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The effect of the deuterium depleted water on the biological activity of the eukaryotic cells

A.V. Syroeshkin[a], N.V. Antipova[a,b], A.V. Zlatska[c,d], I.A. Zlatskiy[a,e,*], M.D. Skylska[e], T.V. Grebennikova[a,f], V.V. Goncharuk[e]

[a] Peoples Friendship University of Russia (RUDN University), 6 Miklukho-Maklaya St., Moscow, 117198, Russian Federation
[b] Shemyakin-Ovchinnikov Institute of bioorganic chemistry, Moscow, Russian Federation
[c] State Institute of Genetic and Regenerative Medicine of National Medical Academy of Sciences of Ukraine, Kyiv 04114, Ukraine
[d] Biotechnology Laboratory ilaya.regeneration, Medical Company ilaya®, Kyiv 03115, Ukraine
[e] Dumanskii Institute of Colloid and Water Chemistry, National Academy of Sciences of Ukraine, Kiev, Ukraine
[f] Federal Research Center of Epidemiology and Microbiology named Gamalei, Moscow, Russian Federation

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ABSTRACT

Here we show the dependence of the unicellular biosensor *S.ambigua* lifespan on the water D/H isotopic composition. This dependence is bell-shaped with descents both in case of deficiency or excess of deuterium in water. The influence of the water D/H isotopic composition on the cell culture proliferative potential and colony forming efficiency in vitro was tested on the human dermal fibroblasts. We observed that the deuterium depleted water stimulates cell colony formation at the early passages. The dynamics of the cell doubling index in the deuterium depleted water-based growth medium showed higher proliferation potential compared to the water with normal isotopic composition. Using scratch assay, we have also studied the impact of the growth medium D/H isotopic composition on the cell motility of human cancer cell lines A549 and HT29. We have shown that the deuterium depleted water considerably suppressed cancer cell lines amoeboid movement in vitro.

1. Introduction

Up to 70% of the human body consists of water. The deuterium content in this water is 0.015%. In quantitative content (atomic percents), it takes 12th place among chemical elements that compose human organism [1,2]. In this respect, deuterium may be classified as a microelement among which it takes the first place as the content of such microelements as copper, iron, zinc, molybdenum or manganese in the organism is tenfold and hundredfold less than that of deuterium. If this isotope is considered as a microelement, it is unclear how the deuterium excess or deprivation may alter the organism biological activity.

It is known that the physicochemical characteristics of the deuterium depleted water (DDW) differ from the water of natural isotopic composition [3–5]. The protective qualities of the DDW were supported by the toxicological research that showed that the water depleted of heavy isotopes effectively removed toxins and metabolic waste products from the organism due to its transport properties [6,7].

At the molecular level, it was previously shown that reducing deuterium in water lower than natural concentrations (< 90 ppm) activates and accelerates the mitochondria respiratory chain reaction, which is almost completely inhibited in the excess of deuterium (deuterium concentration up to 99%) [8,9]. It was also observed that different deuterium concentrations have various impact on the proliferative activity of prokaryotic and eukaryotic cell cultures in vitro [10–12].

As a model of mammalian cell line we have chosen human fibroblasts. Human fibroblasts are actively used in the regenerative medicine and cosmetology. These cells possess substantial proliferative potential, immunomodulatory properties, ability of multilineage differentiation. These features together with low invasiveness of the material taking procedure make this cell type an attractive object for using in regenerative medicine and cosmetology [13,14]. Thus we have chosen this cell type for our investigations.

A positive effect of the DDW was previously shown in the experiments with immortalized tumor cell lines on laboratory animals and other systems in vivo and in vitro [15,16]. One of the most important signs of the malignant tumor process is metastasis - migration of tumor cells from the primary focus all over the organism. The presence of metastases determines the disease progress and the patient's destiny. A number of factors influencing this process was described, such as the degree of tumor malignancy, tumor structure, differentiation level

* Corresponding author at: Dumanskii Institute of Colloid and Water Chemistry, National Academy of Sciences of Ukraine, 42 Vernadskogo bv., Kiev, 03680, Ukraine; Peoples Friendship University of Russia (RUDN University), 6 Miklukho-Maklaya St, Moscow, 117198, Russian Federation.
E-mail address: zlatskiy@ukr.net (I.A. Zlatskiy).

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(anaplasia), cancer cell invasion into venous capillaries, amoeboid movement and looseness of cancer cell junctions, lack of calcium ions, increased enzyme function (hyaluronidase), organ physiological mobility (for instance, tongue, lung, stomach etc.) [17,18]. One of these factors - amoeboid movement - can be analyzed in laboratory conditions using cell culture.

Regarding that the deuterium activity in biological systems is poorly defined, the aim of this study was to examine the role of deuterium in the cell biological activity both of free-living cells and of the normal and cancer cell lines in vitro.

2. Materials and methods

2.1. Physicochemical analysis of water with different deuterium content

The next basic water samples with different deuterium content were used in this study:

deuterium depleted water (DDW, light water) with D/H = 4 ± 2 ppm (NPO "Almaz", Tambov, Russian Federation); heavy water with D/H = 99 absolute at. % ("Sigma", USA).

Control of deuterium concentration was conducted on the - Isotopic Water Analyzer-912-0032 (Los Gatos Research Inc., USA).

The milli-Q deionized water with the natural D/H ratio 150 ± 2 ppm served as a control. For the experiments, the light or heavy water was diluted with the milli-Q deionized water. The final deuterium concentration in the deuterium-depleted water-based growth media for the in vitro experiments was D/H = 30 ± 2 ppm (further referred as DDW medium).

The deionized, deuterium depleted and heavy water did not have significant differences in physical parameters [19] and microelement composition, except for the deuterium content. This excluded the multiple-factor influence of physicochemical composition in experimental system for all comparison groups.

The chemical composition of water with different deuterium content was analyzed using mass spectrometry with inductively coupled plasma on ICP-QMS Agilent 7500CE spectrometer [20].

Calibration solutions with a high range of elements' concentration (from 0.1 μg/dm^3 to 100 μg/dm^3) were used for the device calibration. The solutions were prepared based on the international standard 2.74473.0100 "ICP Multi Element Standard Solution XXI CertiPUR®" which contained the following elements: Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn, Hg.

The water samples were analyzed for the following elements: Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn, Hg. The concentration of all above-listed 24 elements in the deionized, light or heavy water did not exceed the upper detection limit (detection limit range −0.1–10 ppm).

2.2. Biological activity determination using unicellular biosensor *Spirostomum ambigua*

The Spirotox method was used for the determination of biological activity of the unicellular free-living eukaryotic organism infusorium *S. ambigua* in the water with varied isotopic D/H composition [21]. The lifespan and the activation energy of the biosensor in the water with different deuterium content and changing temperature rate were defined [22]. The Spirotox method was performed using Lauda A6 thermostat for the stable temperature maintenance in the medium. The MBR-10 binocular microscope was used for the biosensor observation.

2.3. Human dermal fibroblasts cell culture

The experiments with the human cell culture in vitro were carried out in accordance with the human experiment issues of the Code of Ethics of the World Medical Association (Declaration of Helsinki). In all cases the voluntary informed consents were signed by donors [30]. Human fibroblasts were isolated by the enzymatic digestion of the skin biopsy samples. The skin samples were incubated in the 0.1% collagenase IA and 0.1% pronase with the addition of 2% FBS (fetal bovine serum) in DMEM/F12 medium (all from Sigma, USA) for 2 h at + 37 °C. The obtained cell suspension was transferred to the culture dishes and cultured in the 199 medium (Sigma, USA) prepared from the 10x concentrate by the dilution with the DDW or deionized water with natural isotopic composition (further referred as DDW medium and control medium). The culture media were additionally supplemented with 10% FBS, 2 mM glutamine and 1 μg/ml FGF-2 (all from Sigma, USA). The cells were cultured in multigas incubators (Binder, Germany) at + 37 °C in the atmosphere of absolute humidity, 5% CO_2 and 5% or 20% O_2.

The cells lines from three different donors were used for the experiments. The studies of proliferative activity and colony forming efficiency were performed beginning from the 2nd passage (P2).

For the colony forming unit assay 100 cells were seeded at the 100 mm diameter Petri dish and the FBS concentration in the cell culture medium was increased to 20%. In 14 days the cells were fixed with cold ethanol and stained with Romanowsky stain. The colony forming efficiency was assessed by the number of colonies per culture dish. The experiment was performed in triplicate for each cell line at P2 and P4.

The population doubling time (PDT) was calculated as follows:

Doubling time = ln(2) / growth rate;

Growth rate = $(\ln(N_{(t)}/N_{(0)})/t$,where $N_{(0)}$ - cell number at point 0; $N_{(t)}$ - cell number at point t; t - time of culture.

The PDT was estimated at P2, P3, P4, P5, and P6.

2.4. Migration assay for human highly invasive cancer cell lines

Human cancer cell lines A549 (lung carcinoma [23–25]) and HT29 (colon adenocarcinoma [26–29]) were cultured in DMEM medium prepared from the powder by dilution in the DDW or deionized water with natural isotopic content and sterilized by filtering. The media are further referred as DDW medium and control medium. The culture media were additionally supplemented with 10% FBS, 2 mM glutamine (all from Sigma, USA). The cells were cultured in CO_2 incubators (Binder, Germany) at + 37 °C in the atmosphere of absolute humidity and 5% CO_2.

A549 and HT29 cell lines were seeded in 24 well or 6 well plates in a density to reach 90% confluence in 24 h. Afterwards the cells were cultured in serum free medium (to inhibit cell proliferation). Then the cells were washed three times and the culture medium was completely changed for the experimental medium for each group.

The cell monolayer was scratched (0.5 mm), and the photos of the marked scratch wound regions were taken each 30 min during 12–24 h. The scratch wound healing was evaluated as the ratio of the wound area in 12/24 h to the initial wound area.

2.5. Microscopy, software and statistical analysis

Cell culture observation was performed by phase-contrast microscopy with Carl Zeiss Axio Scope.A1 microscope. The photos were taken by Canon Power Shot G9 camera using AxioVision software (all manufactured by Carl Zeiss, Germany). The estimation of scratch wound area was performed with ImageJ program (Wayne Rasband (NIH)).

Statistical analysis was carried out using standard statistical methods [31] in Microsoft Excel (USA) and Origin 8 (USA). The significance of difference between two data groups was assessed by Student's *t*-test with $p < 0.01$ or $p < 0.05$.