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

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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

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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 $p < 0.05$.

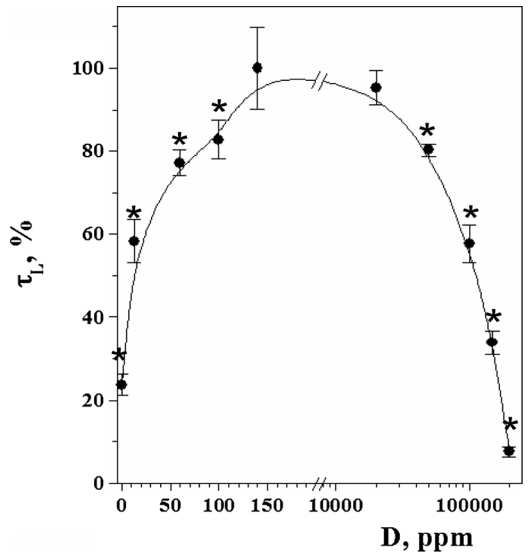

Fig. 1. The dependence of *S.ambigua* lifespan on the deuterium concentration in water. 100% corresponds to 55 ± 5 min (28 °C) (The results are expressed as mean ± SD (n = 18), * - significant differences between the experimental group and the control, $p < 0.01$).

3. Results and discussion

3.1. Estimation of the biological activity of the water with different isotopic D/H ratio using the unicellular biosensor Spirostomum ambigua

Establishing a role of deuterium in the eukaryotic cell metabolism, we observed that the dependence of the cellular biosensor *S.ambigua* lifespan on the deuterium content in water (in the interval from 0 to 99 absolute at. %) was bell-shaped with apparent minimums both in the excess and the lack of the element (Fig. 1). It should be mentioned that in natural geochemical range, where deuterium concentration varies from D/H = 90 ppm (antarctic ice) to D/H = 200 ppm (deep sea water), the difference in cellular biosensor lifespan was not significant. These data indicate that deuterium is necessary for the normal cell functioning.

There was practically no dependence of the cellular biosensor death process activation energy on the deuterium concentration (Table 1). It should be mentioned that the lack of relation between the death kinetics activation energy and the deuterium concentration indicates that the biosensor cellular transitions are due to the kinetic isotope effect [32] but not due to the reaction on the toxin presence.

We suppose that the minor fluctuations in *S. ambigua* lifespan could be the result of the change in the unicellular organisms' subpopulation composition that was caused by the D/H ratio variation. But this issue requires additional research.

3.2. Estimation of the deuterium impact on the human fibroblasts cell culture in vitro

The cell culturing in the conditions of various oxygen content is explained by the different oxygen concentration in the atmosphere and human tissues. Thereby the study of colony forming efficiency of human dermal fibroblasts was performed under the conditions of 5% O_2 and 20% O_2 [33–36].

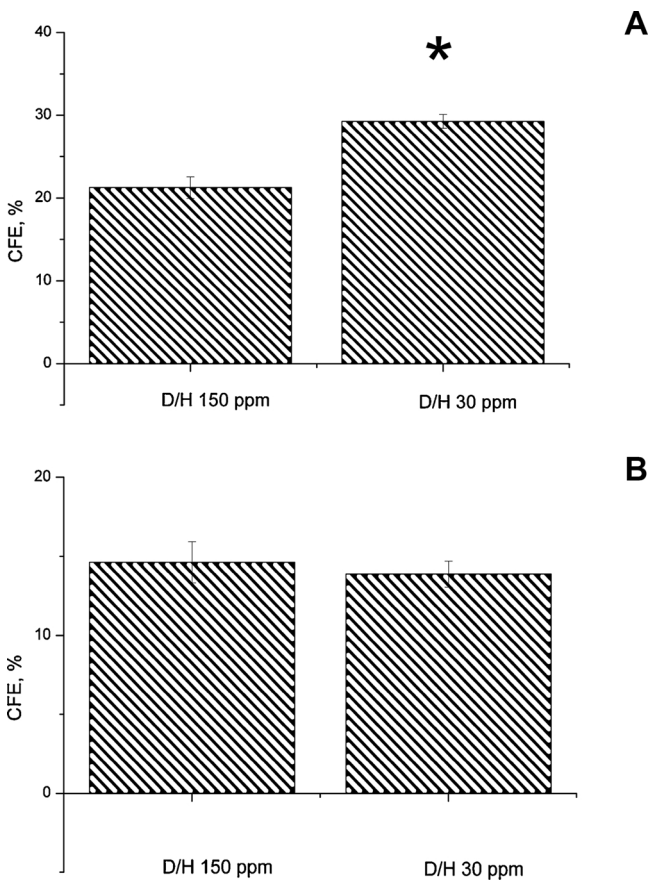

Fig. 2. Human dermal fibroblasts colony forming efficiency (CFE) in the control medium (D/H 150 ppm) or DDW medium (D/H 30 ppm) cultured at 5% O_2 (A) or 20% O_2 (B) at P2 (The results are expressed as mean ± SD (n = 3), * - significant differences between the experimental group and the control, $p < 0.05$).

We observed that the cells cultured at 20% O_2 formed significantly less colonies than the cells at 5% O_2 in both groups (Fig. 2). This fact can be explained by the suggestion that the cells cultured in vitro under 5% O_2 conditions do not undergo such a stress as the cells cultured at 20% O_2 and that 5% O_2 content is normal for most human tissues whereas 20% O_2 cause an oxidative stress. Besides, different oxygen content determines metabolic activity and the kinetics of redox reactions in the cell [35].

If we do not consider 5% O_2 and 20% O_2 conditions, the colony forming efficiency of the cells cultured in the DDW medium at 5% O_2 was higher compared to the cells cultured in the control medium. Interestingly, this tendency was not preserved if the human dermal fibroblasts were cultured at 20% O_2.

As the number of colonies was considerably less under 20% O_2 conditions compared to 5% O_2 the further experiments were performed only at 5% O_2.

However, the data of the human fibroblasts cell culture population doubling time (PDT) in media with different isotopic composition at 5%

Table 1
The values of the true energy of S.ambigua death process activation in the water with different D/H ratio (The results are expressed as mean ± SD (n = 12), * - significant differences between the experimental group and the control, $p < 0.01$).

Concentration D, ppm	4	20	32	61	90	117	137	264
obsEa, kJ/mol	138,6 ± 15,3	108,1 ± 11,2	140,31 ± 19,1	140,31 ± 18,7	135,1 ± 17,3	145,5 ± 21,4	138,2 ± 13,9	103,9 ± 9,6

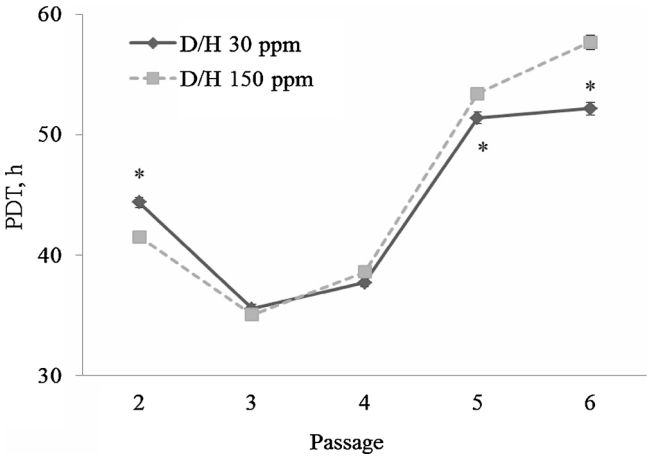

Fig. 3. Human dermal fibroblasts PDT in the control medium (D/H 150 ppm) or DDW medium (D/H 30 ppm) (The results are expressed as mean ± SD (n = 3), * - significant differences between the experimental group and the control, $p < 0.05$).

O_2 appear the most interesting (Fig. 3).

The PDT dynamics was common for both groups: at P2 the values were average for different cell types in vitro (42–45 h), at P3-P4 the PDT decreased (24–28 h) - the active cell proliferation began. However, at P5-P6 the cell cycle duration increased again (52 h), which signified the cell reserve exhaustion and the population senescence [36].

At P2 the PDT in the DDW growth medium was 3 h more compared to the control medium. This indicates the stressful influence of the DDW medium on the human dermal fibroblasts cell culture.

At P3-P4 the PDT was almost equal for both groups (35–36 h). This can be explained by the cell population adaptation to the new culture conditions [37].

The most interesting data were obtained at late passages (P5-P6) were we observed a reliable difference in PDT of human dermal fibroblasts in the DDW medium or control medium. In both comparison groups the PDT increased at later passages which suggested the beginning of the population senescence and the inhibition of proliferation activity. However, in the DDW the PDT change dynamics was more smooth (51–52 h) compared to the water with natural isotopic composition (52–58 h). This means higher proliferative potential reserve of human dermal fibroblasts cell culture in the DDW medium.

The obtained results suggest that deuterium has a direct influence on the proliferative activity and colony forming efficiency of the human dermal fibroblasts in vitro.

3.3. Estimation of the deuterium impact on the migration ability of human highly invasive cancer cell lines

One of the most important cancerogenesis features, which is often associated with the more aggressive phenotype, is the cancer cell migration ability. Cell migration is the complex phenomenon which requires high coordination of multiple cell processes. We have chosen a scratch wound healing assay – a method that imitates in vivo cell migration.

Lung and colon cancer cell lines were chosen from the cell lines collection as the samples of carcinoma and adenocarcinoma - the tumors which give early secondary focuses, i.e. rapidly metastazing tumors [38].

We observed, that the cell monolayer scratch wound healing processed much slower in the DDW medium compared to the control medium (Figs. 4 and 5).

Fig. 5 represents the photos of the A549 cell line scratch wound healing in 0 and 24 h. The minimal cell motility 24 h after scratch was 3%.

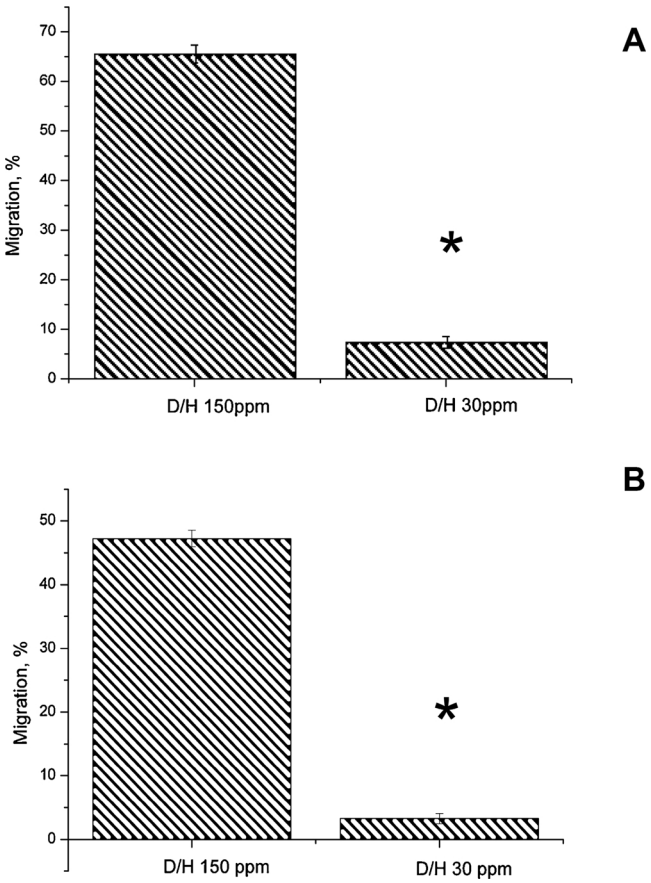

Fig. 4. The evaluation of the amoeboid cell movement of A549 (A) and HT29 (B) cell lines in the media with different deuterium content 24 h after scratch. (The results are expressed as mean ± SD (n = 30), * - significant differences between the experimental group and the control, $p < 0.01$).

Total migration activity analysis showed that in 24 h the cell migration in the DDW medium was 3–6 % compared to 47-65% in the control medium, which is 10 fold less (Fig. 4).

These results demonstrate that deuterium has a direct impact on the A549 and HT29 human cancer cell lines movement ability in vitro.

4. Conclusions

Altogether our data suggest that deuterium as a microelement is indispensable for the normal cell functioning. We have also demonstrated that the human dermal fibroblasts proliferation activity and colony forming ability increase in the DDW medium. However, in a scratch wound healing migration assay for A549 and HT29 human cancer cell lines the monolayer wound healing processed much slower in the DDW medium compared to the control medium.

Thus, considering all above mentioned data, the deuterium role in biological systems is still unclear. Increase or decrease of the deuterium concentration in water may lead both to the increase and decrease of the biological activity. Thereby this issue requires further investigation in the chemistry, physics and biology fields.

Conflict of interest

None.

Acknowledgment

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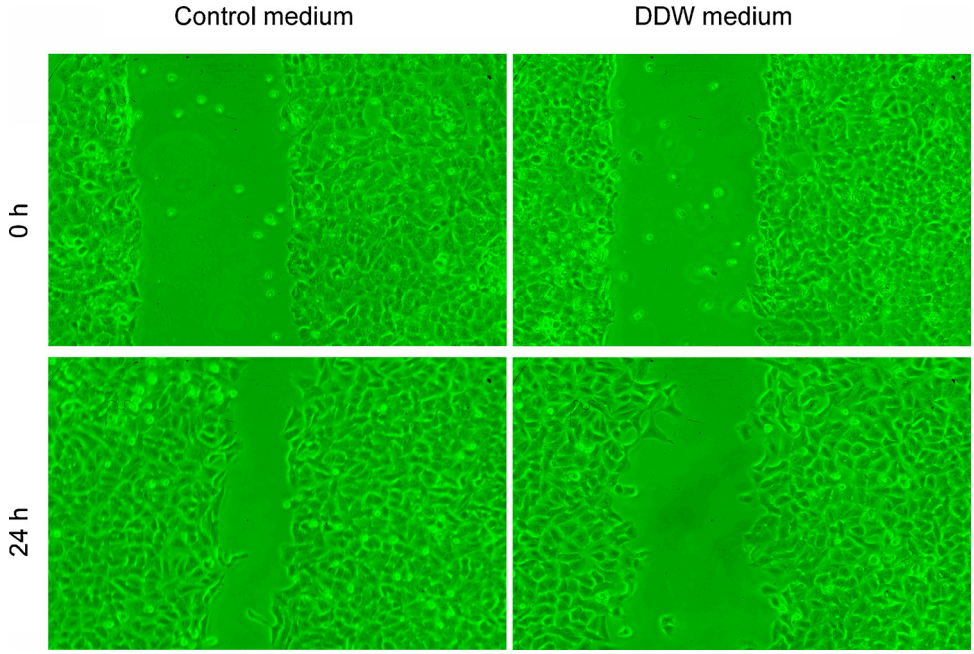

Fig. 5. The scratch wound healing of the A549 cell line in 0 and 24 h. Control medium - D/H 150 ppm, DDW medium - D/H 30 ppm. Magnification x10.

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