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Article

Deuterium Content of the Organic Compounds in Food Has an Impact on Tumor Growth in Mice

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Abstract: Research with deuterium-depleted water (DDW) in the last two decades proved that deuterium/hydrogen ratio has a key role in cell cycle regulation and cellular metabolism. The present study aimed to investigate the possible effect of deuterium-depleted organic compounds (DDOC) alone and in combination with DDW on cancer growth in two *in vivo* mouse models. To produce DDOC, drinking water of laying hens was replaced with DDW (25 ppm) for 6 weeks, resulting in 60 ppm D level in dried egg yolk that was used as deuterium-depleted food additive. In one model, 4T1, a cell line with high metastatic capacity to the lung, was inoculated in the mice's mammary pad. After three weeks of treatment with DDW and/or DDOC, the tumor volume in the lungs was smaller in all treated groups vs. controls with natural D level. Tumor growth and survival in mice transplanted with MCF-7 breast cancer cell line showed that the anticancer effect of DDW was enhanced by food containing the deuterium-depleted yolk. The study confirmed the importance of D/H ratio not only in consumed water but also in metabolic water produced by the mitochondria while oxidizing nutrient molecules. This is in line with the concept that initiation of cell growth requires the cells to generate a higher D/H ratio, but DDW, DDOC, or the naturally low-D lipids in a ketogenic diet, have significant effect on tumor growth by preventing the cells from raising D/H ratio to the threshold.

Keywords: Deuterium-depleted water (DDW); deuterium-depleted organic compounds (DDOC); anticancer drug development; D/H ratio; production of metabolic water; ketogenic diet

1. Introduction

After the discovery of the heavy isotope of hydrogen, deuterium (D), in the early 30's, its possible role in living organisms was not investigated for 60 years [1]. The presence of D was ignored nonetheless its concentration in the human body is about 12-14 mmol/L (equivalent 150 ppm). Data gathered in the meantime, however, suggested that D and its ratio to hydrogen (H) has major impact on several cell processes [2,3]. The first research results, published in 1993, confirmed the key role of D in tumor cell growth and cancer development. Increased D/H ratio in the intracellular space was found to be a key factor to initiate cell growth [4]. Later, results showed the complex nature of the effects of D in living organisms, such as a correlation between drinking water D level and susceptibility of humans to depression [5]; stimulation of long-term memory in rats by deuterium-depleted water (DDW) [6] or reversal of Mn-induced life span decrease in *Caenorhabditis el-*

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egans [7]. Regarding the role of D in cell metabolism, research results confirmed that deuterium depletion enhanced the effect of insulin on GLUT4 translocation in dose dependent manner, and potentiated glucose uptake in diabetic rats, resulting in lower serum glucose, fructose amine, and HbA1c concentrations [8].

Further studies confirmed the role of D in cancer development, in prevention [9] and proved the anticancer effect of deuterium depletion [10-14].

In the experiments revealing the role of deuterium in cell growth, cell metabolism and physiological changes, D level was manipulated by application of DDW.

In the meantime, research confirmed that the D content of organic molecules, such as carbohydrates or lipids, showed significant differences, suggesting that the synthesis pathways of these molecules strongly influence D content. In organic compounds of plants, D/H ratio may substantially differ also from that of the environment. The explanation of that lies in the metabolic processes of various plants. In plants using the so-called C3 or C4 photosynthetic pathways to fix carbon from the atmosphere, D concentration in the glucose molecules will decrease (vs. environmental water) to different extents. In plants using the C3 carbon fixation pathway (e.g., wheat, rice, barley or spinach), glucose D concentration is 135-137 ppm, and in plants using the C4 pathway (maize, sugar cane, millet, and sorghum) it is 141-143 ppm [15,16]. In contrast, plants using the CAM photosynthesis may, under certain circumstances raise the concentration of deuterium in the photosynthesis products. This means that deuterium concentration in the human body is substantially affected by the plants in our diet. Investigating the D content of animal lipids led to similar conclusions. Studies revealed significant depletion (to 90 ppm) of deuterium at certain positions in fatty acids [17,18].

It is obvious that, in case of a normal diet, water content of the food will represent the D concentration of the local environment. But that is not the only factor influencing the final D concentration of the body, since the organic molecules of the nutrients also contain both hydrogen and deuterium which appear in the metabolic water after oxidation by the mitochondria.

So, the final D concentration of the cells depends on the one hand on the D concentration of the fluid intake, including water content of the food. But, on the other hand the D concentration of nutrient molecules also has an effect via the D level of the metabolic water produced in the mitochondria via oxidative utilization of fats, carbohydrates, and proteins.

Recently, ketogenic diet has been used as complementary cancer therapy with high efficacy [19]. Based on the proved anticancer effect of DDW, we postulate that the beneficial impact of ketogenic diet in cancer results from its deuterium-depleting effect, since the mitochondria, when oxidizing fats instead of carbohydrates, produce metabolic water with as low as 118 ppm D concentration, due to the above-mentioned dissimilar D content of the various nutrients [20].

The study presented here aimed to investigate the effect of D concentration of organic nutrient compounds on tumor development. For this purpose, foods with artificially modified, depleted D content were produced, and their effect was tested in two *in vivo* mouse model systems to evaluate the role of altered D/H ratio of organic molecules, and their effect on tumor growth.

2. Materials and Methods

2.1. Production of deuterium-depleted nutrients

Mouse and rat studies indicated that replacing normal drinking water with heavy water increased the D content of organic molecules within short time [21]. Based on that, production of deuterium-depleted organic compounds (DDOC) was attempted by using DDW as drinking water for laying hens, supposing that during egg formation the D content of proteins and lipids will decrease. Seventeen weeks old Tetra SL hens (n=90) were kept in a lay house exposing them to an increased day length with artificial light for 14 hours per day and feeding with a mixture of grains (corn, wheat, soybean). D concentration of the drinking water was 25 ppm. The eggs were collected from day zero and the D concentration in both the water content and the dry substance of albumen and yolk fraction was determined. D concentration reached equilibrium 6–7 weeks after the DDW consumption had started. After 7 weeks of DDW treatment of hens, the eggs were collected and used for the experiments. Albumen and yolk were separated, freeze-dried (to 4.6–5.6% residual moisture) and the yolk was used as food component for mice.

2.2. Preparations made for per os treatment of the mice

Two different *in vivo* studies using mice were performed (see 2.4. for details). For the first one, lasting four weeks, dried yolk (deuterium-depleted and control) was dissolved in distilled water to 0.25 g/mL concentration at 50°C. The mice received *per os* by gavage 400μL of the solution daily in the first two weeks of the four-week-long experiment. The mice in the groups receiving the yolk-containing food lost vitality, attributable to the avidin content of yolk causing biotin deficiency. So, in the second two weeks the yolk solution was treated at 80°C for 10 minutes and the adverse effects disappeared.

In the second experiment, the stress possibly caused by the gavage application was avoided by mixing the dried yolk to powdered VRF1 food at 7%. The mix was re-tableted and sterilized for feeding the mice.

2.3. Cell lines

Metastasis-specific mouse mammary carcinoma cell line 4T1 with high metastatic capacity to the lung (purchased from ATCC) was grown at 37°C under 5% of CO_2 and 100% humidity in RPMI-1640 Medium (Gibco BRL, Carlsbad, CA, USA) containing penicillin (50 IU/mL) (Gibco BRL, Carlsbad, CA, USA), streptomycin (50 mg/mL) (Gibco BRL, Carlsbad, CA, USA) and 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA).

MCF-7 human breast cell line (ATCC) was grown at 37°C under 5% of CO_2 and 100% humidity in Eagle's Minimum Essential Medium (EMEM) (Gibco BRL, Carlsbad, CA, USA) containing penicillin (50 IU/mL) (Gibco BRL, Carlsbad, CA, USA), streptomycin (50 mg/mL) (Gibco BRL, Carlsbad, CA, USA) and 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA).

Of both cell lines, third and fourth passages were used for inoculation. Before inoculation, the cells were trypsinized, washed and resuspended in sterile PBS. 10^5 (4T1) or 5 x 10^6 (MCF7) cells were counted and resuspend in appropriate serum-free medium and injected into the mammary pad in 50 μL volume.

2.4. Description of the in vivo studies

In the first, 4-week study, 8-week-old female and male BALB/cJ mice (Charles River, Innovo Kft., Hungary) were used. The mice (20±4 g weight) were group-housed (5/cage), fed VRF1 (Akronom Kft., Budapest, Hungary) commercial diet *ad libitum*, housed in an animal facility under a 12 h light/dark cycle at constant temperature (22 °C), and humidity. The mice were randomly divided into six groups (n = 10, 5 males and 5 females) (Table 1). Water was provided *ad libitum*, with 150 ppm deuterium content (natural level) for the control animals, and with 25 ppm deuterium content for the DDW-treated mice. In this study, 4T1 cells (metastasis-specific mouse mammary carcinoma model cell line; [22]) were used.

On day 0, the cells (100,000/animal in 50 μL) were inoculated into the mice's mammary pad, and DDW treatment was started. Administration of the yolk solution started on day 1.

Since feeding by gavage with the 400 μL solution of dried yolk was stressful for the mice, an additional control group was set up (treated with 400 μL normal yolk) to compare the data of the other two treated groups receiving DDW and/or yolk. DU283, a compound with documented anticancer effect, was administered (3 mg/kg bw in 150 µL volume, intraperitoneal, once a day) as positive control [23]. The growth of the primary tumor was followed on the 6th, 8th and 10th day of treatment. At the end of the experiment lungs were prepared for weight measurement after incubation in formalin. Lung weights were used to indicate the presence and size of the metastatic tumors compared to the average lung weight of five healthy, untreated mice.

Table 1. Groups and treatments in the first mouse study using the 4T1 cell line.

Group	**Type of treatment**		
	Drinking fluid	Food	Drug
Untreated control	normal water	VFR-1	
Treated	DDW	VFR-1	
Treated	DDW	VFR-1 + deuterium-depleted yolk	
Treated	normal water	VFR-1 + deuterium-depleted yolk	
Untreated control	normal water	VFR-1 + normal yolk	
Positive control	normal water	VFR-1	DU283

In the second mice study (Table 2), 8 weeks old NSG immunodeficient mice (Charles River, Innovo Kft., Hungary) were used. The mice (20±4 g weight) were fed a sterile VRF1 commercial diet and sterile water *ad libitum* and were housed in individual ventilated cages under sterile circumstances in an animal facility under a 12 h light/dark cycle at constant temperature (22℃) and humidity. The mice were randomized and three groups (n=10, 5 males and 5 females) were created, and five million MCF-7 cells were injected into the mice's mammary pad. The control group consumed normal water *ad libitum* and dried VRF1-based food supplemented with 7% normal dried yolk (see 2.2.). One of the treated groups consumed DDW with 25 ppm D concentration and received the same food as the control group. The second treated group consumed DDW with 25 ppm D concentration and VRF1-based food supplemented with 7% deuterium-depleted dried yolk. Five million MCF-7 cells were inoculated at start, and tumor volume and survival were followed up for three months. Tumor volume became measurable around day 14 and was measured every 2-3 days. At each time when one mouse perished, the average tumor volume in the three groups was calculated using previous day's volume data, divided by the number of mice being alive.

Table 2. Groups and treatments in the second mouse study using the MCF-7 cell line.

Groups	Drinking fluid	Food
Untreated control	normal water	VFR-1 supplemented with normal yolk
Treated	DDW	VFR-1 supplemented with normal yolk
Treated	DDW	VRF-1 supplemented with deuterium-depleted yolk

2.5. Ethical considerations

The study was performed according to the Institutional and National Animal Experimentation and Ethics Guidelines in possession of an ethical clearance (XXIX./128/2013.; Title: Investigation of effects of macromolecules).

All animals were treated moribund and were euthanized at the observation of the first sign of torment. All operative procedures and animal care conformed strictly to the

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Hungarian Council on Animal Care guidelines. The animals after treatment were randomly divided into different groups and kept under standard conditions conforming to ARRIVE guidelines [24] and the "Guide for the Care and Use of Laboratory Animals [25]

3. Results

3.1. Production of deuterium-depleted food

It was supposed that the replacement of the laying hens' normal drinking water with DDW of 25 ppm D concentration will influence the D content of the molecules synthesized in the developing eggs. The time course of D levels in water and dry substance of egg white and yolk is shown in Figure 1.

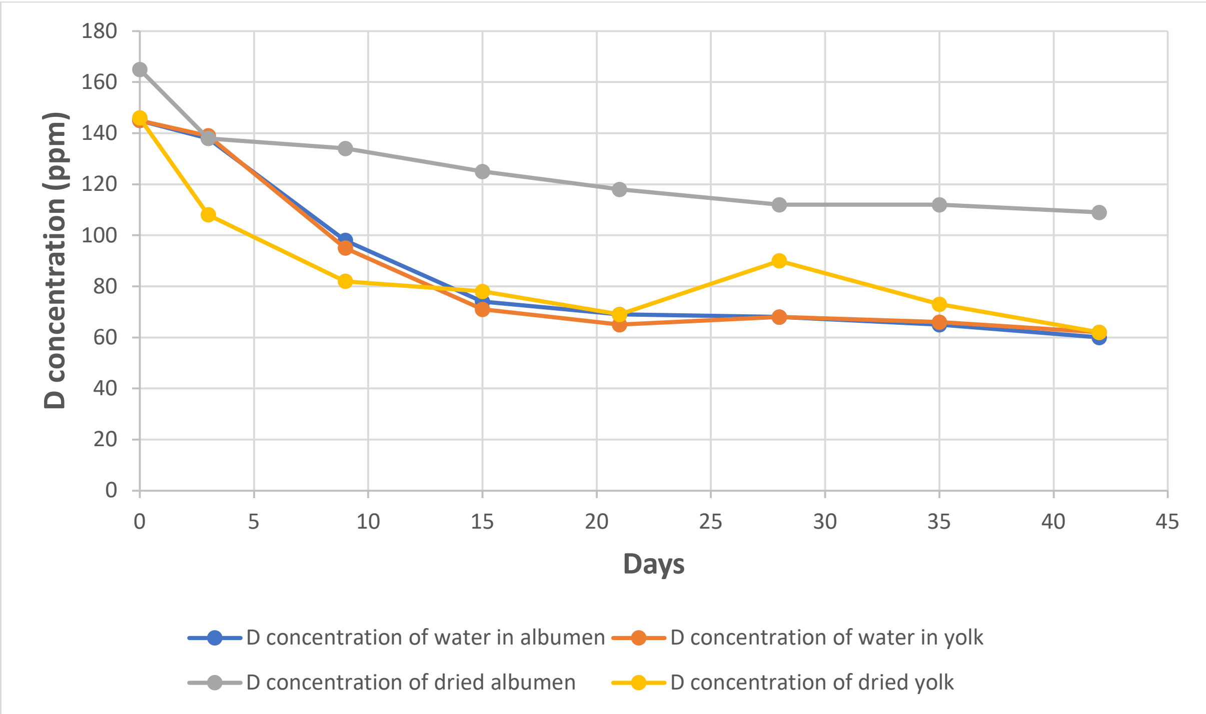

Figure 1. Changes of the D concentration in the water and the organic compounds of eggs after replacement of normal drinking water with 25 ppm DDW (The data represent the average of measurements on two independent samples.).

D concentration of the water extracted from the yolk and egg-white was equal suggesting an equilibrium of D within the liquid phase. There was a sharp, 50 ppm decrease in the first 9 days, and further 30 ppm decrease during the next 12 days, but only a few ppm decrease in the subsequent 3 weeks. The kinetics of the change of D concentration in egg white and yolk was quite different. D level of the albumen was higher already before the hens started drinking DDW, and it decreased from 160 ppm to 110 ppm by the 42nd day of the study. The D concentration of the yolk started to decrease rapidly right after the hens started consuming DDW and decreased much faster than the D concentration of the water content of the yolk, reaching equilibrium after only two weeks. By the time of collecting the eggs for the experiments, there was 50 ppm difference between the D concentration of the egg-white (110 ppm) and the yolk (60 ppm).

3.2. Tumor growth in mice inoculated with 4T1 breast carcinoma cells

The 4T1 model system was chosen as a cell line with *high metastatic* capacity to the lung. Therefore, the size of the primary tumor and the weight of the lung metastases was followed.

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In the group consuming DDW and DD-yolk, the average primary tumor size was smaller vs. control, but the difference was not significant due to high standard deviation (data not shown). Comparing the weight of the metastases in the lungs in the different groups provided more convincing data (Table 3). In all four treated groups, the tumor weight was smaller compared to the two control groups, and the difference was significant in the group treated with deuterium-depleted yolk. These differences may be explained with earlier findings showing that DDW inhibits the migration of tumor cells [14].

Table 3. Average weight of the metastases in the formalin fixed lungs in the control and treated groups.

Treatment	Weight of the metastasis (milligrams; mean±SEM, n=10))
normal water	45.20 ± 4.02
normal water + normal yolk	46.96 ± 5.30
DDW	32.13 ± 6.81
DDW + deuterium-depleted yolk	37.86 ± 5.47
normal water + deuterium-depleted yolk*	32.19 ± 4.80*
normal water + DU283	33.78 ± 6.71

* The tumor volume was significantly decreased compared to normal food and water (p<0,05, unpaired t-test).

3.3. Tumor size and survival in mice inoculated with human MCF-7 breast cancer cell line

The data of tumor volume per mice and survival data are shown in Figure 2 and 3.

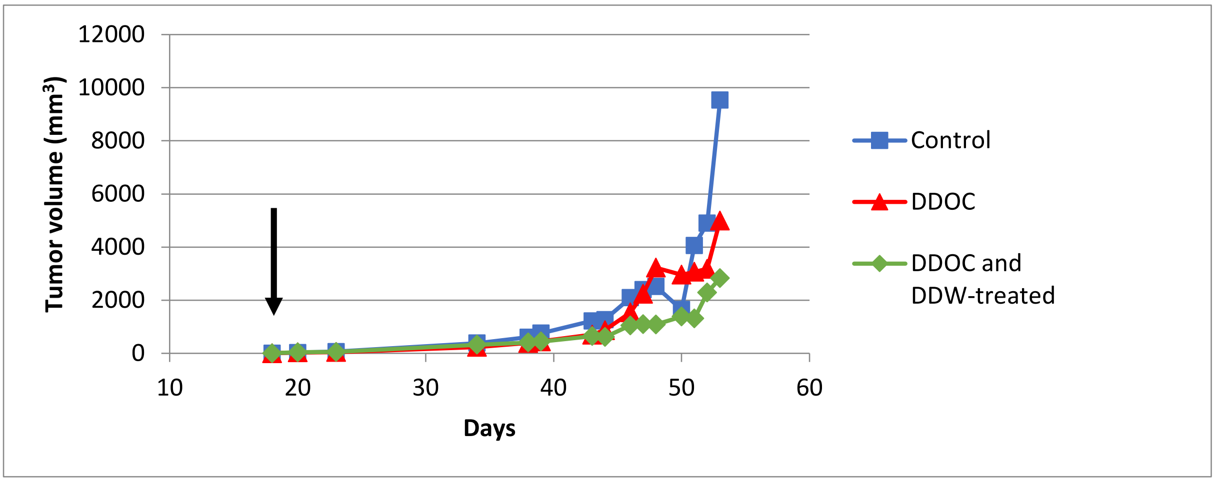

Figure 2. The changes of primary tumor volume in the control and the two treated groups inoculated with the MCF-7 cell line. Arrow represents the first day when primary tumor in the mammary pad was palpable.

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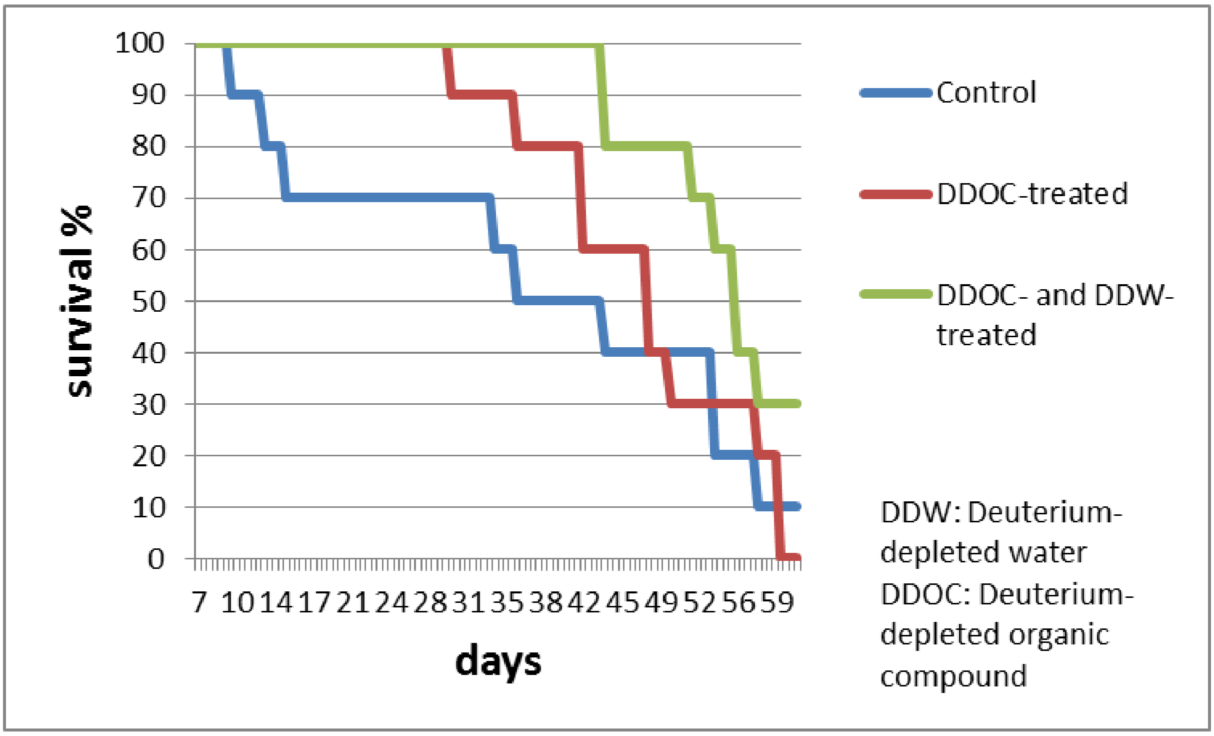

Figure 3. Survival curves of the control and the two treated groups inoculated with the MCF-7 cell line.

These results confirm on the one hand the anticancer effect of DDW, and on the other hand our assumption that, by addition of nutrients as deuterium-depleted organic compounds (DDOC) to normal food, the anticancer effect of deuterium depletion can be boosted. In contrast, in the control group without any depletion of deuterium, the measured tumor volumes kept increasing, except one outlier at day 49, over the whole courses of the experiment.

4. **Discussion**

The consumed food has an undoubtedly significant impact on cell metabolism and physiological processes in living organisms. The effects are typically attributed to the composition of the major nutrients (carbohydrates, lipids, proteins) in foods whereas the possible role of heavy isotope of hydrogen, deuterium, has not been investigated. To preserve health, the most common dietary approach is to reduce fats and increase carbohydrates within the total caloric intake, arguing that the burden of cardiovascular diseases can be reduced this way.

Our study aimed to investigate the impact of varying D/H ratio in food on tumor growth. To obtain deuterium-depleted nutrients, the drinking water of laying hens was replaced with DDW and the decrease of D concentration in the eggs was followed. Interestingly, the kinetics of D level change in yolk and egg-white was different which suggested that the distribution of D is strongly determined by biochemical pathways.

The mice studies presented here confirmed the distinct antitumor effect of DDOC. The significant weight decrease of lung metastases generated by the 4T1 in the first study indicated that deuterium depletion may have inhibited the migration of 4T1 cancer cells. Inhibition of lung cancer cell migration by DDW *in vitro* has been described earlier [14]. Tumor growth and survival data of mice inoculated with the MCF-7 breast cancer cell line confirmed our hypothesis that alterations in D concentration in organic compounds of the food have an impact on tumor growth. This is related to the role of D in cell growth, namely that well-known molecular metabolic processes lead to an increasing D/H ratio which is responsible for entering of the cells from G1 to S phase [4]. A recent study proved

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that higher D/H ratio increased the expression of hundreds of genes with key role in cell cycle regulation. It was concluded that by keeping D concentration at a low level using DDW, the expression of these genes, and so cell growth, can be kept under control [9].

Application of ketogenic (very low in carbs and high in fats) diet was tested in cancer patients with convincing clinical evidence on the anticancer effect of this type of nutrition. The changes of metabolic parameters in patients during such a diet, and the beneficial antitumor effects, are well-documented [26-28]. Our results up to now suggest a common link between the antitumor effect of DDW and of ketogenic diet; the deuterium-depleting effect of both.

A carbohydrate-rich diet will result in production of metabolic water with D concentration close to the SMOW (Standard Mean Ocean Water) value of 155.75 ppm. But the more complex the molecules are, such as lipids, the lower is their D content, because the biochemical processes in the synthesis of these molecules show preference to the lighter isotope of hydrogen due to isotopic effect [2,3,18]. Consequently, increasing the ratio of fats in the food intake will result in lower D concentration of the metabolic water produced by the mitochondria.

The data in Table 4 on D level in the dry matter of certain common foodstuffs provide evidence to the effect of nutrition on the D concentration within the human body, which in turn affects tumor growth [18,30].

Table 4. D concentration of the dry matter of different foods.

Type of foodstuff	D concentration of dry matter
Wheat flour	150 ppm
Table sugar	146 ppm
Cottage cheese	136 ppm
Olive oil	130 ppm
Butter	124 ppm
Pork fat	118 ppm

A high-carbohydrate and low-fat diet alone results in a higher average D concentration of the body. Consumed carbohydrates are converted into glucose for immediate energy, and into glycogen and fat as stored energy. Oxidation of glucose, immediately or after storage as glycogen, yields metabolic water with higher D level, whereas lipids become deuterium-depleted during synthesis and yield lower D in metabolic water. The D atoms not included into the lipids will be enriched in other molecules, preferably molecules with hydrogen in the exchangeable position, such as amino- and carboxyl groups of amino acids. This may explain the dissimilar D level in egg yolk and white of the DDW-treated hens (Figure 1).

Malignancies are not the only chronic disease positively influenced by reduced D levels. A recent animal study proved that the optimal D concentration of blood for reducing blood sugar level in rats was between 125 and 140 ppm stimulating the GLUT4 translocation from the cytosol to the cell membrane [8]. The beneficial effect of D depletion was also confirmed in a human phase 2 clinical trial. DDW (105 ppm D) significantly reduced the fasting glucose level and decreased insulin resistance [31]. Another *in vitro* study confirmed the impact of the light isotopes of not only hydrogen, but also carbon, oxygen, and nitrogen on enzyme activity [32].

To reduce the incidence of chronic diseases, including malignant tumors, in the human populations is a paramount aim. The data presented here prove that the isotopic composition of foods has a major impact on cancer cell growth. These observations raise the need to *reconsider* current *dietary recommendations*, putting the impact of D concentration of nutrients into focus in order to reduce the average D level in the population from 145-150 ppm to 125-140 ppm.

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Abbreviations: D = deuterium; DDW = deuterium-depleted water; DDOC = deuterium-depleted organic compounds; SMOW =Standard Mean Ocean Water

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