2ND INTERNATIONAL CONGRESS ON DEUTERIUM DEPLETION

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The 2nd International Congress on Deuterium Depletion was held on 17-18 May 2012 in Budapest, Hungary (www.deuteriumdepletion.com), as a follow-up to the 1st International Symposium on Deuterium Depletion held in Budapest in the spring of 2010. The goal of the organizers was to provide a global and comprehensive update of the newest results and developments in the research of deuterium depletion, and to give the researchers and medical practitioners a new opportunity to get acquainted with this expanding field of science, share their results and experience. Researchers and practitioners from several countries (Hungary, USA, France, Romania, Russia and China) presented 16 lectures on the various biological effects of deuterium depletion in the field of cancer research, diabetes research and anti-aging science.

Deuterium content of natural materials focusing on water: an overview

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This paper provides an overview on the deuterium content of materials found in nature, focusing on water as the most important deuterium source for living organisms. Deuterium contents of the water pools on Earth vary from about 90 ppm up to 160 ppm. Deuterium depleted water can primarily be found on the Earth's poles (e.g. Greenland, Antarctica, North Pole ice sheet) in the form of ice, while the water richest in deuterium are of lakes and the oceans.

The deuterium content of the precipitation shows a global pattern: D-content decreases from the Equator to the Earth's Poles and from sea level towards high elevations. The main driver in D-content variations in precipitation is air temperature. As a consequence D-content in precipitations bears prominent seasonal variations; e.g. in Hungary D-content in precipitation varies from cca. 127 ppm in cold winters up to cca. 153 ppm in hot summers.

The D-content of subsurface waters mostly reflect the global pattern of D-content of precipitation with the exceptions of some large scale regional water flow systems, and of ground waters older than 10000 years, which infiltrated during the Ice Age. Lakes that collect water from regions of higher altitude and cooler precipitation contain water, again, with lower deuterium ratios.

Other natural materials: Plants (marine and terrestrial) contain several per cent less deuterium than the water on which these plants grew. The widely used fuels as coal and crude oil are further depleted in deuterium with respect to plants.

Keywords: deuterium, D-content variations, natural materials

Natural mechanisms by which deuterium depletion occurs in specific positions in metabolites

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Deuterium is depleted in specific positions in metabolites due to natural processes. This talk will present examples of how this phenomenon can be used to investigate metabolic activities. The information about site-specific deuterium depletion is obtained by using quantitative isotopic ^{2}H NMR spectrometry. In this technique, the ^{2}H NMR spectrum of the molecule of interest is acquired under carefully established quantitative conditions. By reference to a calibrated internal standard, the areas under the peaks in the spectrum can be used to obtain the ^{2}H/^{1}H ratio at each resolved hydrogen position.

In the first example of the exploitation of this technique, the analysis of the redistribution of hydrogen during the metabolism of glucose by lactic acid bacteria will be developed. It will be shown how, by appropriate slight labelling of specific positions in the glucose to be fermented, the quantitative affiliation between all non-exchangeable positions in the substrate and all positions in the product can be obtained. A linking-factor can be calculated that will indicate whether positions will become enriched or depleted during metabolism. It will be shown how the fermentation of glucose to lactic acid via glycolysis or to lactic acid plus ethanol via the reductive pentose phosphate pathway leads to different isotope patterns in the products.

The second example will present our work on understanding fatty acid metabolism. During the biosynthesis of fatty acids, H atoms can be introduced from acetate, water and NAD(P)H. Thus, the origin of the H, isotope effects during the reduction reactions, and non-enzymatic exchange causes variations in the $^2H/^1H$ ratios at the different positions, leading to an alternating enriched/impoverished pattern. This is further altered by the action of desaturases and other enzymes that modify the chain. We have shown that depletion in the residual ^2H at the sites of desaturation is due to the non-equivalence of the $^2H/^1H$ ratio in the CH_2 groups that are subjected to desaturase activity.

How such analyses give insight into intramolecular H distributions and their underlying causes will be discussed.

Keywords: deuterium, isotopic ^2H NMR spectrometry, $^2H/^1H$ ratio, redistribution of hydrogen

Deuterium depletion From tissue culture to human clinical studies

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The growth-altering role of naturally occurring deuterium (D) in living organisms has been examined using deuterium-depleted water (DDW) (25-105 ppm D) and compared with that of natural deuterium containing water in control cultures (150 ppm). DDW significantly decreased the growth rate of L_{929} fibroblasts, HT-29 colon, A4, MDA and MCF-7 breast, PC-3 prostate, as well as M19 melanoma cells. This growth-inhibiting effect was more pronounced when the D-concentration of growth medium was gradually decreased in 3 to 5 steps. In order to reveal the molecular background of the inhibitory effect of deuterium depletion the expression of different genes was investigated. It was found that deuterium depletion inhibited COX-2 expression and there were positive correlations among the degree of growth retardation, COX-2 gene expression and D-concentrations. DDW also influenced the expression of genes encoding different kinases using nanocapillary quantitative real-time PCR analysis. Phosphatidylinositol 3-kinase and hexokinase-2 expressions were stimulated by DDW, whereas glutathione peroxidase 2 and peroxiredoxin 1, which play key roles in regulating electron transport, were suppressed.

Mammary tumors in 81 dogs and 14 cats showed a response rate above 70%; yet, in more than 50% of the animals a complete recovery was achieved in response to DDW treatment. Similar efficacies were observed in 43 dogs and 3 cats bearing rectal tumors. Local treatment with an injectable DDW preparation regressed tumors, which otherwise were resistant to oral DDW administration. Microscopic evaluation of the tumors verified that DDW injection caused a disappearance of tumor cell infiltration, demarcation and a consequent rejection of invasive tumors, while DDW injections were harmless for healthy tissues in the surroundings of tumor sites.

A four-month double blind, phase II, placebo controlled human clinical trial was conducted in 44 prostate cancer patients. Prostate size during the 4-month DDW administration period showed a net decrease of 160.3 cm^3 but only that of 54 cm^3 in the control group. Two patients (9.1%) died in the treated and 9 patients (40.9%) in the placebo group, which is consistent with a decrease in mortality in the treated group (Fisher's Exact Test, p=0.034).

Beside the phase II clinical trial in prostate cancer, a human data base was created from 1992 involving all patients who had been subjected to DDW administration. This data base includes 1,450 patients who consumed DDW for longer than 90 days (654 male and 796 female patients), between October 1992 and March 2012. The cumulative time from diagnosis to the end of the follow-up period was 5,601 years; the cumulative time of DDW administration was 1,964 years. The median age of the investigated population was 55 years. The distribution of the main tumor types among the examined patients was almost identical to the data of the National Cancer Registry (Hungary). Median survival time (MST) was 9.5 years in the entire cohort of 1,450 patients, of which 1,320 patients started consuming DDW with detectable tumor(s), with 130 patients already in remission. Four hundred ten (410) patients died (one in every 12.1 years) out of 1,320 patients within 4,980 years covering the cumulative time from diagnosis to the end of the follow-up period. The MST of the 1,320 patients was 8.3 years. The cumulative follow-up period of 130 patients starting DDW consumption in remission was 615 years. Cohort statistical evaluations show that 8 patients (i.e.: one in every 76.8 years) died, which strongly suggests that integrating D-depletion into conventional therapies may prevent relapses of cancer with a complete recovery in some patients.

It is suggested that cells are readily able to regulate their D/H ratios, while its changes trigger distinct molecular processes. One possibility to modify intracellular D/H ratios is the activation of the H^+-transport system, which preferentially eliminates H^+, resulting in increased D/H ratios within cells.

Altered D/H ratios strongly regulate the expression of distinct genes and the activity of enzymes having key roles in cell cycle regulation and also regulate various molecular mechanisms. We contemplate that naturally occurring D is a key element of a still obscure sub-molecular growth-regulatory system (SMRS).

Keywords: deuterium, deuterium depletion, deuterium-depleted water, cell cycle regulation, gene expression, Phase II clinical trial

Biological effects of deuterium content variation in water

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The aim of the work is to study the nature of biological effects of deuterium depleted and slightly deuterium enriched water on living cell. It was shown in our previous works that increasing of deuterium to 500 ppm resulted in twice hydrolytic activity of the membrane specimens of Na,K-ATPase and Ca-ATPase, increasing of hydroid regeneration velocity [1-3]. On contrary this activation effect was not obtained on Na,K-ATPase at temperature lower than temperature of lipid phase transition and on Na,K-ATPase from muscles. The results obtained proved that this anomalous effect of deuterium is not consistent with biochemical reactions themselves (primary kinetic isotope effect), but occurred due to the cooperative ternary complex of water-protein-lipid.

Natural fractionation of deuterium occurs during phase transitions vapor-liquid-ice. The experiments carried out on the SP-22 North Pole drifting station show that both depleting and increasing deuterium content around the isotopic content of the ancient ocean leads to the increasing of single-cell algae grown inside the ice mass [4-5]. In [6] one can see both activation of small and inhibition of large concentration of deuterium on the growth of bacteria.

The experiments with Na,K-ATPase from nasal salt glands of a duck were followed recently with the depleted light water of various deuterium concentration. Hydrolytic activity of the samples containing 4, 24 and 150 ppm is obtained to have 181±9, 158±12 and 179±10 arbitrary units, respectively. The result shows that depleted water containing 4 ppm of deuterium does not differ from the ordinary water, but 24 ppm depleted water leads to the inhibition of Na,K-ATPase activity.

The experiments with fertilized roe of loach *Misgurnus fossilis* show that the number of live embryos after six days of incubation is twice higher in depleted water with 2, 9 ppm of deuterium in comparison to ordinary water. The dying dynamics of non-fertilized roe does not depend on isotopic content of deuterium.

Our preliminary experimental result on motility of human sperm cells indicates that in light water (4 ppm) motility is 40% higher during five hours of the registration. However the effect depends on the initial properties of a sperm sample.The results obtained clearly shows that deuterium content variation in water including deep deuterium depletion produce various nonlinear isotopic effect on key processes in a cell as enzyme action of Na,K-ATPase, regeneration, motility, fertilizing effectiveness and embryo developing. It should be noted that in any case concentration dependence is needed to find an optimal condition for the best result. Sometimes small variation of deuterium in frame of natural fractionation on the Earth may be the most effective. Due to the fact that heavy isotope of hydrogen tends to be accumulated in an organism, deuterium depleted water seems to be preferable in practical use.

We are thankful to the “Light water” Russian company which gives us an opportunity to study isotopic effects in wide concentration range of deuterium and for light water samples which are used in the experiments.

Keywords: deuterium, deuterium-depleted water, natural fractionation, DDW, Na,K-ATPase, Ca-ATPase, nonlinear isotopic effect

Physiological effects of drinking water enriched with $^{1}H_{2}{}^{16}O$

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The quality and purity of drinking water are determinative factors for life quality and human health. In natural water the residual concentration of isotopomer molecules, containing the heavy isotopes ^{2}H, ^{17}O, ^{18}O, can amount to 2.97 g/l. Light and heavy water isotopomers differ appreciably in their physical properties such as boiling point, freezing point, and density.

Molecules containing heavy isotopes in the mammalian organism can lead to changes in normal biochemical processes and to a decrease of functional resources of the organism.

In our studies, water enriched with $^{1}H_{2}{}^{16}O$ was used, which is the main ingredient of the commercial product AquaSLAP™. Such water was produced by the company ZAO “Light water”. The level of the light isotopomer was 99.757 % (D/H= 90 ppm), which corresponds closely with water of the Antarctic, the one with the least concentration of heavy water isotopomers in Nature.

The goal of the work was to reveal biological effects of water enriched with $^{1}H_{2}{}^{16}O$ on quantitative blood chemistry values and physical challenges in 20 volunteers (healthy persons aged 18-34 years). At the P. K. Anokhin Institute of Normal Physiology, subjects were tested for their psychological and vegetative statuses, lung function and gas

exchange, general health and physical performance, while blood tests were conducted to assess clinical and biochemical parameters, immunological and hormonal statuses, as well as antioxidant activities).

Results of the 28 days intake period of light water revealed in every subject an improvement in health and reduced anxiety with improved psychological response.

The positive influence of drinking light water on blood chemistries included a significant reduction of glucose, cholesterol, erythrocyte sedimentation rates, leukocyte counts and cortisol (stress hormone) levels, while also revealed an increase in antioxidant capacities.

Volunteers who consumed 1 liter of light water per day improved their hemodynamic parameters (i.e.: increased stroke volume and cardiac output, decreased total peripheral vascular resistance), which had a positive impact on overall physical performance and aerobic metabolism during an exercise.

These findings evidence the significance of light water to increase energy resources even in a healthy cohort, while decreasing risks of psycho-emotional stress, which is known to pose a negative influence on blood biochemistries that often lead to psychosomatic diseases and shorten life.

Keywords: deuterium, heavy isotopes, isotopomer molecules

Comparative study concerning deuterium depletion in two laboratory animal species

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Deuterium depletion is a cutting-edge tool used especially in cancer research and oncotherapy, diabetes and anti-aging studies. The aim of this paper was to investigate comparatively how a deuterium-depleting agent (deuterium depleted water) lowers the isotopic concentration of deuterium, acquired via bioaccumulation processes, in Wistar rats and Swiss mice. Control animals were maintained on standard food and drinking water, while experimental rats and mice were maintained on standard food and deuterium depleted water (produced at ICIT, according patent WO/2006/028400 "Process and installation for obtaining the deuterium depleted water"). The water extracted from biological samples of the sacrificed animals was analyzed by mass-spectrometry. Results showed that deuterium depleted water efficiently decreased deuterium isotopic content at systemic level in both species. It can be stated that the grade of deuterium depletion was directly dependent on two extrinsic factors: deuterium concentration of the depleting agent, and duration of administration. Moreover, deuterium depletion seems to depend on genotype, at both species and individual level, and on behavioral influences by each specimen.

Keywords: deuterium, deuterium depletion, deuterium-depleted water, mass spectrometry

Tracer substrate-based metabolic profiling, phenotypic phase plane and regression matrix analyses of pancreatic cancer cells under deuterium depleted growth environment

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The current state of metabolomics art involves the increasing use of stable ^{13}C isotope tracers in medicine. Main advantages of ^{13}C tracer-based metabolomics include functional, real time and phenotype related surrogate markers of cell function, drug response, and to determine drug efficacy for flux control in the metabolic network. This presentation covers important milestones in ^{13}C tracer substrate based metabolomics, its business projections for medical research, as well as significant new applications for deuterium depletion. Phenotypic phase plane analyses and regression matrices of the ^{13}C stable isotope labeled metabolome, especially its energy yielding products, intermediary metabolites, and cell membrane/nucleic acid components in cultured tumor cells, already indicate a major System response to deuterium depletion primed by limited reductive synthesis.

One prominent effect of deuterium depletion is to inhibit fatty synthesis, chain elongation and desaturation. These anabolic reactions utilize acetyl-CoA, as well as hydrogen of water for new fatty acid pools. Fatty acids then are used for new membrane formation in the rapidly proliferating cell. The complex structure and molecular organization of the mammalian fatty acid synthase offer remarkable opportunities with altered morphology and flux handling properties [*Nature Chemical Biology* **2**, 232-234 (2006)].

Thus, fatty acid synthase (FAS; EC 3.2.1.85) is an attractive novel drug development target site in oncology where major pharmaceutical companies have already positioned themselves. For example, GSK837149A by GlaxoSmithKline controls the b-ketoacyl reductase subunit of human fatty acid synthase [FEBS Journal 275 (2008) 1556–1567] with potential clinical candidacy. This is also the site where hydrogen/deuterium ratios readily affect enzyme reactivity as well as reaction elasticity.

Besides affecting reductive (proton dependent) synthesis, our previous ^{13}C glucose experiments indicate that there is a deuterium-dependent decrease in *de novo* fatty acid ^{13}C net labeling in multiple tumor cells, with a decreased contribution of glucose-deriving acetyl-CoA to newly formed palmitate and sterols. This indicates an important role for limiting malonyl-CoA's carbon skeleton trafficking towards the consecutive acyl chain-elongating reductive synthesis steps within the FAS complex. Therefore, it is evident that reactivity and elasticity are also limited for ^{13}C-acetyl-CoA trafficking towards several membrane-bound newly formed lipids by DDW administration. This is consistent, on one hand, with a limited role of the Copenhagen model for malonyl-CoA processing (decarboxylation) but, on the other, that of an alternate, activated water and deuterium-dependent rate limiting mechanism [Biochemistry, Vol. 41, No. 35, 2002]. As the decarboxylation reaction is initiated by the attack of an activated water molecule, or hydroxide, on the malonyl C-3 target, the significant mass difference between deuterium and hydrogen readily alters reaction kinetics for acetyl-CoA trafficking, with a major impact on fatty acid synthesis and cell proliferation, as one of DDW's growth limiting mechanisms with pharmaceutical appropriateness.

Keywords: deuterium, deuterium depletion, ^{13}C isotope tracers, metabolomics, phenotypic phase plane analyses, tumor cells, fatty acid synthesis

Effects of deuterium depleted water alone and in combination of known chemotherapeutic agents on different tumor cells

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Consumption of deuterium depleted water can be used as a supplement for anti-cancer treatments and could also serve as a tumor prevention strategy. The anticancer effects of the optimal concentration of deuterium in water, the duration of treatment, the gradient decrease of deuterium content and the possible synergistic effects of deuterium depleted water with known chemotherapeutic agents were analyzed on different tumor cell lines by using a real-time cell analysis method, Excelligence.

The Excelligence RTCA SP (Acea-Roche) is a microelectronic cell sensor method, where microelectrodes are integrated in the bottom of a microtiter plate (96-well E-plate) and measures adhesion and proliferation. The real-time measurement can detect changes continuously, which means that the system can give information at any stages of the experiment. Real-Time Cell Analyzer (RTCA) DP is a novel cell migration and invasion assay system that uses the Boyden Chamber principle but does not involve any fixation, labelling or counting of the cells. The core of the system is the CIM-Plate device, composed of an upper chamber and a lower chamber. The upper chamber has 16 wells that are sealed at the bottom with a micro-pore-containing polycarbonate or polyester membrane. The membrane contains microelectronic sensor arrays that are integrated on its bottom surface. Migration of cells will occur through these electrodes, which changes impedance, and will increase cell index.

In the present paper we report the effects of different concentrations of deuterium depleted water (155 ppm 135 ppm, 125 ppm, 115 ppm, 105 ppm, 85 ppm, 65 ppm, 40 ppm) on the proliferation of HT199 melanoma, A549 lung cancer and MCF7 breast cancer cells. In addition we studied the synergetic effect of deuterium depleted water with known chemotherapeutic agents, namely etoposide, taxol, doxorubicin and cisplatin.

Deuterium depletion interfered with cell proliferation at the concentration of 105 ppm. The 40 ppm concentration showed the most significant effects in A549 and MCF7 cultures, while the proliferation of HT199 cells was most efficiently controlled by the 65 ppm preparation. During the combined treatment modalities deuterium depleted water enhanced the growth controlling effects of anti-cancer drugs. Combining–450 nM doxorubicin with 85 ppm deuterium depleted water decreased cell proliferation, yet combining 85 ppm deuterium depleted water with 900 nM doxorubicin induced cell death in all cultures. The 85 ppm deuterium depleted water significantly increased the effect of 65 μM cysplatin in A549 and HT199 cultures.

In conclusion, deuterium depletion inhibits tumor cell proliferation and decreases their migration. Furthermore, in combination with known anti-cancer drugs deuterium depletion had a synergistic effects. Based on these results deuterium depletion therapy could be used alone as well as in combination with different chemotherapeutic treatment protocols in the clinics. Further studies are needed to confirm the effects of combination therapy in animal experiments as well as in human clinical trials.

Keywords: deuterium, deuterium-depleted water, real-time cell analyzer, cell proliferation, cell migration, cancer cell lines, chemotherapeutic agents

Effects of deuterium depletion on proliferation and apoptosis in cultured murine haemopoietic cells

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The purpose of the study was to investigate the effects of deuterium depletion (DD) on proliferation and apoptosis in a murine cell model system. The murine haemopoietic cells (cell line FDCP-Mix clone A4) used in this study require the growth factor Interleukin-3 (IL-3) for survival and proliferation. In the absence of IL-3 these cells not only undergo cell cycle arrest at the G_1/S checkpoint but also enter apoptosis. Therefore, this model system offers a unique opportunity to study the effects of different conditions on both proliferation and apoptosis.

DD conditions brought about an extension of the lag phase of the growth curve of murine haemopoetic cells when cultured in the presence of IL-3. DD conditions also decrased the rate of recovery of the cytoplasmic pH of the cells from acid load, an indicator of the activity of the Na^+/H^+ antiport. Moreover, DD conditions increased the rate of apoptotic cell death in the IL-3-deprived samples, with no effect on cell viability in the normal, IL-3-supplied population.

The results indicate that DD conditions a/ interfere with the onset of cell proliferation by extending the period during which the cells enter the phase of intense divisions, b/ modulate the activity of the Na^+/H^+ antiport, and c/ promote apoptosis after being induced by a physiological inducer. The implications of these findings will be discussed in the light of the two distinct signalling pathways employed by IL-3 with respect to the two cellular decisions: the Shc-Ras-MAPK pathway to induce proliferation and the PI3-P-Akt-Bax-P cascade to suppress apoptosis.

The authors wish to express their thanks to Professor T.M. Dexter and Dr. D. Allan (Paterson Institute for Cancer Research, Manchester, U.K.) for making it possible to conduct the study.

Keywords: deuterium, deuterium depletion, proliferation, apoptosis, hematopoietic cells, Na^+/H^+ antiport, signalling pathways

Anti-aging effects of deuterium depletion on Mn-induced toxicity in a C. elegans model

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Sub natural concentrations of deuterium (D) in water have been shown to have several health effects. Herein, we tested the hypothesis that deuterium-depleted water (DDW) can reverse the effects of manganese in a *Caenorhabditis elegans* (C. elegans) experimental model. A synchronous population of N2(wild type) worms was treated with 35mM $MnCl_2$ for 30 minutes followed by 48 h treatment with 150, 120 or 90 ppm of DDW. Mn reduced DAF-16 (a transcription factor strongly associated with life-span regulation) levels in the worms and this effect was restored by 90 ppm DDW treatment. Superoxide dismutase (SOD) and AKT, downstream and upstream proteins in the DAF-16 pathway, respectively, were altered by Mn exposure, and their expression was also restored by DDW treatment. These findings demonstrate that DDW may play a protective role against Mn toxicity and aging, likely by attenuating Mn-induced reactive oxygen species (ROS) generation. The effect of DDW is likely mediated by the DAF-16 pathway, as a transcriptional factor that increases the expression of antioxidants proteins and increase in lifespan in *C. elegans*. Additional studies are necessary in order to clarify the precise molecular mechanisms of the anti-aging activity of DDW.

Keywords: sub-natural deuterium, Caenorhabditis elegans, life-span, manganese, DAF-16, SOD-3, anti-aging effect

Deuteronation and ATP Synthase: A Stochastic Mechanism of Aging

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Although the ratio of deuterium to hydrogen is ~1/6600 in nature, deuteron/proton ratio is ~1/15000 due to the lower ionization of deuterium that has a twofold mass of hydrogen. Therefore in any biological molecular reaction/interaction where proton is involved, there is a ~1/15000 chance of deuteronation.

Chemical bond formed by deuteron is more stronger, shorter and have a different angle than hydrogen bond. The same conditions apply for the F0 part of ATP synthase also where deuteronation increases the pKa of its Asp61 by 0.35. This increase can make difficult the dissociation of deuteron from Asp61 by stator subunit's Arg210 and likely causes the rotation of F0 in both directions in a futile manner that stops ATP synthesis at least transiently. Each second, one of ~15 ATP synthases is likely transiently deuteronated at Asp61 site. If a critical percentage of ATP synthases is stochastically deuteronated at a certain time point in a mitochondrion, this can lead to local ATP deficiency with reversible or irreversible consequences in maintenance and repair. As examplified by ATP synthase, the role –if any- of deuteronation in the mechanisms of aging and age related pathologies needs and deserves to be clarified.

Keywords: deuterium, deuteronation, ATP synthase, aging

Deuterium-depleted water inhibits human lung carcinoma cell growth by apoptosis

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Objective: To explore the *in vivo* and *in vitro* inhibitory effects of deuterium-depleted water (DDW) on human lung cancer, and to explore the possible mechanisms.

Methods: The inhibitory effect of DDW on the proliferation of human lung carcinoma A549 cells and human embryonic lung fibroblast HLF-1 cells was examined by MTT assay; apoptosis of A549 cells was examined by TUNEL; and cell cycle was analyzed by flow cytometry. Mouse model of lung carcinoma was established by inoculating human lung carcinoma H460 cells into BALB/c nude mice, and the growth of implanted tumors was observed after DDW treatment for 60 days.

Results: Compared with control group, A549 cells treated with DDW containing 25, 50 or 105 ppm deuterium DDW showed significantly decreased proliferation at 10 h ($P < 0.01$). Then the inhibitory effects of DDW gradually disappeared, but re-appeared 48 h later, becoming significant at 72 h ($P<0.05$). DDW showed no inhibition on the proliferation of HLF-1cells($P<0.05$). TUNEL assay verified DDW-induced apoptosis in A549 cells, which was significantly higher than that of the control group ([45.30±4.21]% vs ([22.25±0.30]%, $P<0.01$). Cells in S phase were significantly increased in DDW-treated A549 cells compared with those in the control group ($P<0.05$). Life quality of H460 cell-inoculated nude mice treated with DDW was greatly improved, with a tumor inhibition rate of 30.08%. Conclusions: DDW inhibits the proliferation of lung cancer cells at a wide dosing range, but with a fluctuation pattern; its mechanism might be associated with induction of apoptosis and S-phase cell cycle arrest in tumor cells.

Keywords: deuterium, deuterium-depleted water, A549 human lung carcinoma cells, apoptosis, tumor inhibition

The effect of DDW on the kinetics of tumor clone in *in vitro* experiments

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Acute leukemia represents a group of heterogeneous malignant blood disorders the key role in the development of which is played by the disbalance between proliferation and differentiation of hematopoiesis progenitor cells. Recent studies have shown that deuterium depleted water (DDW) can affect both the proliferation and apoptosis of the malignant cells. DDW may become a potentially perspective drug in the prevention of cancer and blood disorders.

In this study we investigated the effect of DDW on the rate of spontaneous apoptosis by incubating leukemic cells in culture media with deuterium concentration of 10 or 50 ppm. The leukemic cells were extracted from bone marrow of patients with first diagnosed leukemia. In all experiments, the number of viable cells before incubation was 95%. The cells were incubated in RPMI-1640 medium, based on bi-distilled DDW with deuterium concentration 10 or 50 ppm, for 24, 48 and 72 hours. Control cells were incubated in a medium with 143 ppm deuterium concentration. After incubation, the cells were washed in phosphate-buffered saline in the centrifuge at 1000 rpm for 5 minutes at 18°C. The pellet was resuspended in 200 ml saline, and the cell suspension was put into 2 test tubes. To one, 15 μL of annexin V/FITS and 10 μL of propidium iodide was added. Unstained cells were used as negative control and for determining growth rate. The samples were shaken on a vortex and incubated in the dark for 15 minutes at room temperature. After that, 400 μL phosphate-buffered saline was added to the samples, and these were analyzed by means of a FACScan cytofluorimeter (Becton Dickinson, USA) with fluorescence excitation in channel FL1 (525 nm) and red range excitation in FL2 channel (525 nm). Evaluation was done with the CELLQuest program

according to the characteristics viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells.

Cells of acute leukemia patients (M1 subtype in the FAB classification; marrow, blast cells 95%), cells of patients with acute lymphoblastic leukemia (marrow, blast cells 98%) and mononuclear cells of a healthy donor were investigated. The sensibility of the analyzed cells to the decrease of deuterium concentration in the medium and its rate was different for all periods of incubation. For example, after incubating the M1 cells at 10 ppm deuterium level for 24 hours, the number of viable cells was not significantly different from the control (46.8 vs. 46.7%), while in the medium with 50 ppm deuterium the number of viable cells was 10% lower than in control. Cells of acute lymphoblastic leukemia were, in contrast, more sensitive to decreased deuterium concentration (10 ppm). Prolongation of the incubation period regularly led to decrease of cell viability and increase of the number of cells in different apoptosis stages.

Mononuclear cells of the healthy donor were also sensitive to the deuterium concentration. At the beginning of culturing the cells, the difference vs. control was significant and reached 20%.

DDW affected the viability of leukemic cells in *in vitro* experiments. The reaction of the cells on decreased deuterium concentration in the medium depended on the cell type, deuterium level, and length of cultivation.

Keywords: deuterium, deuterium-depleted water, cell culture, leukemic cells

Lung cancer patients, who consume deuterium depleted water, have extended survival

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Lung cancer is the leading cause of cancer mortality. In Hungary, the median survival time (MST) of lung cancer patients is 7.5 months for men and 11.3 months for women, based on the data obtained between 2002 and 2005.

While smoking is the primary risk factor of lung cancer, environmental pollution and genetic factors can also promote formation of lung cancer in non-smoker population, too. Moreover, regular lung cancer therapy is less effective than patients and physicians wish. Deuterium depletion slows cell division and suppresses tumour-related genes and longer survival of breast and prostate cancer patients is observed respectively. We followed more than 3 hundred patients with diagnosed lung cancer. These Hungarian patients voluntary consumed deuterium depleted water (DDW) beside regular chemo and radiotherapy. The calculated MST was dependent mainly on gender and histological subtype but it was significantly, 2-7 times longer in the group of patients, who drank DDW than it was calculated in the entire Hungarian lung cancer population. Tissue samples were not obtained from patients, because DDW consumption was beginning after surgery, but in an animal model we could measure diminished expression of several cancer-related genes in the lung tissues of DDW-drinking animals. We assume being correlation of silenced expression of those genes and extended cancer survival of DDW-drinking patients having lung cancer at the beginning of follow-up.

Keywords: Deuterium depletion, lung cancer, median survival time, MST

A retrospective study of survival in breast cancer patients undergoing deuterium-depletion in addition to the conventional therapies

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Breast cancer is among the most prevalent forms of malignancies worldwide. It ranks as the second most common fatal cancers in women. Although population screening by mammography and rapidly developing new therapies (i.e.: surgery, radiotherapy, hormonal therapy, chemotherapy and targeted drugs) improve early diagnosis and multidisciplinary treatment options, morbidities for breast cancer remain relatively high.

The possible role of naturally occurring deuterium (D) and the impact of its shortage on cell proliferation have already been established for numerous biological systems. Anticancer effects of D-depletion have been demonstrated in *in vitro* cell growth studies and in animal models of tumor xenografts. Deuterium depleted water (DDW) in a human phase II clinical trial of prostate cancer and retrospective evaluations all showed anticancer- and preventive effects as a novel treatment modality.

The aim of the present study was to investigate the impact of deuterium depletion (D-depletion) on breast cancer outcome. The daily water intake of patients was replaced with DDW (105-25 ppm D) at least for 91 days, without restrictions for the continued conventional treatment regimen. The DDW treatment started with 105 ppm D, which was gradually decreased to preparations with 85 ppm, 65 ppm and 45 ppm, (i.e.: 20 ppm lower D contents than the

starting preparation) every 1 to 3 month, reaching a total of 6 to 10 months treatment periods. DDW treatments are discontinued with 2 to 3 month intervals, and then repeated several times for 4 to 6 months periods.

The data base of 232 breast cancer patients (median age: 50 years) was retrospectively evaluated, the median follow up time was 49.7 months. Patients were continuously enrolled between February 1993 and April 2011. Patients consumed DDW for a median time period of 14.1 months. Median survival time (MST) from the diagnosis was 148 months (12.3 years) for the population of the DDW treated cohort. According to the staging at initial diagnosis, patients with primary breast cancer achieved 217 months (18.1 years) MST, which was 52 months (4.3 years) in patients with advanced cancer. Since the median time from the initial diagnosis to the start of DDW treatment reached 13.2 months (1.1 years), MST was also calculated from the initiation of DDW treatment. Most patients underwent conventional therapies before entering the trial; therefore a new study arm was created for restaging at the time of inclusion in the study. Due to the extremely long survival of patients already in remission at the start of DDW treatment MST calculations are still pending: one patient out of the 48 patients died during the cumulative total follow-up of 289 years (median: 47.8 months). Patients with primary breast cancer achieved 89 months (7.4 years) MST, while in advanced breast cancer MST is achieving 39 months (3.3 years).

In comparison with published data DDW treatment in combination with, or as an extension of, conventional therapies noticeably prolonged MST in subgroups of breast cancer patients. The method proved to be safe, and D-depletion may act as a highly effective therapy both in preventing the recurrence of breast cancer and for the treatment of primary or advanced breast cancers. We conclude that D-depletion offers additional benefits to standard treatment regimens in breast cancer.

Keywords: Deuterium depletion, breast cancer, median survival time, MST, retrospective study

Effect of reduced deuterium (D) content of drinking water in STZ-induced diabetic rats and in humans with altered glucose metabolism

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Deuterium, a stable heavy isotope of hydrogen, binds to oxygen to form D_2O. D_2O exist in the environment at 1/6700 of H_2O (150 ppm) and is expected to have biological effects. Several lines of evidences suggest that D_2O inhibits insulin release from pancreatic islets. Very little or no data is available on the complex actions of lowering D_2O content of the cellular environment. Some experimental and clinical observations suggest that depletion of D_2O has anti-mitotic effect in various tumor cells. Some clinical observations also suggest that depletion of D_2O interferes with glucose metabolism in diabetic patients. In our experiments we wanted to test the effect of removal of D_2O on glucose metabolism in sreptozotocin (STZ)-induced diabetic rat model. After 2 weeks, animals were randomly distributed in to several groups to test the effect of D_2O (25-150ppm) on glucose metabolism in diabetic animals with or without 2x1U/day insulin treatment. Our results indicate that STZ treatment significantly increased serum glucose, fructoseamine, HbA_{IC} and TBARS concentrations. Depletion of D_2O did not influence any of the measured parameters in animals not receiving insulin. However the measured parameters were significantly lower in those animals that received lower D_2O containing drinking water and insulin treatment. Membrane associated GLUT-4 was significantly higher in the soleus muscle of these animals. These observations suggest that D_2O depletion enhances insulin effect on GLUT-4 translocation and potentiate glucose uptake in diabetic animals. The mode of action of D_2O depletion is not fully understood and needs further experiments to be elucidated in insulin-requiring Type1 diabetes.

To investigate the effect of DDW in humans on metabolic diseases a 90 day-long open label study was completed using a) the hyperinsulinemic euglycemic clamp technique, b) the homeostasis model assessment methods (HOMA-IR) and c) HOMA-B% and S%. The study was approved by the Regional and Institutional Ethical Committee before prospective patients were enrolled and screened. A total of 30 patients with established insulin resistance and/or with glucose intolerance (IFG or IGT) were selected for inclusion in this study, screened within two weeks prior to the first drug administration. Male and female patients, between age 18-60 years consumed 1.5 liters DDW per day with 110 ± 5 ppm. Before and after the treatment period blood samples were collected and stored at -80°C to determine HbA1c, FFA, triglicerid, lipidfractions, leptin, adiponectin, osteocalcin, kathepsin, sRANKL, osteoprotegerin, IL6, IL1ß, TNFalfa, usCRP, ApoA, Apo B, vitamin D_3, oestradiol, tesztosteron, FSH. Serum D-concentration decreased from 148 ppm (146-150 ppm) to 134 ppm (125-143 ppm) during the treatment. The average body mass increased from 85.55 kg to 86.71 kg (p=0.0071).

Insulin resistance decreased in 11 volunteers (36.6%), total body glucose uptake increased with 0.2 to 4.2 mg/bwkg/min. The possible cause-effect relationship will be discussed based on volunteer IFG, IGT, type 2 DM status, insulin concentrations, body weight, fasting glucose levels and HDL concentrations. The results clearly show that consumption of DDW reduces fasting glucose levels, increase HDL concentrations, and, in early disease stages, reduce insulin resistance.

Keywords: deuterium, deuterium depletion, GLUT-4, insulin resistance, diabetes, HDL