Results obtained with Hydrogen-rich Water on the Experimental *Graffi* Tumor of Hamsters

Reneta Toshkova, Ignat Ignatov, Nikolai Neshev, Fabio Huether, Ivan Angushev

Abstract

This paper presents the results of an in vivo study of the antitumor effect of hydrogen-rich water in hamsters with experimental Graffi tumors. Hydrogen rich-water (HRW) with a molecular hydrogen (H2) concentration of 1.2 ppm, pH=7.3 and an oxidation reduction potential (ORP) of 390 mV as basic parameters was obtained with the EVObooster apparatus. The Graffi tumor was implanted subcutaneously on the back of Syrian Golden hamsters used for the experiment. The effect of Hydrogen-rich Water on biometric parameters of tumor growth, namely 19 hematological indices 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 Hydrogen-rich Water seen by a decrease in tumor uptake rate, a decrease in mortality, an increase in survival, a normalizing effect on hematological parameters and biomarkers (NLR, WBC/LR, PLT/LR), and an increase in energy between water molecules in blood serum in hamsters with Graffi's experimental myeloid tumor.

These results indicate that HRW can be administered as a safe adjuvant therapy in cancer treatment, alone or in combination with conventional chemotherapeutics.

Key words: hydrogren-rich water, *Graffi* tumor, NES and DNES spectral analyses

1. Introduction

Molecular hydrogen (H_2) is administered in practice by inhalation as a gas (1, 2), orally dissolved in water as hydrogen-rich water (HRW) (3, 4), and intravenously as hydrogen-rich saline [Y. Zhang, Q. Sun, B. He, J. Xiao, Z. Wang, X. Sun, Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion, Int. J. Int. Cardiol. 148 (1) (2011) 91-95].

Hydrogen-rich water has antioxidant properties against hydroxyl radical and hydrogen peroxynitrite [12, 13]. The active species of HRW are hydrogen molecules and atoms.

H_2 can penetrate cell membranes and reach subcellular components to protect nuclear mitochondria and DNA [7]. H_2 reduces oxidative stress and free radicals

with antioxidant action (8). It has anti-inflammatory effects (8, 9).
Studies illustrate the findings on the role of H2 and 2H+ in relation to oxidative stress (OS) and highlight the potential anticancer activity of this endogenous ion and different types of H2 donors (7).

The cell membrane conductance for hydrogen ions in healthy human cells is (-140 mV) and (-70 mV) (5) in cancer patients.

In cellular processes, the following reactions with H2 and H+ are valid (6):

$2H^+ + 2e^- \rightarrow H_2$ (1)

$H_2 \rightarrow 2H^+ + 2e^-$ (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 (10). 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 (11).

Hydrogen-rich water has antioxidant properties against hydroxyl radical and peroxynitrite of hydrogen (12, 13).

At first, Hydrogen rich-water (HRW) was obtained using the following chemical reaction (9):

$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$ (3)

Hydrogen rich-water (HRW) is also produced by the Polymer Electrolyte Membrane (PEM) (14).

For our research we used the EVObooster with Polymer electrolyte membrane (PEM) and additional activation for hydrogen water enrichment.

The aim of the present study was to investigate the effects of HRW as an antitumor agent in an experimental tumor model system in vivo, by evaluating tumor growth parameters, hematological parameters and indices, and hydrogen bonding parameters between water molecules in blood serum through spectral analysis.

2. Materials and Methods

2.1. Preparation and characterization of hydrogen-rich water (HRW)

2.2 Effect of oral administration of hydrogen-rich water on hamsters with experimental Graffi tumor

2.2.1. Experimental animals

Golden Syrian hamsters weighing 80-100 g, two months old and from both sexes were used for the experiments. The hamsters were supplied by the BAS Breeding facility, Kostinbrod, Bulgaria. The animals were kept in individual plastic cages in the vivarium of IEMPAM in accordance with the standard conditions adopted by the Bulgarian Veterinary Medical Control Service. Free access to food and water was ensured.

2.2.2 Experimental graphic tumor model.

Graffi's murine leukemia virus-induced myeloid tumor in newborn hamsters was adapted and maintained until adulthood in the Institute of Experimental Morphology, Pathology and Anthropology with Museum (IEMPAM) - Bulgarian Academy of Sciences (BAS) (Toshkova, 1995)(17). The tumor was maintained monthly in vivo by subcutaneous inoculation of 105 viable tumor cells. In the present experiment, 100% transplantability of the tumors was achieved by s.c. inoculation of 2x104 viable tumor cells into the interscalene field of the animals. Using this amount of cells, an inevitable 100% mortality of the animals was established in our previous studies (Toshkova, 1995).

2.2.3. Experimental design

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

The experimental animals were randomly divided into three groups (n =6 in each group) and treated as follows: (1) Graffi-TBH hamsters drinking hydrogen-rich water (HRW); (2) Graffi-TBH hamsters drinking deionized water (control) (DW) and (3) healthy hamsters without any treatment. Five days later, Graffi tumor cells (2 × 104) were aseptically obtained from tumor pieces, suspended in PBS (200 μl), and injected subcutaneously into the dorsal region of hamsters from groups 1 and 2. In experimental group 1 and 2, hamsters drank HRW and DW, respectively, starting 5 days before Graffi tumor cell engraftment and continuing for 25 days (daily) after transplantation. Each day HRW and DW were replaced with freshly prepared water. Redox potential was measured. Water with an ORP between -380 and -405 mV was used.

On day 25 of the experiment, hamsters from each group were randomly selected for analysis of hematological parameters, biomarkers and spectral analysis of blood sera. Tumor growth parameters were determined in the remaining hamsters of the groups.

2.2.4. Protective effect - Evaluation of biometric parameters

The efficacy of HRW therapy was assessed by tumor growth parameters (17, 18,19,20). Tumor appearance (transplantability) in hamsters was recorded daily by skin palpation at the injection site. Tumor size (mm) was measured at regular intervals using a digital caliper in two perpendicular dimensions. Survival and mortality rates were expressed as percentages. Median survival time was also determined. Tumor parameters were compared with those of controls.

2.2.5. Evaluation of hematological parameters and biomarkers

Blood samples of hamsters were collected on day 25 after tumor transplantation and divided into whole blood and serum for further analysis. Nineteen blood parameters were measured with a BC-2800 Vet machine blood cell analyzer (Mindray, China). Hematometric biomarkers/indexes reflecting the quantitative ratio of neutrophils and lymphocytes (NLR), leukocytes and lymphocytes (WBC/LR) and platelets/lymphocytes (PLT/LR) were calculated (determined)

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

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2.3. Statistical analysis

Student's t test was used to calculate the significance of differences between the means of specific parameters at the $\alpha = 0.01$ and 0.05 agreement levels, respectively.

2.4. Ethical aspects

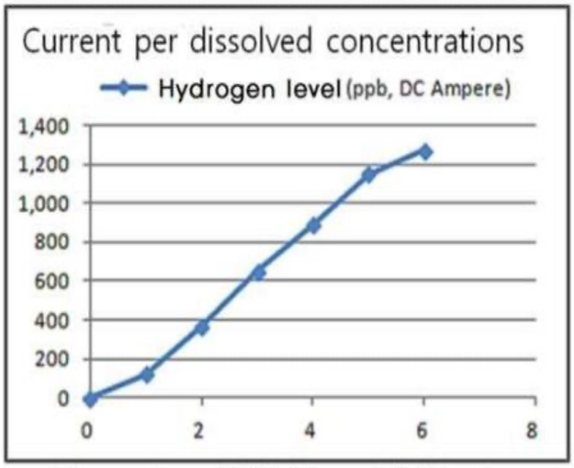
All experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (OJ L 222) and approved by the National Veterinary Service, Bulgaria.

3. Results

3.1. Parameters of hydrogen-rich water (HRW)

Hydrogen-rich water was prepared by a ...-minute electrolysis of deionized water. The characteristic parameters of the obtained HRW were as follows.

The EVOdrop hydrogen-rich water has a concentration of 1.2 ppm (Fig. 1)



The ORP decreased from +560 mV at the beginning of electrolysis to –270 mV after 30 minutes of electrolysis, indicating the intensification of the reducing power (Figure 1B?).

The pH evolved within a narrow physiological range of approximately 7.0 to 7.3 (Figure 1C?), compatible with the human body, in contrast to pH values above 8 that have been observed for most other hydrogen-rich water production devices.

3.2. Effect of oral administration of hydrogen-rich water on tumor-related parameters

The tumor model had 100% transplantability, 100% mortality and lack of spontaneous regression. Typically, after 7 to 15 days of inoculation, tumors developed as a solid subcutaneous formation approximately 5 mm in diameter. The tumor reached 5-6 cm in diameter and caused the hamster death at 30-35 days.

In the present experiments, tumor appearance was recorded by skin palpation on the hamster's back at the injection site daily from day 7 after the beginning of the experiment until the day the tumor was detected in all animals. The data are shown in Fig. 1.

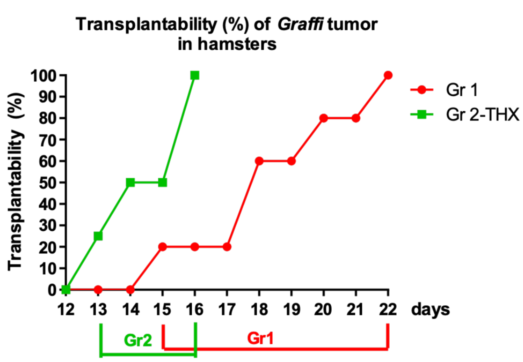


Fig. 1. Transplantability (%) of the Graffi tumor, in hamsters receiving H2-enriched water (Group 1) and hamsters receiving deionized water (Group 2 TNH-hamsters-control group)

In the control group of hamsters (Group 2) receiving deionized water, tumors appeared between days 13 and 16. In hamsters of group 1 receiving H2-enriched water (HRW), tumors were recorded between days 15 and 22.

Tumor compaction (1-2 mm tumor) was measured at regular intervals until day 30 after Graffi tumor cell transplantation. The average data for the two groups are presented graphically in Fig. 2**.**

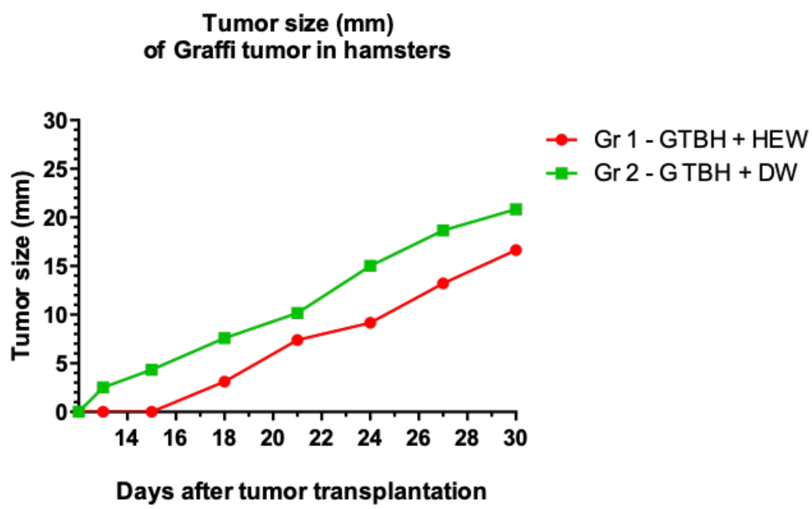
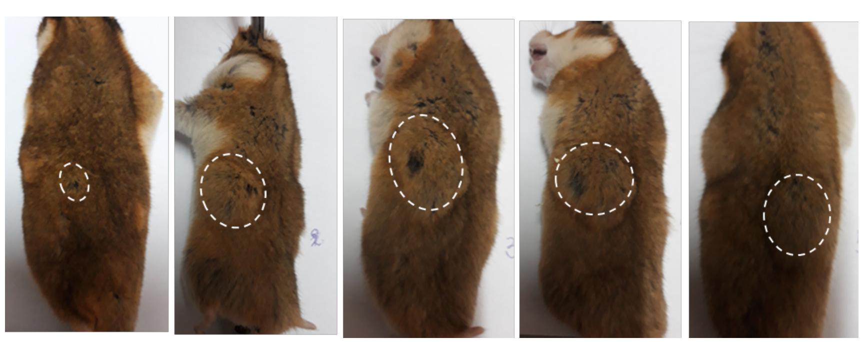


Fig. 2. Size (mm) of Graffi tumor, in hamsters receiving HRW (group. 1) and hamsters treated with deionized water (control group 2)

In the hamsters of the control group (group 2) (getting deionized water), the tumors grew to a size of about 20.8±3.3 mm, while in hamsters of group 1 (accepting HRW), the tumors reached a size of about 16.7±4.7 mm on day 30. The graph illustrates that in both groups the tumor progressively increased. The size in hamsters of group 1 receiving HRW was smaller, which can be explained by the therapeutic effect of the HRW used.
The photographic material shown in Fig. 3 supports the metric data obtained.



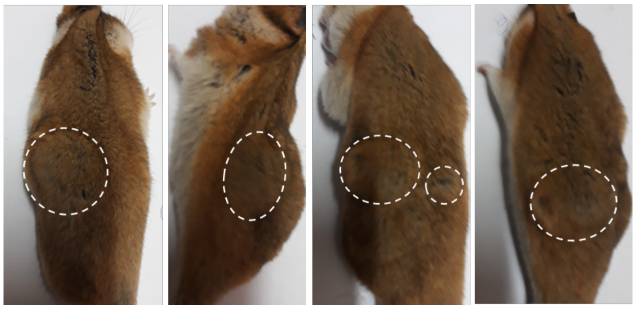


Fig. 3. Photographs of hamsters from Group 1 (first row) getting HEW. The second row is of control group 2 receiving deionized water. Photos are from day 24 after tumor transplantation. Tumors in each hamster are delineated with dotted lines.

Mortality data for the two experimental groups are presented in Fig. 4.

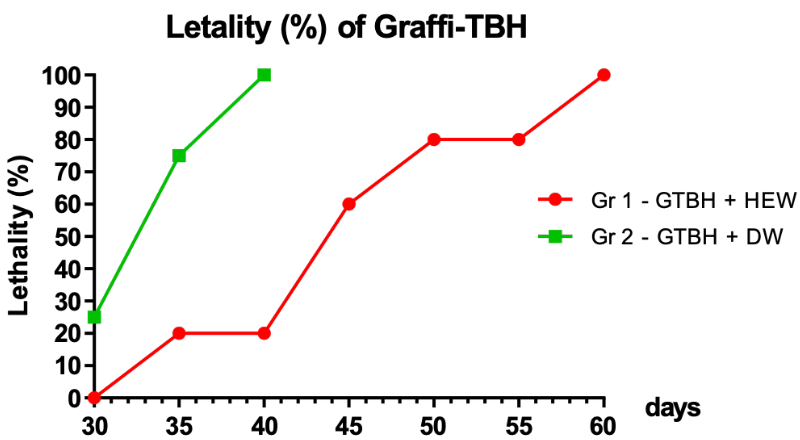


Fig. 4. Lethality (%) of Graffi tumor-bearing hamsters (GTBH) getting H_2-enriched water (Group 1) and hamsters receiving deionized water (Group 2 TNH-hamster-control group)

There was lower mortality in Group 1 hamsters getting H_2-enriched water (HEW) compared to Group 2 control hamster absorbing deionized water. In control group 2, a mortality of 100 was observed by day 40 after Graffi tumor cell transplantation. In group 1 hamsters, mortality was 20% at day 40 and increased to 100% at day 57. The 17-day difference in mortality indicates an effect of HEW.

A higher mean survival time (MST) was found for group 1 with a value of 46.4±8.1 days compared with control group 2 with a value of 35.0±4.1 days (Fig. 5).

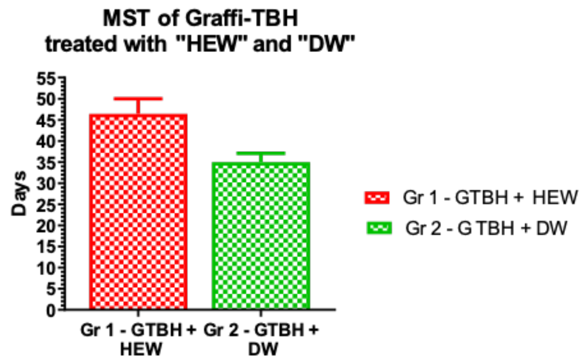


Fig. 5. Median survival time (in days) of Graffi tumor-bearing hamsters (GTBH) receiving HEW (Group 1) and Group 2 hamsters. The control group 2 consisted of hamsters receiving deionized water.

Higher individual survival was reported for Group 1 hamsters. Twenty percent (20%) of the hamsters survived 57 days, while control group 2 survived 40 days (Fig. 6).

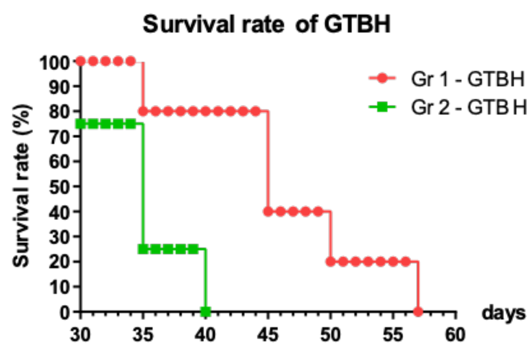


Fig. 6. Individual survival rate (in days) of Graffi tumor-bearing hamsters (GTBH) receiving H_2-enriched HEW (Group 1) and hamsters receiving deionized water (Group 2 - control)

3.3. Effect of hydrogen-rich water on blood values and blood indices

The concentration of circulating peripheral blood cells determined using a hematology analyzer is presented in Tab. 1.

Table 1 Blood values on day 25 of the experiments

Parameters	Units	Hamsters with Graffi tumor, drinking Deionized Water Group 2	Hamsters with Graffi tumor, drinking Hydrogen EVOdrop water Group 1	Healthy hamsters Group 3
WBC (Leukocytes)	$10^9.L^{-1}$	94.8	102.9	102.7
Lymph	$10^9.L^{-1}$	7.8	9.8	9.5
Mon	$10^9.L^{-1}$	2.2	2.4	2.3
Gran	$10^9.L^{-1}$	84.8	90.7	85.1
Limph	%	8.2	9.5	8.5
Mon	%	2.3	2.4	2.3
Gran	%	89.5	88.1	88.3
RBC (Erythrocytes)	$10^9.L^{-1}$	5.40	5.16	5.03
HGB (Hemoglobin)	$g.L^{-1}$	279	269	265
HCT (Hematocrit)	$L. L^{-1}$	0.291	0.273	0.282
MCV (Mean red blood cell volume)	fL	53.9	53.1	55.8
MCH (Average HGB content in erythr)	pg	51.6	50.7	53.2
MCHC (mean con cog Hb)	$g.L^{-1}$	958	959	947
RDW	%	13.5	13.1	12.7
PLT (Platelets)	$10^9.L^{-1}$	315	122	117
MPV(means volume platelets)	$f.L^{-1}$	6.1	6.3	7.4

PDW	**%**	**19.6**	**18.6**	**18.5**
PCT	**%**	**0.192**	**0.076**	**0.071**

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 and 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 [10], while higher platelet concentration increases the risk of lethal thromboses [32]. Thus, also in clinical practice, some basic hematometric indices, NLR,WBC/LR, PLT/LR are used as diagnostic and prognostic tools in patients with cancer and leukemia (9,10,11). For example, quantitative neutrophil to lymphocyte ratio - NLR index is important for predicting higher survival of cancer patients. NLR values ≤ 2.0 have been associated with higher survival for breast cancer patients, and NLR ≥ 5.0 has been associated with lower survival for patients with breast cancer, gastric cancer, etc. (12,13,14). In recent years, new results have been reported for WBC/Lymph (30), PLT/Lymph (30), and Neut/Lymph (31).
The results in Table 1 show about 3-fold higher platelet values in group 2 hamsters (drinking deionized water) compared with those in healthy hamsters (group 3) and group 1 hamsters (drinking HEW). Statistically significant differences were found between WBC, PLT, PDW and PCT blood parameters values of hamsters from g.1 and g.2. The data are presented in Table 2.

Table 2. Blood parameters for Group 1 (Hydrogen-rich EVOdrop water for hamsters with Graffi tumor) and Group 2 (Deionized water for hamsters with Graffi tumor)

Parameter	**WBC**	**PLT**	**PDW %**	**PCT %**
α	**0.01**	**0.01**	**0.05**	**0.01**

The statistical significance of the difference in means for three of these indicators is high: 99% and for one it is 95%.

In our experiments, the WBC/Lymph index was increased in hamsters of group 2 (drinking deionized water) and had a value of 12. In hamsters of group 1 (drinking HEW) it was 10.5, and this value was close to that reported in group 3 (healthy hamsters) -10.8. A similar correlation was found for the Neutrophil/Lymph index, which in g.2 had a value of 10.9, and in hamsters of group 1 and group 3 similar values were reported, 9.3 and 9.0, respectively. A similar relationship was also observed for the third PLT/Lymph index we monitored. The highest values were recorded in the hamsters of gr.2, 40.4, while in the hamsters of group 1 and group 3 similar values were recorded -12.4 and 12.3, respectively.

3.4 Effect of hydrogen-rich water on serum parameters obtained with spectral methods NES and DNES

The established findings for the effects of hydrogen Evodrop water (HEW) in the sera of Graffi tumor bearing hamsters were as follow:
DNES of Group 1 (GTBH, drinking HRW) and Group 2 (GTBH drinking DW) were (-0.1236)-(0.1201) =-0.1236+0.1201 eV= (-3.5) meV . The result was reliable.
DNES of Group 1 and Group 3 (Healthy hamsters) were (-0.1236)-(-0.1228) =-0.1236+0.1228 eV= (-0.8) meV. The result was not reliable
This led to a very important conclusion, that Hydrogen Evodrop water (HEW) stabilized the hydrogen bonds in the blood serum of TBH drinking HRW (Gr.1 to healthy state).

Table 3 and figure 7 illustrate the results of blood sera from experimental and control hamsters obtained with NES and DNES spectral methods.

Table 3 Blood serum results of hamsters with NES and DNES spectral methods.

The established results for the effects of hydrogen Evodrop water (HEW) in the sera of *Graffi* tumor-bearing hamsters were as follow:
DNES of Group 1 (GTBH, drinking HRW) and Group 2 (GTBH drinking DW) were (-0.1236)-(0.1201) =-0.1236+0.1201 eV= (-3.5) meV. The result was reliable.
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Table 3 Results of blood serum of hamsters with NES and DNES spectral methods

-E(eV) x-axis	f(eV^{-1}) Hydrogen EVOdrop water in Hamsters with Graffi tumor Group 1	f(eV^{-1}) Deionized water in Hamsters with Graffi tumor Group 2	f(eV^{-1}) Healthy hamsters	-E(eV) x-axis	f(eV^{-1}) Hydrogen EVOdrop water in Hamsters with Graffi tumor Group 1	f(eV^{-1}) Deionized water in Hamsters with Graffi tumor Group 2	f(eV^{-1}) Healthy hamsters
0.0912	0	0	0	0.1162	23.5	46.1	0

0.0937	0	0	0	0.1187	23.5	15.5	0
0.0962	0	0	0	0.1212	**70.6**[2]	**30.8**[2]	**66.7**[1]
0.0987	0	0	0	0.1237	23.5	30.8	33.3
0.1012	0	0	0	0.1262	0	30.8	33.3
0.1037	0	61.5	0	0.1287	47.1	0	0
0.1062	23.5	0	66.7	0.1312	23.5	30.8	33.7
0.1087	30.8	30.8	0	0.1337	23.5	46.1	33.7
0.1112	**0**[1]	**30.8**[1]	**0**[1]	0.1362	11.8	15.5	16.7
0.1137	47.1	0	66.7	0.1387	**58.8**[3]	**30.8**[3]	**50.3**[1]

Blue line – Hamsters with Graffi tumor and Evodrop hydrogen water
Red line – Hamsters with Graffi tumor
Green line – Healthy hamsters

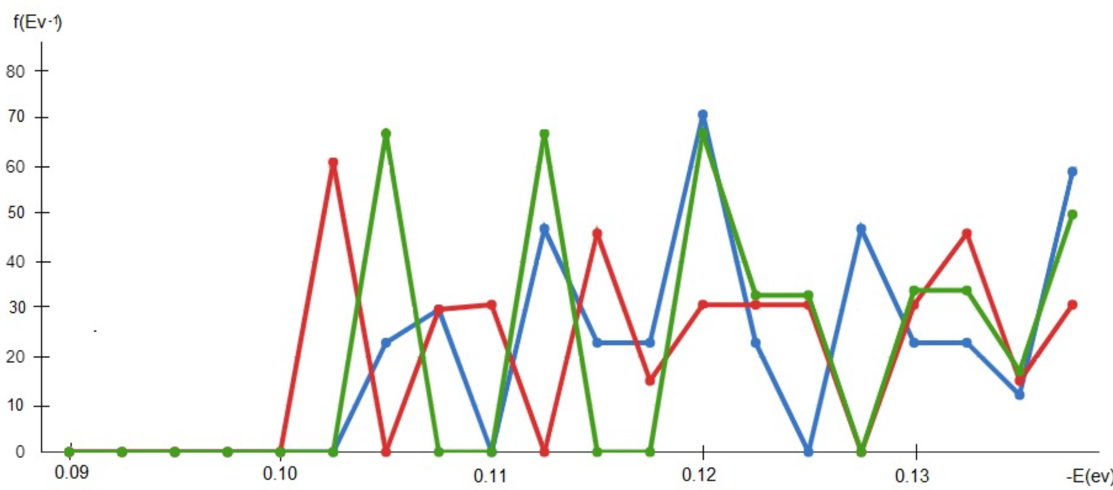

Fig. 7 Results of blood serum of hamsters with NES and DNES spectral methods

The research was performed with blood sera from three experimental groups with the NES and DNES methods. What is very important was that for E=-0.1212 eV the distribution function of energies f in eV^{-1} was similar for healthy hamsters and hamsters, which drank HEW. For the Gr.1 was 70.6 and for the Gr.3 was 66.7 eV^{-1}. For the Gr.2 (control) was 30.8 eV^{-1}.

The statistical significance of the difference in means between Groups 1 and 2 is high. In Student's t-test it was $p<0.01$ for 10 measurements. It is very important to note that for E=-0.1387 eV the distribution function of energies f in eV^{-1} was similar. For the first group it was 58.8 and for the third group it was 50.3 eV^{-1}. For the control group was it 30.8 eV^{-1}.

The statistical significance of the difference in means between Groups 1 and 2 is high. In Student's t-test it was p<0.01 for 10 measurements.

4. Conclusions

The following conclusions can be drawn from the application of (H2)-enriched water obtained with the EVOdrop booster as an antitumor agent.

1. Daily HEW ingestion for 35 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) (Fig. 1),
- A reduction in the tumor growth rate (Fig. 2 and Fig. 3),
- A decrease in the mortality rate (Fig. 4)
- An increase in the average (Fig. 5) and individual (Fig. 6) survival of optic hamsters with Graffi's experimental myeloid tumor.
2. Statistically significant results were obtained for the normalizing effect on PLT and WBC blood indices and on hematological indices- NLR, 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 blood serum of hamsters with Graffi tumor drinking Hydrogen EVOdrop Water.
4. The positive effects observed in hamsters with experimental Graffi tumor may be explained by a therapeutic antitumor effect of H2-enriched water (HEW).

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