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EFFECTS OF HYDROGEN-RICH WATER ON HAMSTERS WITH EXPERIMENTAL MYELOID TUMOR

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Summary

This paper presents the results of an *in vivo* study of the antitumor effect of hydrogen-rich water in hamsters with experimental myeloid tumors. Hydrogen-rich EVOdrop water (HEW) with a molecular hydrogen (H_2) concentration of 1.2 ppm. pH=7.3, and oxidation-reduction potential (ORP) of (-390 mV) as basic parameters were obtained with the EVObooster apparatus. The myeloid tumor was implanted subcutaneously on the back of *Golden Syrian* hamsters used for the experiment. The effect of HEW on biometric parameters of tumor growth, namely 18 hematological parameters and some hematological biomarkers (indices) was evaluated. The blood sera from experimental and control animals were analyzed using the Non-equilibrium energy spectrum (NES) and the Differential non-equilibrium energy spectrum (DNES) spectral methods. The results showed a protective antitumor effect of HEW seen by a decrease in tumor uptake rate, a decrease in mortality, an increase in survival time, a normalizing effect on hematological parameters and biomarkers (N/LR, WBC/LR, PLT/LR), and an increase in energy between water molecules in blood serum in hamsters with experimental myeloid tumor. The results show that HEW has anticancer potential that could be used with conventional chemotherapeutics to develop new treatment strategies as well as cancer prevention.

KEYWORDS: *hydrogen-rich water, myeloid tumor, NES and DNES spectral analyses*

INTRODUCTION

Cellular redox (oxidation-reduction) homeostasis is associated with aging processes and various diseases such as hypertension, neurodegenerative diseases, atherosclerosis, diabetes, cardiovascular diseases, reproductive system diseases, and oncological diseases(1). With an increase in the level of reactive oxygen species (ROS) or oxidants, there is a deviation from the redox balance, whereby the cell cannot have a stable antioxidant defense(2). Cytoplasmic pH and hydrogen transport influence cell growth and tumorigenesis. Protons (H^+) and molecular hydrogen (H_2) are involved in several different processes(3). The studies proved the anti-inflammatory, anti-oxidative, and anti-tumor effects of molecular hydrogen (H_2) (4). The role of H_2 and H^+ in oxidative stress outlines the potential anticancer activity of this endogenous ion and different types of H_2 donors(3).

The hydrogen (H^+) and hydroxyl (OH^-) ions play a critical role in the cells for biological processes(5). Cells maintain intracellular pH (pH_i) in a narrow range (7.1–7.2) by controlling membrane

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Technical data	
Weight	2500 g
Size	260/185/100 mm
Hydrogen concentration	900-1200 ppb 0.9-1.2 ppm
Oxidation-reduction potential (ORP)	-450 ~ -580 mV
Maximal water production	720 L/24 hours
Maximal capacity	20 000 L
Water supply pressure	4.2 kgf.cm^{-1}
Electrical parameters	U=110; 220 V v=50; 60 Hz

Fig. 1. EVOdrop booster for hydrogen-rich water (HEW)

proton pumps and transporters whose activity is determined by intra-cytoplasmic pH sensors. The transport of protons (H+) through the plasma membrane is a key process for maintaining the pH value. For cancer cells, the extracellular pH is 6.7-7.1 while the cytoplasmic pH is greater than 7.4(3,6). Mitochondrial function is related to the transfer of hydrogen (H^+) ions across its inner membrane and the formation of a charged layer of H^+ ions in the cytosol around mitochondria. The actual electric potential of the fully functional mitochondria evaluated from the transfer of H^+ ions is about −140 mV, and that of the dysfunctional mitochondria is about −70 mV(7).

Molecular hydrogen (H_2) is administered in practice by inhalation as a gas(8,9), orally, dissolved in water as Hydrogen-rich water (HRW) (10-12), and intravenously, as a hydrogen-rich saline solution(13,14). Hydrogen-rich water has antioxidant properties against hydroxyl radicals (·OH)(15,16).

The active species of HRW are hydrogen molecules and atoms. H_2 can penetrate cell membranes and reach subcellular components to protect nuclear and mitochondrial DNA(3). In cellular processes, the following reactions with H_2 and H^+ is valid(3):

$$2H^+ + 2e^- \leftrightarrow H_2.$$

Increased intracellular Ca^{2+} and changes in H^+ transport are critical for tumor development and metastasis. The acid–base balance is controlled by a different approach in cancer cells than in normal cells(12). Carbon monoxide dehydrogenase (CODH) of biological reactions oxidizes CO, and electrons are released. As a result of this reaction, coupled hydrogenase reduces the released electrons to H_2(17). HRW has antioxidant properties against hydroxyl radicals of hydrogen(18,19).

Hydrogen-rich water can be produced by placing a metal magnesium rod in drinking water where the following chemical reaction occurs(15):

$$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$$

HRW can also be produced using the Proton Exchange Membrane (PEM)(20). We used EVO-booster with PEM and additional activation to enrich water with hydrogen in our research(12).

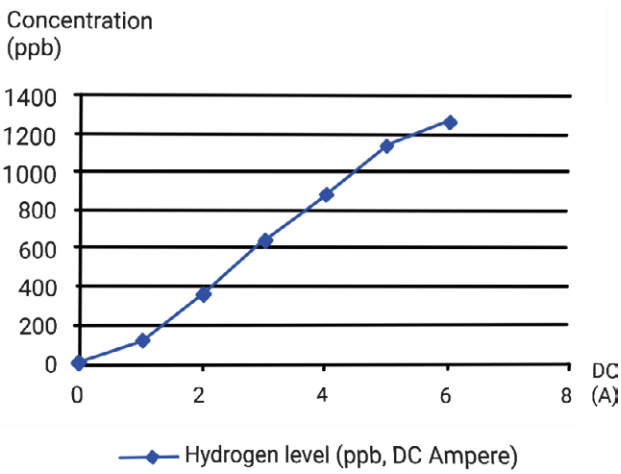

Fig. 2. Hydrogen EVOdrop water (HEW). Relation of electric current per dissolved concentration.

The present study aimed to investigate the effects of hydrogen-rich EVOdrop water (HEW) as a potential antitumor agent in an experimental tumor model system *in vivo*, by evaluating tumor growth parameters, hematological parameters, indices, and hydrogen bonding parameters between water molecules in blood serum through spectral analysis.

MATERIALS AND METHODS

Preparation and characterization of Hydrogen-rich EVOdrop water (HEW)

Hydrogen-rich EVOdrop water was prepared by electrolysis of deionized water using EVO booster apparatus with a Proton exchange membrane (Figure 1). Hydrogen EVOdrop water (HEW) is enriched with hydrogen molecules in a concentration of 1.2 ppm (Figure 2).

Deionized water was obtained by filtering tap water on a TOB-DBW-SYS-10L/H device with parameters of 99.99% desalination and electrical conductivity of 3μS.cm-1.

Effect of oral administration of HEW on hamsters with experimental myeloid tumor

Experimental animals

Golden Syrian hamsters weighing 80-100 g, two months old, and from both sexes were used for the experiments. The Bulgarian Academy of Sciences, Breeding Facility, Kostinbrod, Bulgaria, supplied the hamsters. After a 7-day acclimatization period in the vivarium of the Institute of Experimental Morphology, Pathology, and Anthropology with Museum, Bulgarian Academy of Sciences (IEMPAM-BAS), the animals were randomly assigned to plastic cages and reared with free access to chow pellets and water with 12 hour light/dark cycles. All procedures were carried out in accordance with the national regulation № 20/01.11.2012 regarding laboratory animals and animal welfare and European directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Experimental Myeloid tumor model

Graffi's virus-induced(21,22) myeloid tumor in newborn hamsters was adapted and maintained in sexually mature hamsters at the IEMPAM-BAS (Toshkova, 1995)(23). The tumor was maintained monthly *in vivo* by subcutaneous (*s.c.*) inoculation of 10^6 viable tumor cells. In the present experiment, tumor transplantability was achieved by *s.c.* inoculation of $2x10^4$ viable tumor cells into the interscapular area of the animals(24,25). This amount of cells ensures 100% occurrence of tumor and inevitable 100% animal mortality as established in our previous studies(23-27).

Experimental design

All animal experiments were approved by the Animal Experiments Ethics Committee of IEMPAM-BAS.

The experimental animals were randomly divided into three groups (n =8 in Group 1 and Group 2, n=3 in Group 3) and treated as follows: (1) Group 1 - Tumor-bearing hamsters (TBH) drinking Hydrogen-rich EVOdrop water (HEW) ad libitum; (2) Group 2 - TBH drinking deionized water (positive control, DW) and (3) Group 3 - healthy hamsters without tumor drinking deionized water (negative control). Five days later, myeloid tumor cells were aseptically obtained from tumor pieces, suspended in PBS, and 2×10^4 cells/200 μl PBS were injected *s.c.* into the dorsal region of each hamster from Groups 1 and 2. In experimental Groups 1 and 2, hamsters drank HEW and DW, respectively, starting 5 days before myeloid tumor cell engraftment and continuing for 25 days after tumor cell inoculation. Fresh HEW and DW water were prepared daily, and the oxidation redox potential (ORP) of HEW was -405 mV < ORP < -390 mV.

On day 25 of the experiment, three hamsters from each group were randomly selected regardless of gender for analysis of hematological parameters, biomarkers, and spectral analysis of blood sera. Tumor growth parameters were determined in the remaining five hamsters of Group 1 and Group 2.

Protective effect - Evaluation of biometric parameters

The efficacy of HEW therapy was assessed by measuring the tumor growth parameters(16,20). Tumor appearance (transplantability) in hamsters was recorded daily by skin palpation at the injection site. Tumor size (mm) was measured at regular intervals until day 30, using a digital caliper in two perpendicular dimensions. Survival and mortality rates were expressed as percentages. Mean survival time was also determined. Tumor parameters of experimental hamsters were compared with those of controls.

Evaluation of hematological parameters and biomarkers

Hamsters were euthanized after administration of general anesthesia, and peripheral blood

was collected from the jugular vein on day 25 after tumor transplantation.

A portion (2.0 ml) of peripheral blood was collected in a vacutainer containing anticoagulant and analyzed using an automatic hematology analyzer (BC-2800 Vet machine blood cell analyzer, Mindray, China). The serum was separated from a portion of the blood after the blood had clotted. The clot was removed by centrifugation, and the serum (supernatant) was carefully removed using a Pasteur pipette.

Hematometric biomarkers/indexes reflecting the quantitative ratio of neutrophils and lymphocytes (N/LR), leukocytes and lymphocytes (WBC/LR), and platelets/lymphocytes (PLT/LR) were calculated(28-31).

Evaluation of physicochemical parameters of blood serum from control and experimental hamsters by spectral methods NES and DNES

NES and DNES Spectral Analyses

The methods of Non-equilibrium Energy Spectrum (NES) and Differential Non-equilibrium Energy Spectrum (DNES) for the measurement of hydrogen bond energy distribution were applied to research serums from hamsters. The device invented by A. Antonov(32-34) for spectral analysis with NES and DNES methods is based on an optical principle(35,36).

The energy (E) of hydrogen O...H-bonds among H_2O molecules in the water sample is measured in eV. The function f(E) is called the spectrum of distribution according to energies. The energy spectrum of water is characterized by a non-equilibrium process of water droplets evaporation and this is a non-equilibrium energy spectrum (NES) and is measured in eV^{-1}. DNES is defined as the difference

$$\Delta f(E) = f(\text{sample of water}) - f(\text{control sample of water})$$

where *f(*)* denotes the evaluated energy(32-37).

Statistical analysis

Student's t-test was used to calculate the significance of differences between the mean values of hematological parameters of experimental and control hamsters, with p-values set at $p < 0.01$ and $p < 0.05$.

Statistical analysis of the NES and average hydrogen bond energies was performed with the Mann-Whitney U test at $p < 0.05$ due to the experimental and control samples' deviation from the normal distribution. According to the established practice, the reported results include the medians of the compared samples (groups), the value of the calculated U statistics, the p-value, and the acceptance (H_0) or rejection (H_1) of the null hypothesis(38).

The null hypothesis H_0 is formulated as follows: the two samples are from the same general population. Alternative H_1 is the opposite. The SPSS package was used for the evaluation.

Ethical aspects

All experiments were performed following the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (OJ L 222) and approved by the national regulation No. 20/01.11.2012 regarding laboratory animals and animal welfare of the National Veterinary Service, Bulgaria.

RESULTS AND DISCUSSION

Parameters of hydrogen-rich EVOdrop water (HEW)

HEW was prepared for this research by electrolysis of deionized water (DW) and had the following parameters: concentration of hydrogen molecules was 1.2 ppm (Figure 2); the oxidation-reduction potential (ORP) has changed from +260 mV in DW before electrolysis to –390 mV after 30 minutes of electrolysis (indicating an intensification of the reducing power), the pH evolved within a narrow physiological range of approximately 7.0 to 7.3.

Effect of oral administration of HEW on tumor-related parameters

This tumor model had 100% transplantability, 100% mortality, and a lack of spontaneous regression. Typically, after 7 to 15 days of inoculation, tumors develop as a solid subcutaneous mass approximately 5 mm in diameter, which usually grows to about 5-6 cm in diameter and causes the death of hamsters on days 30 to 35.

In the present experiments, tumor appearance was daily recorded by skin palpation on the hamster's back at the injection site from day 7 after the beginning of the experiment until the day the tumor was detected in all animals. The data are shown in Figure 3.

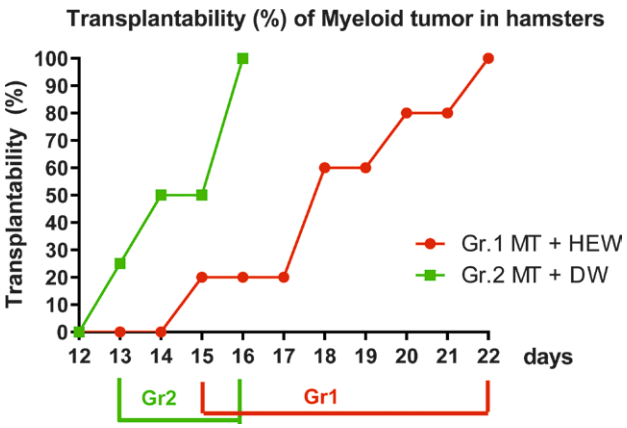

Fig. 3. Transplantability (%) of the myeloid tumor (MT) in hamsters receiving Hydrogen EVOdrop water - HEW (Group 1) and hamsters receiving deionized water - DW (Group 2)

In Group 1 of hamsters receiving HEW, tumors were palpable between days 15 and 22. In the positive control group of hamsters (Group 2) receiving DW, tumors appeared between days 13 and 16. Tumor compaction (tumor 1-2 mm) was measured at regular intervals until day 30 after myeloid tumor cell transplantation, and the average data are presented in Figure 4.

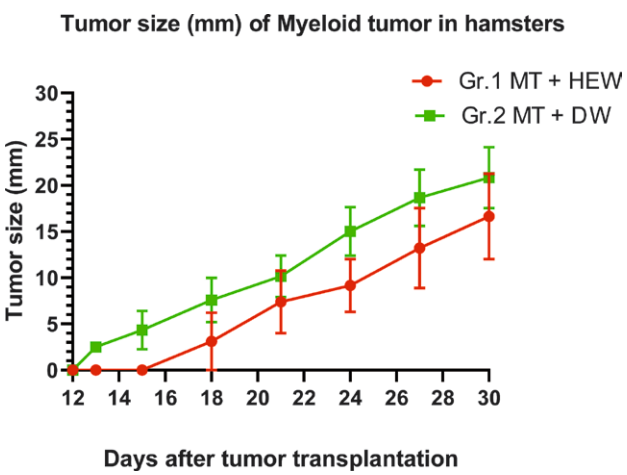

Fig. 4. Myeloid tumor size (mm) in Hydrogen EVOdrop water (HEW)-treated hamsters (Group 1) and deionized water (DW)-treated hamsters (Group 2)

In the hamsters from positive control Group 2 (receiving DW), the tumors grew to a mean size of 20.8±3.3 mm, while in the hamsters of Group 1 (drinking HEW) the tumors reached a mean size of 16.7±4.7 mm on day 30. The graph illustrates that in both groups the tumor progressively increased. The mean tumor size in Group 1 receiving HEW was smaller, which can be explained by the therapeutic effect of the HEW used. The photographs shown in Figure 5 support the obtained metric data.

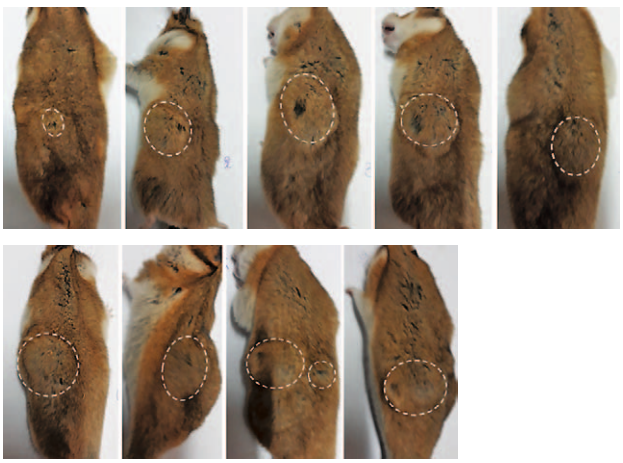

Fig. 5. Photographs of hamsters from Group 1 drinking HEW (first row). The second row is of positive control Group 2 drinking DW. Photos are from day 24 after myeloid tumor transplantation. Tumors in each hamster are delineated with dotted lines.

Mortality data for the two experimental groups are presented in Figure 6.

There was lower mortality in Group 1, hamsters drinking HEW compared to positive control hamsters receiving DW (Group 2). In Group 2,

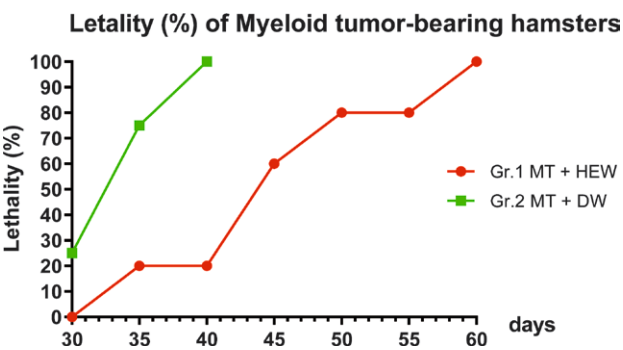

Fig. 6. Percent lethality (%) of myeloid tumor-bearing hamsters (MT) drinking Hydrogen EVOdrop water - HEW (Group 1) and hamsters drinking deionized water- DW (Group 2)

100% mortality was observed by day 40 after myeloid tumor cell transplantation. In Group 1, mortality was 20% at day 40 and increased to 100% at day 57. The 17-day difference in mortality rate indicates an effect of HEW.

A higher mean overall survival time (OS) was found for Group 1, with a 46.4±8.1 days compared with 35.0±4.1 days in positive control Group 2 (Figure 7).

The Kaplan-Meier Long-rank test shows a statistically significant difference ($p<0.05$).

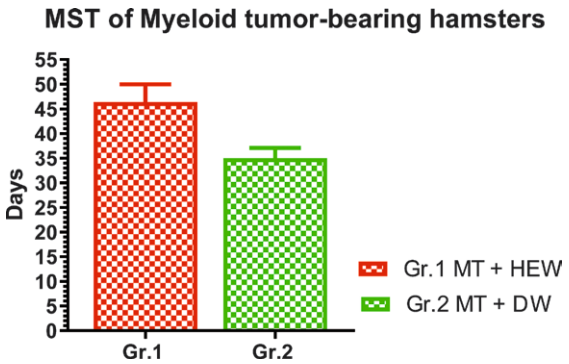

Fig. 7. Mean survival time (in days) of myeloid tumor-bearing hamsters (MT) receiving Hydrogen EVOdrop water - HEW (Group 1) and deionized water - DW (Group 2).

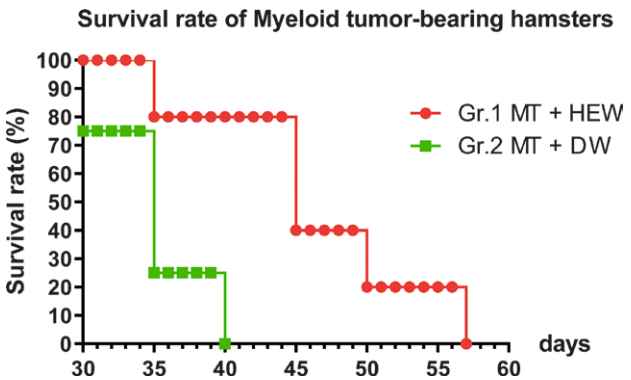

Fig. 8. Individual survival rate (in days) of myeloid tumor-bearing hamsters (MT) receiving Hydrogen EVOdrop water - HEW (Group 1) and hamsters receiving deionized water - DW (Group 2, positive control). Twenty percent (20%) of the hamsters survived 57 days in Group 1 while in Group 2 they survived 40 days ($p<0.05$)

Effect of HEW on blood values and indices

Table 1 illustrates the values of blood parameters(43) of hamsters (Groups 1, 2, and 3) on day 25 of the experiments with Hydrogen EVOdrop water.

From Table 1 it can be seen 3-fold higher platelet values in Group 2 (hamsters, drinking DW) compared with those in Group 1 (hamsters drinking HEW) as well as in the group of healthy hamsters (Group 3). Statistically significant differences were found between WBC, PLT, PDW, and PCT blood parameters values of hamsters from Group 1 and Group 2, represented by asterisks. The difference in means for three of these indicators is high: 99% (0.01^{**}) and for one it is 95% (0.05^{*}).

To evaluate the impact of various antitumor drugs and therapeutic regimens applied in cancer treatment, various parameters related to tumor occurrence and development, survival, mortality, changes in hematological indices, etc. are most commonly measured. Total WBC (white blood cell) count, neutrophil count, and lymphocyte count can be statistically significant predictors of 5-year survival (or mortality) in individuals with malignancy(29), while higher platelet concentration increases the risk of lethal thromboses(40).

In our experiments, the WBC/LR, N/LR, and PLT/LR indices(36) with Hydrogen-rich water (HRW) of type Hydrogen-rich EVOdrop water (HEW) ~~indices~~ were monitored and data are presented in Figure 9.

It could be seen that the WBC/LR index of Group 1, Group 2, and Group 3 had values of 10.5, 12.0, and 10.8, respectively. A similar correlation was found for the N/LR index, which in Group 2 had a value of 10.9, and in hamsters of Group 1 and Group 3, similar values were established (9.3 and 9.0). The same relationship was found also for the PLT/LR index, with the highest value of 40.4 in Group 2, which was about 3.5 times higher than values in Group 1 and Group 2, respectively (12.4 and 12.3) (Figure 9). It is noteworthy, however, that the values of all three indices in hamsters from Group 1 and Group 3 (healthy hamsters) were approximately the same. This fact can be explained by the normalizing effect of HEW on blood parameters during tumor progression.

Thus, also in clinical practice, some primary hematometric indices like N/LR, WBC/LR, and PLT/LR are used as diagnostic and prognostic tools in patients with cancer and leukemia(28-30). For example, the quantitative neutrophil to lymphocyte ratio – N/LR index is important for predicting higher survival of cancer patients. N/LR values ≤ 2.0 have been associated with higher sur-

Table 1.

Values of blood parameters of hamsters (Groups 1, 2, and 3) on day 25 of the experiments.

Parameters	Units	**Hamsters with myeloid tumor, drinking Hydrogen EVOdrop water (Group 1)**	**Hamsters with myeloid tumor, drinking Deionized Water (Group 2)**	**Healthy hamsters without tumor (Group 3)**
WBC (Leukocytes)	$10^9.L^{-1}$	102.9	**94.8****	102.7
Lymphocytes	$10^9.L^{-1}$	9.8	7.8	9.5
Monocytes	$10^9.L^{-1}$	2.4	2.2	2.3
Granulocytes	$10^9.L^{-1}$	90.7	84.8	85.1
Lymphocytes	%	9.5	8.2	8.5
Monocytes	%	2.4	2.3	2.3
Granulocytes	%	88.1	89.5	88.3
RBC (Erythrocytes)	$10^9.L^{-1}$	5.16	5.40	5.03
HGB (Hemoglobin)	$g.L^{-1}$	269	279	265
HCT (Hematocrit)	$L. L^{-1}$	0.273	0.291	0.282
MCV (Mean red blood cell volume)	fL	53.1	53.9	55.8
MCH (Average HGB content in erythr)	pg	50.7	51.6	53.2
MCHC (mean con cog Hb)	$g.L^{-1}$	959	958	947
RDW (Red cell distribution width)	%	13.1	13.5	12.7
PLT (Platelets)	$10^9.L^{-1}$	122	315**	117
MPV (means volume platelets)	$f.L^{-1}$	6.3	6.1	7.4
PDW (Platelet distribution width)	**%**	18.6	**19.6***	18.5
PCT (Procalcitonin)	**%**	**0.076**	**0.192****	**0.071**

* Statistically significant difference is at $p<0.05$
** Statistically significant difference is at $p<0.01$

vival for breast cancer patients, and N/LR ≥ 5.0 has been associated with lower survival for patients with breast cancer, gastric cancer, etc.(41). In recent years, new results have been reported for WBC/LR(31), PLT/LR(29), and N/LR(42).

The biological effects of hydrogen-rich water with concentrations of 0.08-1.5 ppm have been described(44, 45). Feeding mice with H_2-water (0.08 - 1.5 ppm [w/w] H_2) significantly reduced the loss of dopaminergic neurons in this chemically-induced mouse(44). Hydrogen-rich water administered to improve mood, anxiety, and autonomic nerve function in everyday life had an H_2 concentration of 0.08-1.5 ppm(45).

Effect of HEW on blood serum hydrogen bonds energy distribution investigated with NES and DNES spectral methods

The Non-equilibrium Energy Spectrum (NES) of 5 blood serum samples from each group: experimental myeloid tumor-bearing hamsters receiving Hydrogen EVOdrop water - HEW (Group 1), myeloid tumor-bearing hamsters receiving deionized water - DW (Group 2), and healthy hamsters without tumor (Group 3) is shown in Figure 10.

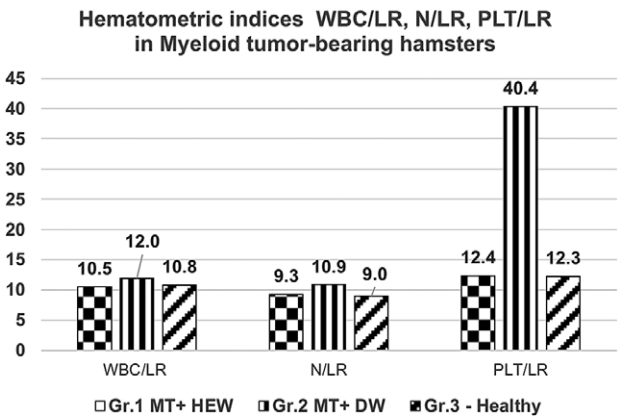

Fig. 9. Hematometric indices of myeloid tumor-bearing (MT) hamsters receiving Hydrogen EVOdrop water - HEW (Group 1), MT hamsters receiving deionized water - DW (Group 2), and healthy hamsters without tumor (Group 3). Hematometric indices represent the quantitative ratio of leukocytes and lymphocytes (WBC/LR), neutrophil and lymphocytes (N/LR) and platelets and lymphocytes (PLT/LR)

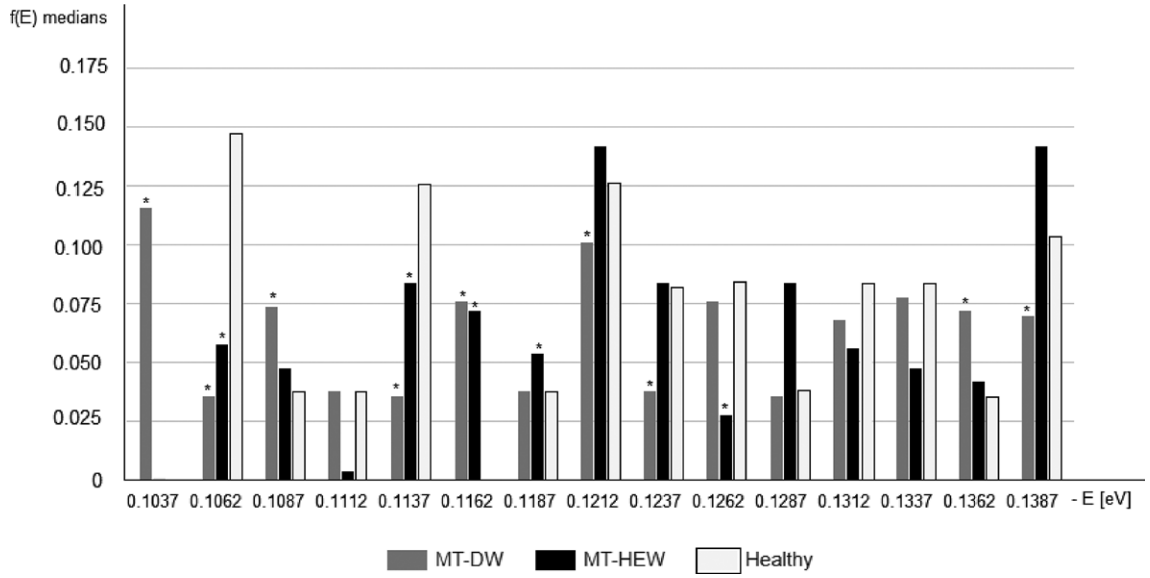

Fig. 10. Non-equilibrium Energy Spectrum (NSE) at each value of hydrogen bond energy. Medians of the experimental MT-HEW (Group 1), control group MT-DW (Group 2), and group of healthy hamsters (Group 3). Statistically significant differences compared to the control group, with p<0.05, are marked with an asterisk

Non-equilibrium Energy Spectrum (NES) and Differential Non-equilibrium Energy Spectrum (DNES)

The comparison between the Non-equilibrium Energy Spectrum (NES) of the control MT-DW and the healthy animals in Group 3 (Figure 10) shows a general shift of hydrogen bond energy distribution towards lower levels. It is markedly expressed as depletion of (-E) at 0.1387, 0.1362, 0.1237, 0.1212, 0.1137, and 0.1037 eV levels as well as the appearance of (-E) at 0.1162 and 0.1037 eV ones in the lower half of the spectrum due to the myeloid tumor. Thus, statistically significant differences between these NES are observed at 9 out of 15 energy levels.

On the other hand, the comparison between the NES of the experimental MT-HEW and the healthy group (Figure 10) demonstrates the effect of hydrogen-rich water consumption as an absence of the lowest at E= -0.1037 eV or (λ=11.96 μm; $\tilde{\nu}$=836 cm^{-1}) energy level as well as less depletion of the at E=-0.1087 (λ= 11.41μm; $\tilde{\nu}$= 877 cm^{-1}) and -0.1137 eV ones. Moreover, the highest measured at E=-0.1387 eV or (λ=8.95 μm; $\tilde{\nu}$=1119 cm^{-1}) level shifts from statistically significant depletion in the case of the MT-DW control group into an absence of statistically significant difference. In general, differences between the MT-HEW and healthy NES are observed below at E=-0.1287 eV or (λ= 9.63 μm; $\tilde{\nu}$= 1038 cm^{-1}), thus pointing to a stabilizing effect of hydrogen-rich water consumption on hydrogen bonds energy distribution (there are statistically significant differences at 5 out of 14 energy levels).

It should be pointed out that the -0.1162 eV energy level, not being populated in the control group, becomes equally populated in both experimental groups. That is why it could be considered a myeloid tumor diagnostic marker in hamsters.

The comparison between the NES of both MT groups (Figure 10) shows a tendency towards a greater population of the hydrogen bonds energy levels in the upper half of the spectrum due to hydrogen-rich water consumption. Along these lines, statistical analysis was performed on the average hydrogen bond energy of all samples calculated with their corresponding NES. The results are shown in Table 2.

These findings clearly demonstrate that the average hydrogen bond energy in the MT-DW control group is lower than that in the healthy group, while that of the MT-HEW group is higher. And, the comparison of both experimental groups confirms a markedly higher average hydrogen bond energy in the hydrogen-rich water-consuming group than that in the deionized water-con-

Table 2.

Statistical comparison of the average hydrogen bond energy of all samples

SPL No.	1	2	3	4	5				
Average Energy [-eV]						Median	U	p	Hyp
MT-DW	0.121	0.120	0.121	0.120	0.121	0.121	0	0.01	H1
Healthy	0.122	0.123	0.122	0.123	0.123	0.123			
MT-HEW	0.124	0.124	0.125	0.123	0.124	0.124		0.02	H1
Healthy	0.122	0.123	0.122	0.123	0.123	0.123	1.5		
MT-HEW	0.124	0.124	0.125	0.123	0.124	0.124	0	0.01	H1
MT-DW	0.121	0.120	0.121	0.120	0.121	0.121			

suming one. Consequently, the consumption of hydrogen-rich water appears as an active factor to counteract the lowering of average hydrogen bond energy in the blood serum due to myeloid tumor development.

DISCUSSION

The study conducted in 2012 compared groups of oncology patients and healthy individuals. The results showed significant differences in energy levels related to hydrogen bonds in the blood serum of these two groups. The energy levels were measured in millielectronvolts (meV), with oncology patients having E=-1.6 meV and healthy individuals having a result of E=-9.1 meV ($p < 0.05$)(46).

Furthermore, our previously published study also looked at hamsters with myeloid tumors, comparing them to healthy hamsters. Statistically significant differences were observed in energy levels (-E)=0.0937; 0.1187; 0.1212; 0.1337; 0.1387 eV(39). Additionally, there were observations regarding the effects of hydrogen-rich water (HRW) consumption on hydrogen bond energy distribution. Specifically, consumption of hydrogen-rich water appeared to stabilize the energy distribution of hydrogen bonds, with statistically significant differences observed at 5 out of 14 energy levels(47).

The spectral results and parameters suggest that hydrogen-rich water may have preventative applications(48,49). The results of the effects of HRW on patients with chemotherapy were published. Results demonstrated that hydrogen-rich water (HRW) could mitigate oxaliplatin-induced hyperalgesia. HRW reduces microbial diversity while altering the gut microbiota structure. It restores the balance of inflammatory cytokines and oxidative stress. HRW decreases the expression of LPS and TLR4(49,50). There are effects against thioacetamide-induced cholangiofibrosis(51) and ovarian cancer(52).

Our research shows that the anti-inflammatory effects of hydrogen-rich water are primarily associated with the peak at E=-0.1212 eV(39). The same peak was observed in the medical drugs for diabetes(53). The findings indicate that consuming hydrogen-rich water (HRW) effectively reduces elevated levels of glucose and total cholesterol(54,55).

CONCLUSIONS

The following conclusions can be drawn from the application of HEW obtained with the EVOdrop booster as an antitumor agent.

1. Daily HEW ingestion for 25 days after tumor cell transplantation resulted in a protective antitumor effect in the experimental hamsters that was expressed by:
   - An extension of the latency period and a decrease in the tumor uptake rate (transplantability) (Figure 3),
   - A reduction in the tumor growth rate (Figure 4 and Figure 5),
   - An increase in the average (Figures 6 and 7) and individual (Figure 8) survival of hamsters with experimental myeloid tumor.

2. Statistically significant results were obtained for the normalizing effect on PLT and WBC blood values and on hematological indices- N/LR, WBC/LR, PLT/LR.

3. Statistically significant results were obtained with NES and DNES spectral methods, showing an increase in energy between water

molecules in the blood serum of hamsters with myeloid tumor drinking HEW.

4. The positive effects observed in hamsters with experimental myeloid tumor may be explained by a therapeutic antitumor effect of HEW.

REFERENCES

1. Kruk J, Aboul-Enein H Y, Kladna A, Bowser J E. Oxidative stress in biological systems and its relation with pathophysiological functions: the effect of physical activity on cellular redox homeostasis. Free Rad. Res. 2019;53(5):497-521.
2. Podsednik A, Jacob A, Lin Z Li, Xu He N. Relationship between optical redox status and reactive oxygen species in cancer cells. React Oxyg Species. 2020;9(26): 95-108.
3. Rochette L, Zeller M, Cottin Y, Vergely C. Antitumor activity of protons and molecular Hydrogen: Underlying mechanisms. Cancers. 2021;13(4):893.
4. LeBaron T W, Kura B, Kalocayova B, Tribulova N, Slazak J. A new approach for the prevention and treatment of cardiovascular disorders. Molecular hydrogen significantly reduces oxidative stress. Molecules. 2019;24(11):2076.
5. Agmon N, Bakker H J, Campen R K, Henchman R H, Pohl P, Roke S, Thaemer, M, Hassanali A. Protons and hydroxide ions in aqueous systems. Chem Rev. 2016; 116(13)7642–7672.
6. Damaghi M, Wojtkowiak J W, Gillies R J. pH sensing and regulation in cancer. Front. Physiol. 2013. doi.org/ 10.3389/fphys.2013.00370
7. Pokorný J, Pokorný J, Kobilková, J, Jandová, A, Holaj, R. Cancer development and damped electromagnetic activity. Appl. Sci. 2020;10(5):1826.
8. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata, K, Katsura, K, Katayama, Y, Asoh, S, Ohta, S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat. Med. 2007;13:688–694.
9. Dixon B J, Tang, J, Zhang J H. The evolution of molecular hydrogen: a noteworthy potential therapy with clinical significance. Med Gas Res.2013;3(10). doi: 10. 1186/2045-9912-3-10
10. Kasuyama K, Tomofuju T, Ekuni D, Tamaki N, Azuma T, Irie K, et al. Hydrogen-rich water attenuates experimental periodontitis in a rat model. J. Clin. Periodontol. 2011;38(12):1085-1090.
11. Sim M, Kim Ch-S, Shon W-J, Lee Y-K, Choi E Y, Shin D-M. Hydrogen-rich water reduces inflammatory responses and prevents apoptosis of peripheral blood cells in healthy adults: a randomized, double-blind, controlled trial. Sci. Rep. 2020;10:12130.
12. Zanini D, Todorovic N, Stajer V, Huether F, Ostojic S M. (2023) Short-term intake of hydrogen-rich water positively affects neuropsychological performance in young adults. Clin. Nutr. ESPEN. 2023;54:P715
13. Zhang Y, Sun Q, He B, Xiao J, Wang Z, Sun X, Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion. Int. J. Int. Cardiol. 2011;148(1):91-95.
14. Ikeda M, Shimizu K, Ogura H, Kurakawa T, Umemoto E, Motooka D, Nakamura S, Ichimaru N, Takeda K, Takahara Sh, Hirano Sh.-i., Shimazu T. Hydrogen-rich saline regulates intestinal barrier dysfunction, dysbiosis, and bacterial translocation in a murine model of sepsis. Shock. 2018;50(6):640-647.
15. Nakao A, Toyoda Y, Sharma P, Evans M, Guthrie N. Effectiveness of hydrogen-rich water on antioxidant status of subjects with potential metabolic syndrome - an open-label pilot study. J Clin Biochem Nutr. 2010; 46(2):140-149.
16. Ostojic S M. Hydrogen-rich water as a modulator of gut microbiota? J Funct Foods. 2021;78,104360.
17. Muramatsu Y, Ito M, Oshima T, Kojima S, Ohno K. Hydrogen-rich water ameliorates bronchopulmonary dysplasia (BPD) in newborn rats. Pediatr Pulmonol. 2016;51(9):928-935.
18. Harguindey S, Alfarouk K, Orozco J P, Hardonniere K, Stanciu, D, Fais S, Devesa J A. New and integral approach to the etiopathogenesis and treatment of breast cancer cased upon its hydrogen ion dynamics. Int J Mol Sci. 2020:21:1110. doi: 10.3390/ijms21031110
19. Mohr T, Aliyu H, Kuchlin R, Polliack S, Zwick M, Neumann A, Cowan D, de Mayer P. CO-dependent hydrogen production by the facultative anaerobe Parageobacillus thermoglucosidasius. Microb Cell Fact. 2018;17,108. doi.org/10.1186/s12934-018-0954-3
20. Zeng N, Hu C, Lv Ch and al. Large-current density and high-durability proton exchange membrane water electrolysis for practical hydrogen isotope separation. Separation and Purification Technology. 2023;310: 123148.
21. Chieco-Bianchi L, Fiore-Donati L, Tridente G, Penneli N. Graffi virus and immunological tolerance in leukemogenesis. Nature. 1967;214:1227-1228.
22. Charfi C, Voisin V, Levros, Jr., L-Ch., Edouard, E., Rassart, E. Gene profiling of Graffi murine leukemia virus-induced lymphoid leukemias: identification of leukemia markers and Fmn2 as a potential oncogene. Blood. 2011;117(6):1899–1910.
23. Toshkova, R. Attemps for immunomodulation in hamsters with transplanted Mieloid tumor, previously induced by Graffi virus. Bulgarian Academy of Sciences. PhD thesis. 1995.
24. Toshkova R, Ignatov I, Gluhchev G, Dinkov G. Protective and antitumor effects of Oxidal and Pyrucet in hamsters with experimental Graffi tumor. J Pharm Res Int. 2021; 33(12):1-19.
25. Georgieva, A K, Toshkova, R A, Todorova, K S, Tzoneva, R D. Antineoplastic effects of Erufosine on Graffi Myeloid tumour in hamsters. Bulg J Vet Med. 2021; 24(3):442-449.

26. Popov G, Shkondrov A, Toshkova R, Krasteva I, Kondeva-Burdina M, Manov V. Antiproliferative and antitumour activity of saponins from Astragalus glycyphyllos on myeloid Graffi tumour. J Ethnopharmacol. 2021;267. doi.org/10.1016/j.jep.2020.113519
27. Toshkova R, Manolova N, Gerdeva E, Ignatova M, Yossifova L, Rashkov I, Alexandrov M. Antitumor activity of quaternized chitosan-based electrospun implants against Graffi myeloid tumor. Int J Pharm. 2010; 400(1-2):221-233.
28. Azab B. et al. Pretreatment Neurophil/lymphocyte ratio is superior to platelet/lymphocyte ratio as a predictor of long-term mortality in breast cancer patients, Med. Oncol. 2013;30:432
29. Zvetkova E, Fuchs D. Medical significance of simultaneous application of red blood cell distribution width (RDW) and neopterin as diagnostic biomarkers in clinical practice. Pteridines. 2017;28(3-4):133-140.
30. Hirahara N. et al. Prognostic values of hematological parameters in patients with esophageal squamous carcinoma. Int J Clin Oncology. 2016;21(5):909-919.
31. Tang Ch, Cheng X, Yu Sh, Wang Y, Hou J, Li Q, Shen Zh, Liu T, Cui Y. Platelet-to-lymphocyte ratio and lymphocyte-to-white blood cell ratio predict the efficacy of neoadjuvant chemotherapy and the prognosis of locally advanced gastric cancer patients treated with the oxaliplatin and capecitabine regime. Onco Targets Ther. 2018;11:7061-7075.
32. Todorov S, Damianova A, Sivriev I, Antonov A, Galabova T. Water energy spectrum method and investigation of the variations of the H-bond structure of natural waters. Comptes Rendus de l 'Academie Bulg. des Sci. 2008;61(5251):857-862.
33. Gramtikov P, Antonov A, Gramatikova M. A study of the properties and structure variations of water systems under the stimulus of outside influences. Fresenius J Anal Chem. 1992;343(1):134-5.
34. Antonov A. Research of the Non-equilibrium processes in the area in allocated systems. DSc Thesis, Southwest University Neofit Rilski, Blagoevgrad, Bulgaria. 1995.
35. Mehandjiev D, Ignatov I, Neshev N, Vassileva P, Gluhchev G, Huether F, Drossinakis Ch. History-dependent hydrogen bonds energy distributions in NaCl aqueous solutions undergoing osmosis and diffusion through a ceramic barrier. J Chem Technol Metall. 2023;58(2):340
36. Ignatov I, Gluhchev G, Neshev N, Mehandjiev D. Structuring of water clusters depending on the energy of hydrogen bonds in electrochemically activated waters Anolyte and Catholyte. Bulg Chem Commun. 2021;53(20):234-239.
37. Mehandjiev D, Ignatov I, Neshev N, Huether F, Gluhchev G, Drossinakis Ch. Formation of clusters in water and their distribution according to the number of water molecules. Bulg. Chem. Commun. 2022;54(3):211.
38. Ignatov I, Huether F, Neshev N, Kiselova-Kaneva, Y, Popova T P, et al. Research of water molecules cluster structuring during Haberlea rhodopensis Friv. hydration. Plants. 2022;11(19):2655.
39. Neshev N, Ignatov I, Toshkova R, Drossinakis Ch, Gluhchev G. (2022) Hydrogen bonds energy distribution and information-theoretic analysis of blood serum from hamsters with experimental Graffi tumor. Libr Oncol. 2022;50(2-3):52-61.
40. Ma J, Kuzman J, Ray A, Lawson B O, Khong B, Xuan S, Hahn A W, Khong B. Neutrophil-to-lymphocyte Ratio (NLR) as a predictor for recurrence in patients with stage III melanoma. Sci Rep. 2018;8: 4044. doi.org/10.1038/s41598-018-22425-3
41. Fujita K, Seike T, Yutsudo N, Ohno M, Yamada H, Yamaguchi H., Sakumi K, Yamakawa Y, Kido M A, Takaki A, Kawaguchi T, Tanaka Y, Tanaka Y, Noda M. Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. PLoS ONE. 2009;4(9): e7247.https://doi.org/10.1371/journal.pone.0007247
42. Deng H, Meng T, Li C, Xiaojun W, Shuguo Zh, Yong C. Risk factors and anticoagulation effects of portal vein system thrombosis after laparoscopic splenectomy in patients with or without cirrhosis. Surg Laparosc Endosc Percutan Tech. 2019;29(6): 498-502.
43. Toshkova R, Zvetkova E, Ignatov I, Gluhchev G. Effects of Catholyte water on the development of experimental Graffi tumor on hamsters. Bulg.J.Public Health. 2019;11(3):60-73.
44. Nicolson G L, Ferreira de Mattos G, Settineri R, Costa C, Ellithorpe R, Rosenblatt S, La Valle J, Jimenez A, Ohta Sh. Clinical effects of hydrogen administration: from animal and human diseases to exercise medicine. Int J Clin Med. 2016;7:62945,45. DOI: 10.4236/ijcm.2016.71005
45. Mizuno, K., Sasaki, A. et al. Hydrogen-rich water for improvements of mood, anxiety, and autonomic nerve function in daily life. Med Ga. Res. 2017;7(4):247-255.
46. Ignatov I, Mosin O V, Naneva K. Water in the human body is information bearer about longevity. Euromedica, Hanover. 2012; 110-111.
47. Ignatov I. Research of the factors of health and longevity of for the population in Bulgaria. Bulg J Public Health. 2018;10(3):52-65.
48. Asgharzadeh F, Tarnava A, Mostafapour A. Hydrogen-rich water exerts anti-tumor effects comparable to 5-fluorouracil in colorectal xenograft model. World J Gastrointest Oncol. 2022;14(1):242-252.
49. Lian N, Shen N, Zhang K et al. Drinking Hydrogen-rich water alleviates chemotherapy-induced neuropathic pain through the regulation of gut microbiota. J Pain Res. 2020;14:681-691.
50. Yang Q, Guangdong J, Pan R et al. Protective effect of hydrogen-rich water on liver function of colorectal

cancer patients treated with mFOLFOX6 chemotherapy. Mol Clin Oncol. 2017;891-896.
51. Li Ch, Zhao X, Gu X et al. The preventive role of Hydrogen-rich water in Thioacetamide-induced cholangiofibrosis in rat assessed by automated histological classification. Front. Pharmacol. 2021; 12. doi.org/10.3389/fphar.2021.632045
52. Shang L, Xiw F, Li J et al. Therapeutic potential of molecular hydrogen in ovarian cancer. Transl Cancer Res**.** 2018;7(4). doi: 10.21037/tcr.2018
53. Ki J, Mukherjee A, Rangasamys S. Insuline-mimetic and anti-inflammatory potential of a vavadyl-shift base complex for its application against diabetes. RSI Adv. 2016;6:57530-57539.
54. Zheng M, Yu H, Xue Y et al. The protective effect of hydrogen-rich water on rats with type 2 diabetes mellitus. Mol Cell Biochem. 2021;476:3089–3097.
55. Le Baron T, Singh R, Ostojic S et al. The Effects of 24-Week, High-concentration hydrogen-rich water on body composition, blood lipid profiles and inflammation biomarkers in men and women with metabolic syndrome: A randomized controlled trial. Diabetes Metab Syndr Obes. 2020;13. doi.org/10.2147/DMSO.S240122

Sažetak

UČINCI VODE OBOGAĆENE VODIKOM NA HRČKE S EKSPERIMENTALNIM MIJELOIDNIM TUMOROM

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U radu su prikazani rezultati *in vivo* istraživanja antitumorskog učinka vode obogaćene vodikom na modelu hrčka s mijeloidnim tumorom. U eksperimentu je korištena voda obogaćena molekularnim vodikom (H_2) u koncentraciji od 1.2 ppm, pH=7.3, s oksidacijsko-redukcijskim potencijalom od -390 mV dobivena elektrolizom deionizirane vode pomoću uređaja EVObooster. Suspenzija stanica mijeloidnog tumora injicirana je subkutano u područje leđa hrčaka koji su nasumično svrstani u dvije skupine: 1) skupina u kojoj su hrčci pili vodu obogaćenu vodikom (HEW); 2) skupina u kojoj su hrčci pili deioniziranu vodu (DW); dok su 3) skupina zdrave životinje (kontrola). 25. dan nakon inokulacije mijeloidnog tumora žrtvovane su po tri životinje za procjenu učinka HEW na biometrijske parametre rasta tumora i 18 hematoloških parametara. Ostale životinje su ostavljene za praćenje veličine i brzine rasta tumora te ukupnog preživljenja. Serumi životinja analizirani su i spektralnim metodama neravnotežnog energetskog spektra (NES) i diferencijalnog neravnotežnog energetskog spektra (DNES). Rezultati su pokazali antitumorski učinak vode obogaćene vodikom kroz smanjenje stope rasta tumora, produljenje preživljenja te normalizaciju hematoloških parametara. 30. dan eksperimenta prosječna veličina tumora u skupini HEW je 16.7±4.7 mm, u usporedbi s 20.8±3.3 mm u skupini DW. Ukupna smrtnost životinja postignuta je 40. dan eksperimenta u skupini DW te 57. dan u skupini HEW. Dobiveni su statistički značajni rezultati učinka vode obogaćene vodikom na normalizaciju vrijednosti limfocita i trombocita u krvi te hematoloških indeksa (PLT/LR). Spektralnim metodama utvrđeno je statistički značajno povećanje energije između molekula vode u serumu hrčaka s mijeloičnim tumorom koji su pili HEW. Možemo zaključiti da voda obogaćena vodikom ima antikancerogeni potencijal koji bi se mogao koristiti s konvencionalnim kemoterapeuticima za razvoj novih strategija liječenja, kao i za prevenciju raka.

KLJUČNE RIJEČI: *voda obogaćena vodikom, mijeloidni tumor, krvni parametri, NES i DES spektralne analize*