	50	60.50 ± 2.01	5.72 ± 0.68	33.78 ± 1.32*
Sune-1	150	73.91 ± 2.18	12.55 ± 0.88	13.55 ± 0.93
	100	65.71 ± 1.98	24.2 ± 1.01	10.08 ± 0.81
	75	60.90 ± 1.90	33.40 ± 1.12	5.70 ± 0.32*
	50	71.45 ± 2.01	24.65 ± 0.68	3.90 ± 0.27*
5-8F	150	24.45 ± 1.05	26.65 ± 1.11	48.90 ± 1.55
	100	30.75 ± 1.50	28.06 ± 1.15	41.19 ± 1.47*
	75	28.10 ± 1.07	30.25 ± 1.27	41.65 ± 1.43*
	50	30.20 ± 1.13	31.75 ± 1.26	38.05 ± 1.31*
MC3T3-E1	150	39.70 ± 1.47	12.30 ± 0.91	48.00 ± 1.52
	100	49.80 ± 1.65	9.29 ± 0.85	40.90 ± 1.41*
	75	49.00 ± 1.63	7.33 ± 0.70	43.60 ± 1.49*
	50	46.90 ± 1.55	9.99 ± 0.87	43.10 ± 1.49*

SD: standard deviation.
* $P < 0.05$ compared to cells treated with 150 ppm of DDW in 1640 medium.

2.4. Colony formation assay

Logarithmically growing cells (500) were seeded in 6-well culture plates and were cultured in the presence of media containing different concentrations of deuterium (150, 75 and 50 ppm). After 2 weeks of incubation, the cells were washed twice with PBS, fixed with methanol and stained with crystal violet. The number of colonies, containing greater or equal to 50 cells, was counted under a microscope. Colony number and colony formation were determined by comparing the values to those obtained from NPC cells that were grown in normal culture medium. Each experiment was performed in triplicate.

2.5. Transwell assay

Logarithmically growing cells were treated with trypsin and resuspended as a single-cell solution. A total of 5×10^4 cells/mL were seeded onto fibronectin-coated polycarbonate membrane inserts in a Transwell apparatus (Corning Inc., Corning, NY). In the lower chamber, media containing different concentrations of deuterium (150, 100, 75 and 50 ppm) was added as a chemo-attractant. After the cells were incubated for 24 h, the inserts were washed with PBS, and the cells on the top surface of the insert were removed using a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with crystal violet, and counted under a microscope in five predetermined fields (×100). All experiments were independently repeated a minimum of three times.

2.6. Cell cycle analysis

Cells were plated in 6-cm culture dishes and grown to 60–70% confluence within 24 h. After overnight culture and cell adherence to the bottom of the culture dish, the culture medium was replaced with FBS-free RPMI1640. The cells were grown in the presence of media containing different concentrations of deuterium (150, 100, 75 and 50 ppm) for 24 h. The cells were harvested with EDTA containing 0.25% trypsin. After being washed, the cells were fixed with 70% ice-cold ethanol and were maintained overnight at 4 °C. The cells were collected in ice-cold PBS and stained with propidium iodide (PI) (500 μl of PBS + 2.5 μl of PI + 2 μl of RNase) in the dark for 30 min at room temperature. The cell cycle phase distribution was analyzed using a FACS flow cytometer (BD Biosciences). Each experiment was performed in triplicate.

2.7. Western blot analysis

Briefly, the cells were grown in FBS-free RPMI1640 for 12 h and then plated in 6-cm culture dishes with media containing different concentrations of deuterium (150, 100, 75 and 50 ppm) for 24 h. The cells were then washed once with ice-cold PBS and lysed in 200 μl of RIPA buffer containing protease inhibitors. Protein concentrations were determined using a NanoDrop Spectrophotometer. Total proteins (100 μg) were resolved using a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 2.5% nonfat dry milk in PBST for 60 min. The membranes were immunoblotted overnight at 4 °C with an anti-NQO1 monoclonal antibody (1:3000) and an anti-β-actin antibody (1:3000), followed by incubation with a goat anti-mouse IgG (H + L)/TRITC secondary antibody. Signals were detected using an LI-COR Odyssey Infrared imaging system. All experiments were repeated three times.

2.8. Immunofluorescence

Logarithmically growing cells were seeded in 24-well culture plates and were cultured in the presence of media containing different concentrations of deuterium (150 and 50 ppm) for 24 h. The cells were washed twice with PBS and then fixed with a methanol/glacial acetic acid mixture (3:1). The cells were then permeabilized with 0.25% Triton X-100 plus 5% DMSO in PBST for 30 min at 37 °C. The samples were washed with PBS and blocked with 2.5% nonfat dry milk in PBST for 60 min. The coverslips were incubated overnight with an anti-PCNA monoclonal antibody (dilution 1:500) and an anti-MMP9 monoclonal antibody (dilution 1:200) in PBS at 4 °C. The cells were rinsed three times in PBS and incubated with a Dylight 594 AffiniPure Goat anti-mouse IgG (H + L) secondary antibody (dilution 1:200) for 1 h at 37 °C. Finally, the coverslips were washed and stained with DAPI. Images were observed under a fluorescent microscope. Each experiment was performed in triplicate.

2.9. Statistical analyses

All data were analyzed for statistical significance using SPSS 13.0 software. One-way ANOVA was used to determine the differences between the groups for all in vitro analyses. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Deuterium-depleted water inhibited the proliferation of nasopharyngeal carcinoma cells in vitro

Using MTT assays, we found that DDW significantly inhibited the proliferation of NPC cells. At 24 h, the inhibition ratio of media containing deuterated water (100, 75, and 50 ppm) in CNE-1 cells was 13.51%, 20.28% and 27.51% compared to cells that were cultured in normal medium. At 48 h, the inhibition ratio of media containing deuterated water (100, 75, and 50 ppm) in CNE-1 cells was 21.35%, 32.54% and 40.25% compared to cells that were cultured in normal medium. At 72 h, the inhibition ratio of media

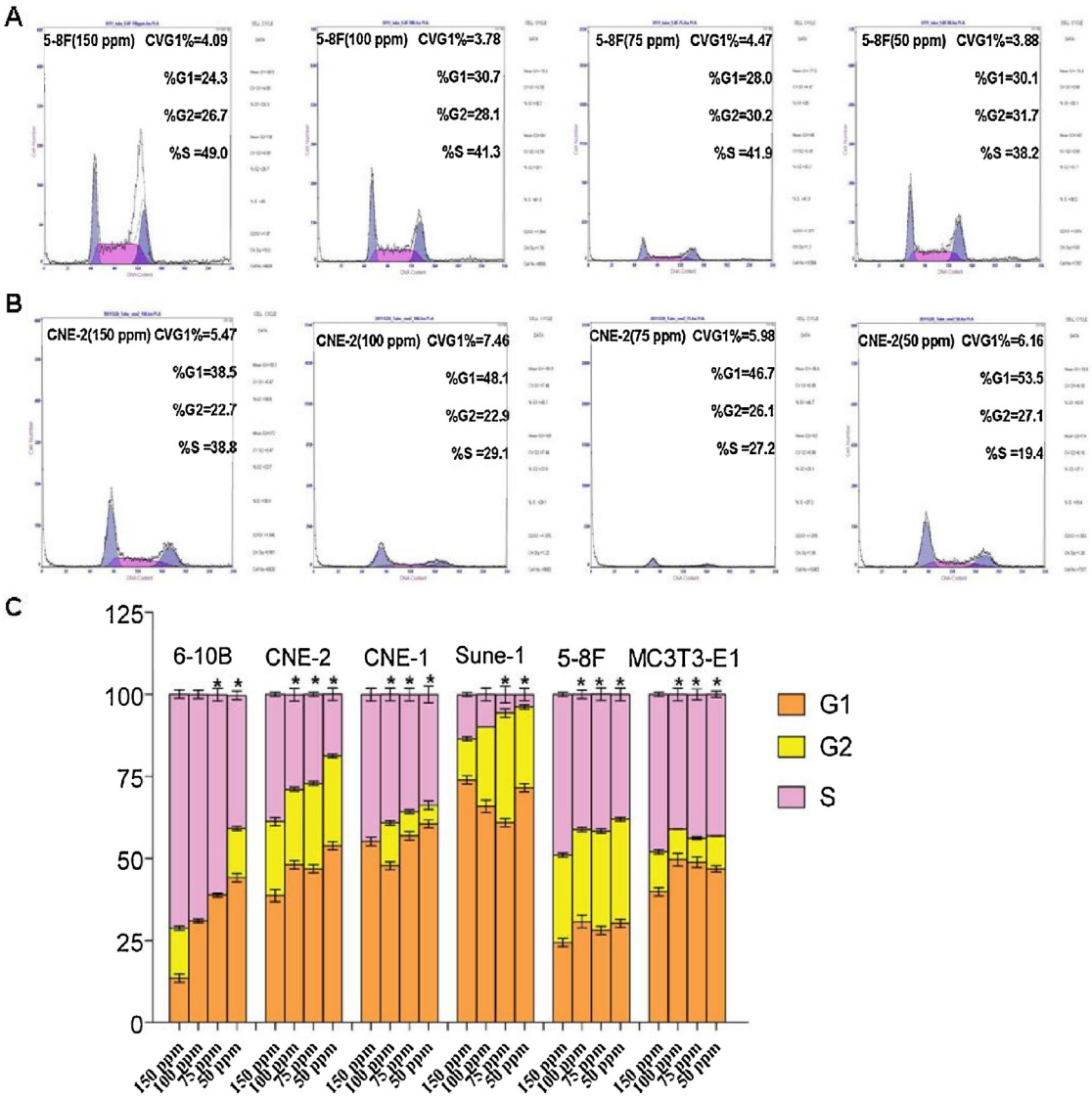

Fig. 3. Deuterium-depleted water (DDW) inhibited cell cycle progression in vitro. A. The cell cycle profile of 5-8F cells that were treated with 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h was determined using FACS Caliber cytometry. B. The cell cycle profile of CNE-2 cells that were treated with 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h was determined using FACS Caliber cytometry. C. Six cell cycle profiles were determined using FACS Caliber cytometry. Data are presented as the means ± SD for three independent experiments. $^{*}P < 0.05$ compared to 150, 100, 75, and 50 ppm of DDW in 1640 medium for 24 h.

containing deuterated water (100, 75, and 50 ppm) in CNE-1 cells was 24.35%, 32.71% and 37.61% compared to cells that were cultured in normal medium. The differences were statistically significant ($P < 0.05$) (Fig. 1A). Similar results were observed in other NPC cell lines (data not shown).

In addition, MTT analysis revealed that at decreasing concentrations of deuterium, DDW significantly promoted normal preosteoblast MC3T3-E1 proliferation. At 24 h, the proliferation rate of normal preosteoblast MC3T3-E1 cells cultured in media containing deuterated water (100, 75, and 50 ppm) was 111.2%, 173.2% and 168.8% compared to cells that were cultured in normal medium. At 48 h, the proliferation rate of normal preosteoblast MC3T3-E1 cells cultured in media containing deuterated water (100, 75, and 50 ppm) was 110.1%, 139.3% and 169.8% compared to cells that were cultured in normal medium. At 72 h, the proliferation rate of normal preosteoblast MC3T3-E1 cells cultured in media containing deuterated water (100, 75, and 50 ppm) was 152.8%, 166.8% and 172.8% compared to cells that were cultured in normal medium. The differences were statistically significant ($P < 0.05$) (Fig. 1A).

3.2. Effects of deuterium-depleted water on colony forming ability

Plate cloning results showed that the ability of NPC tumor cells to form colonies was significantly inhibited by DDW. The colony numbers in the groups exposed to media containing low deuterium concentrations (75 and 50 ppm) and normal medium in CNE-1 cells were 23.5 ± 4.5, 23.0 ± 4.0, and 228.0 ± 18.0, respectively (Fig. 1B), and in 5-8F (Fig. 1C) cells, the colony numbers were 86.0 ± 6.0, 27.0 ± 3.0, and 137.0 ± 7.0, respectively ($P < 0.01$, Fig. 1E). Other NPC cell lines that were treated with DDW exhibited similar results (data not shown). However, decreases in the deuterium concentration in DDW promoted colony formation in normal preosteoblast MC3T3-E1 cells (Fig. 1D). The colony numbers in MC3T3-E1 cells that were exposed to media containing low deuterium concentrations (75 and 50 ppm) and normal medium

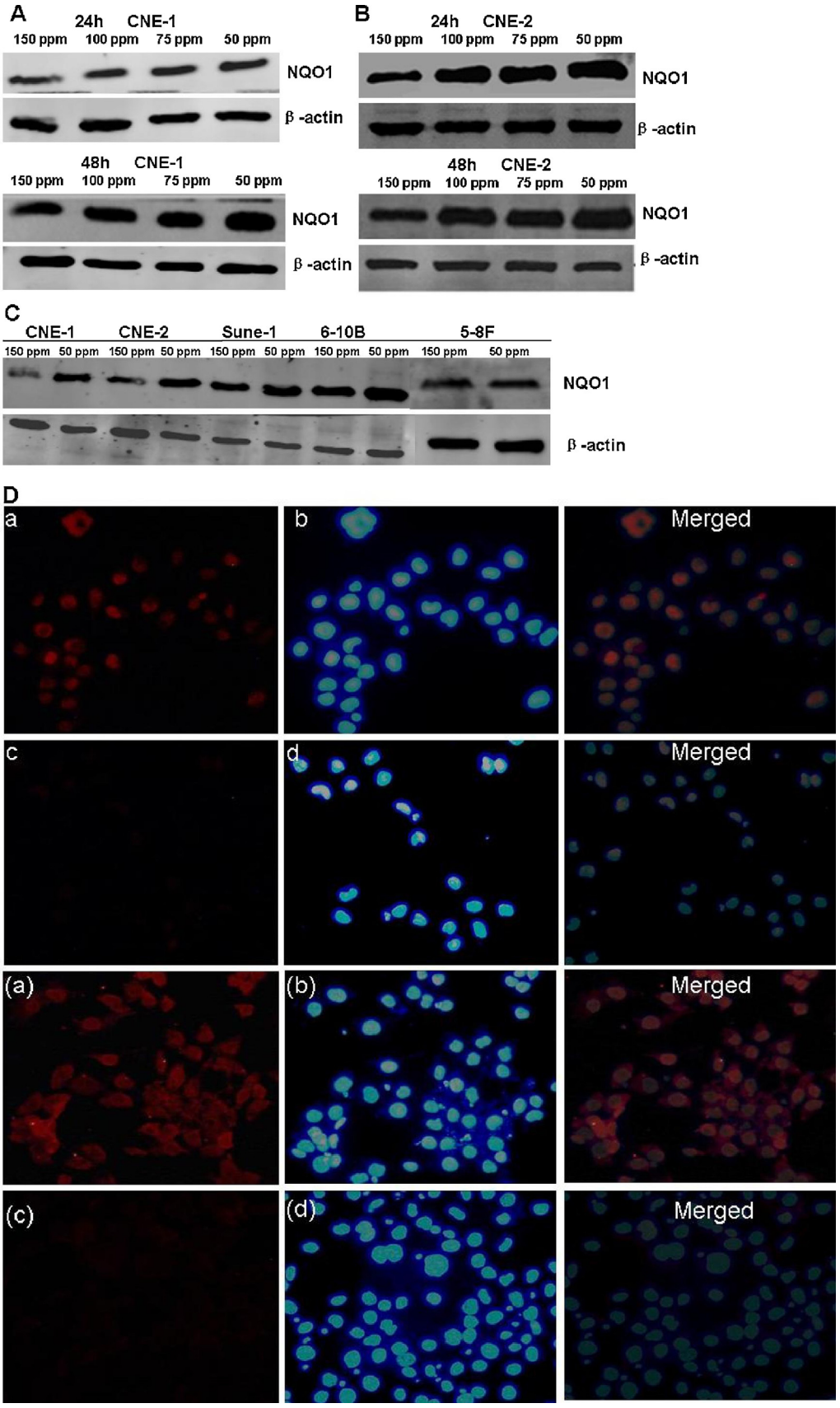

Fig. 4. NADPH:quinone oxidoreductase-1 (NQO1) expression was determined via western blot. A. NQO1 expression was determined in CNE-1 cells that were treated with 150, 100, 75, and 50 ppm of deuterium-depleted water (DDW) in 1640 medium for 24 or 48 h. B. NQO1 expression was determined in CNE-2 cells that were treated with 150, 100, 7, and 50 ppm of DDW in 1640 medium for 24 or 48 h. C. NQO1 expression was determined in five nasopharyngeal carcinoma (NPC) cell lines that were treated with 150 and 50 ppm of DDW in 1640 medium for 24 h. D. Immunofluorescent microcopy of NPC cell lines that were treated with DDW using anti-PCNA and anti-MMP9 monoclonal antibodies. CNE-2 cells were chosen to represent NPC cells. a: CNE-2 cells treated with 150 ppm of DDW in 1640 medium for 24 h were fixed and stained for PCNA-immunoreactivity (red); b: Cell

were 83.0 ± 3.0, 75.0 ± 4.0 and 49.5 ± 4.5, respectively ($P < 0.01$, Fig. 1E).

3.3. Deuterium-depleted water inhibited migration

Cell migration is a key step for tumor development and metastasis. We tested the ability of five NPC cell lines to migrate through 8 μm pores on a polycarbonate membrane and found that as the deuterium concentrations in DDW were reduced, cell migration in the five NPC cell lines significantly decreased ($P < 0.01$) (Fig. 2 A–C). Migration was markedly inhibited in CNE-2, Sune-1 and CNE-1 cells that were treated with DDW (data not shown).

3.4. Deuterium-depleted water inhibited cell cycle progression

We measured alterations in cell cycle progression in five NPC cell lines that were cultured in the presence of media containing deuterium (100, 75 and 50 ppm) and normal medium. Flow cytometric analysis revealed that the NPC cell cycle was altered as the concentration of deuterium in DDW was reduced. Specifically, gradual increases in the number of cells in the G1 phase and gradual reductions in the number of cells in the S phase were observed ($P < 0.05$) (Table 1, Fig. 3 A–C). The results of cell cycle analysis for the 6-10B, CNE-1, Sune-1 and MC3T3-E1 cell lines are shown in Table 1.

3.5. Deuterium-depleted water promoted NADPH:quinone oxidoreductase-1 protein expression in nasopharyngeal carcinoma cells

We measured the protein expression levels of NADPH:quinone oxidoreductase-1 (NQO1) in various NPC cell lines via western blot. As the deuterium concentrations were reduced, NQO1 protein expression was markedly promoted in five NPC cell lines (Fig. 4 A–C). NQO1 protein expression in 6-10B, 5-8F and Sune-1 cells was increased following DDW exposure in both a dose- and time-dependent manner (Fig. 4C).

3.6. Deuterium-depleted water inhibited proliferating cell nuclear antigen and MMP9 immunostaining in vitro

We measured the expression levels of proliferating cell nuclear antigen (PCNA) and MMP9 in NPC cell lines using immunofluorescence. We found that NPC cell lines that were treated with ordinary culture medium displayed strong PCNA and MMP9 fluorescence after immunostaining. As the deuterium concentration was reduced to 50 ppm, PCNA and MMP9 expression decreased markedly in NPC cells, as indicated by fluorescence levels (Fig. 4D).

4. Discussion

Deuterium, a stable, non-radioactive isotope of hydrogen, binds to oxygen to form deuterium oxide (D_2O), which is commonly referred to as heavy water. The naturally occurring deuterium concentration in the environment is only 1/6600 (150 ppm) of H_2O and 12 mM in all-living systems. Interestingly, recent studies have demonstrated that using deuterium-depleted water, decreasing the deuterium concentration of internal solutions, or replacing additional extracellular D can result in tumor regression in vitro or in vivo, but the specific mechanisms remain unclear. Krempels et al. found that when the water consumed by four lung cancer patients with brain metastases was replaced with DDW (10–20 ppm) for at least three months, the deuterium concentrations in the patients' bodies were gradually reduced. DDW consumption in combination with conventional treatments noticeably prolonged the survival time of all the four lung cancer patients with brain metastases [16]. Furthermore, the anti-tumor effects of DDW have been extensively studied in various cell types [16–18]. DDW is able to inhibit tumor growth and prolong xenograft survival in mice. Deuterium concentrations gradually increased in the brain and other tissues in experimental mice drinking heavy water (D_2O). However, when the daily water intake of the mice was replaced with DDW, the increased deuterium that had become integrated into the brain tissue of the experimental mice decreased [19]. This experiment suggested that D/H could rapidly cross the blood-brain barrier. Most anti-cancer chemotherapeutic agents are unable to target brain metastases because they are unable to cross the blood-brain barrier. Thus, DDW may be a useful auxiliary therapeutic in the treatment of central nervous system diseases.

In this present study, we observed that DDW effectively inhibited growth and colony formation in five different human NPC cell lines. This finding was similar to the reports of Somlyai et al. in human prostate cancer, breast cancer, colon cancer, and melanoma in vitro [20]. Interestingly, we found that DDW did not suppress the proliferation of normal preosteoblast MC3T3-E1 cells. On the contrary, DDW promoted the proliferation of these cells. This observation indicated that DDW could selectively inhibit the proliferation of NPC cells but not normal cells, indicating that DDW may be a novel anti-NPC agent. Furthermore, we also found that DDW significantly reduced the migration and invasion ability of NPC cells. These results further demonstrated the anti-tumor effects of DDW in NPC.

The biological functions of DDW observed in this study further supported our hypotheses. Previous studies have provided abundant evidence demonstrating that alterations in the D/H ratio can trigger molecular mechanisms that play key roles in cell cycle regulation. Concomitant increases in the D/H ratio trigger the cells to enter into the S phase [21]. Consistent with these findings, our experiments showed that decreases in the D/H ratio caused remarkable decreases in the number of cells in the S and G2 phases of the cell cycle in all five NPC cell lines. Over the past few years, NADPH:quinone oxidoreductase-1 (NQO1) has been reported to be a regulator of cell cycle factors, including CCND1, p21, and c-Myc [22]. In this study, we found that NQO1 expression became upregulated in NPC cells as the deuterium concentrations in the medium declined. Furthermore, immunofluorescence analysis revealed that the expression of PCNA, a gene marking cell growth speed, declined as the deuterium concentrations in NPC cells decreased. These results further supported the notion that DDW suppresses cell cycle progression, thereby, inhibiting NPC growth.

In addition to the ability of DDW to suppress NPC growth, we also observed that cell migration and invasion were significantly reduced as the deuterium concentrations in NPC cells declined. Based on these findings, we examined the protein expression levels of MMP9, a matrix metalloproteinase that is thought to play a critical role in promoting NPC cell metastasis [23]. Interestingly, immunofluorescence analysis revealed that MMP9 expression decreased significantly in NPC cells as the concentration of deuterium declined. These results suggested that the DDW-mediated suppression of metastasis might be partially attributed to the attenuated expression of MMP9 in NPC cells.

nuclei were stained using DAPI (blue); c: CNE-2 cells that were treated with 50 ppm of DDW in 1640 medium for 24 h were fixed and stained for PCNA-immunoreactivity (red); d: Cell nuclei were stained using DAPI (blue); (a): CNE-2 cells treated with 150 ppm of DDW in 1640 medium for 24 h were fixed and stained for MMP9-immunoreactivity (red); (b): Cell nuclei were stained using DAPI (blue); (c): CNE-2 cells treated with 50 ppm of DDW in 1640 medium for 24 h were fixed and stained for MMP9-immunoreactivity (red); (d): Cell nuclei were stained using DAPI (blue).

In summary, this study provided evidence that DDW suppresses cell growth, migration, invasion, and cell cycle progression in NPC cells but does not affect the growth of normal cells. Furthermore, DDW-mediated suppression of growth and invasion may be partially attributed to the stimulation of NQO1 and the down-regulation of MMP9 expression.

Authors' contributions

Huiling Yang and Weiyi Fang designed the research. Hongqiang Wang and Baohua Zhu performed this research and analyzed the data. Zhiwei He, Hui Fu, Zhong Dai, Dongyun Qin and Xiaoyan Zhang contributed to the experiments. Guoliang Huang, Binbin Li and Lu Tian contributed reagents and analytical tools. Huiling Yang and Hongqiang Wang interpreted the data and wrote the manuscript. Weiyi Fang and Huiling Yang supervised all of the work. All of the authors have read and approved the final manuscript.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgements

This work was supported by two grants from the National Natural Sciences Foundation of China (NO. 30971438 and NO. 81272334).

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