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COMPARATIVE INVESTIGATION EFFECTS ON NERVOUS AND IMMUNE SYSTEMS OF BIOACTIVE WATER NAFTUSSYA SPA TRUSKAVETS' AND STABLE WATER SOLUTION OF BORYSLAV'S OZOKERITE

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Abstract

Background. It is known that biological activity of curative water Naftussya spa Truskavets' (Ukraine) caused by its organic substances, genetically connected with Oil and Ozokerite from Boryslav's layer, located near from Truskavets'. The low stability of water Naftussya defined our trying to reproduce its natural receiving in laboratory conditions. **Aim:** compare effects on nervous and immune systems of natural bioactive water Naftussya (BAWN) spa Truskavets' and stable water solution of the Ozokerite, extracted from the Boryslav's field. **Material and research methods.** The clinical approbation involved 27 volunteers with dysfunction of immune and autonomous nervous system. Immune status evaluated on a set of I and II levels recommended by the WHO. About phagocytic function of neutrophils judged by activity, intensity and completeness phagocytose of Staphylococcus aureus and Escherichia coli. About state of autonomous and central nervous system judged by parameters of Heart Rate Variability ("CardioLab+HRV") and Electroencephalogram ("NeuroCom Standard"). After testing 20 volunteers drank BAWN while 7 others volunteers simultaneously drank Ozokerite extract during 7 days. Daily entering of organic substances (as C organic) for both groups makes 0,3 mg/kg. **Results.** By method of discriminant analysis we detected 7 immune and 3 neural variables changes in which are almost identical in both groups. In particular, increases reduced levels Bacterocidity against Staph. aur., Killing Index against E. coli, Circulating Immune Complexes and Asymmetry of δ -Rhythm as well as normal levels Entropy of Immunocytogram and Immunity Integral Index while decreases raised levels Sympathetic tone, Centralization Index HRV and IgM of serum. Among variables currently not in the model it is necessary to note increasing reduced levels Bacterocidity against E. coli and Vagal tone as well as normal levels Microbial Count for Staph. aur., "active" T-Lymphocytes and Deviation α -Rhythm Frequency while decreasing raised levels Bayevskiy's Stress Index and Activity Regulatory Systems Index HRV. **Conclusion.** Course drinking of stable water solution of the Ozokerite, extracted from the

Boryslav's field, imitates favourable effects of bioactive water Naftussya spa Truskavets' on parameters of Immune and Autonomous Nervous systems at volunteers with their disfunction.

Keywords: bioactive water Naftussya, Ozokerite, Immune and Autonomous Nervous systems.

INTRODUCTION

It is known that curative waters Naftussya spa Truskavets' and Skhidnytsya (Ukraine) specifically inhibites activity of Na,K-ATPase of enteral epithelium at rats in vivo as well as in vitro. This inhibitory effect, accepted as marker of biological activity, turned out highly labil and connected with contents in water carboxylic acids as well as autochtonous microbes, which significantly changed during storage by contact with oxygen. On the other hand, inhibitory effect reproduced by obtained from water Naftussya carboxylic acids (C₁₆-C₂₄) but not bitums. In biotechnological experiment have been shown that carboxylic acids produced during cultivation sowed from water Naftussya hydrocarbonoxydating microbes in medium contained water-bearing dirt as well as Oil (Naphta in Greek) or Ozokerite Boryslav's layer, located near (5-7 km) from Truskavets' [11,15,42]. It is shown that organic substances as well as aucthone microbes containing in water Naftussya causes (probably through macrophages of gat mucosa) modulating effects on neuroendorine-immune complex at healthy rats as well as patients with various chronic diseases [3,17,18,23,25,27-32,35,40]. It is necessary note data about stresslimiting and immunomodulating effects of Ozokerite applications (probably through macrophages of skin) on neuroendocrine-immune complex at rats [12,13].

The low stability of biologically active water Naftusya defined our trying to reproduce its natural receiving in laboratory conditions. In laboratory of the scientific company "Verba" using unique nanotechnology stable water solution of the Ozokerite, extracted from the Boryslav's field has been received. The conducted researches were productive and have confirmed preserving the properties lost by natural water Naftussya and creations of its imitation. At the same time the origin conception of our investigations is the method of analogies and similarities.

MATERIAL AND RESEARCH METHODS

The following methods of the chemical analysis have been applied to determination of the organic substances entering the received concentrate: Luminescence, Extraction, Chromatography and Spectroscopy [22]. The general necessary initial indicator – gross content of organic carbon has been determined by method of dry burning. It was determined that gross constant of organic carbon is 10800 mg/dm³ or 32 000 mg/100g. The constant of organic nitrogen is 2% from C org. [2,41]. Freeze drying was applied to release of polar substances[20].

The following classes of OS are identified: Oxygen-containing combinations (acids, ketones, aldehydes, ethers); High molecular alcohols; Aromatic and nitrogen-containing connections; Connections with unsaturated bonds [5].

2/3 oil products (alkanes, monoolefita, dienes and monocycloolefins, alkylbenzene, alkenylbenzene, polyaromatic hydrocarbons, alkyl-naphthalenes, sulfur-containing connections), a 1/3 – products of processing of oil products microbes (carboxylic acids, sulfur-containing connections, esters of aromatic acids, alkyl phenols). The maintenance of the listed OS in a concentrate makes 240 mg/L, and paraffin is 800mg/L. Carboxylic acids – 20 mg/dm³ (formic,

oil, acetic, kapron, valerian, palmitic, stearin). Natural air of high molecular weight acids due to the lack of standards did not manage to be identified.

For comparison we adduce data by OR Dats'ko et al [6] about organic compounds (in mg/L) water Naftussya obtained by Solid Phase Extraction method and mass-spectroscopy by using as Sorbents Tenacle GC 60/80 and Polysorb-2. Paraffins 4,10 and 4,20; monoolefins 1,67 and 1,75; dienes and monocycloolefins 0,84 and 0,85; alkylbenzene 1,55 and 1,54; alkenylbenzene 0,47 and 0,46; esters of aromatic acids 1,32 and 1,33; alkyl phenols 1,14 and 1,14; polyaromatic hydrocarbons 0,077 and 0,059; oxygene-containing connections (acids) 1,12 and 1,14; sulfur-containing connections 0,30 and 0,31; alkyl naphthalenes 0,53 and 0,53; unidentified polyaromatic hydrocarbons 0,19 and 0,19; connections required subsequent identification 0,48 and 0,50 correspondingly. Early have been shown that detected in Naftussya phenols (0,5-4,1 µg/L) comed from falled leaves [14].

Long ago known favourable effects balneotherapy on spa Truskavets' on diuresis, urinary excretion of nitrous metabolites and electrolytes, choleresis and cholekinetics, gastric and pancreatic secretion as well as inflammation in urinary and digestive systems [4,8,15,31,42] according to contemporary notions [17,29,31] may be the result of modulation of neuroendocrine-immune complex. IL Popovych [29] advanced conception about stresslimiting adaptogene mechanism of biological and curative activity of Water Naftussya that including participation of nervous, endocrine and immune systems closely interacting in the bounds of neuroendocrine-immune complex [18,24,26,33,34]. Based on this conception we enlisted in investigation persons with disfunction of immune and autonomous nervous systems.

The clinical approbation involved 27 volunteers – 14 women (body mass average 71,5 kg, SD 10,0 kg, aged average 50 yrs, SD 15,8 yrs) and 13 men (body mass average 85 kg, SD 7,7 kg, aged average 53,6 yrs, SD 10,7 yrs). In the morning on an empty stomach in portion of blood counted up leukocytogram. Immune status evaluated on a set of I and II levels recommended by the WHO [10]. For phenotyping subpopulations of lymphocytes used the methods of rosette formation with sheep erythrocytes on which adsorbed monoclonal antibodies against receptors CD3, CD4, CD8, CD22 and CD16 from company “Granum” (Kharkiv, Ukraine) with visualization under light microscope with immersion system. We carried out also test of “active” rosette formation [19,23]. The Entropy (h) of Immunocytogram calculated using formula Shannon [cit. by: 29]:

$$h = - [\text{CD4} \cdot \log_2 \text{CD4} + \text{CD8} \cdot \log_2 \text{CD8} + \text{CD16} \cdot \log_2 \text{CD16} + \text{CD22} \cdot \log_2 \text{CD22}] / \log_2 4$$

The state of humoral immunity judged by the concentration in serum immunoglobulins classes G, A, M (ELISA, analyser “Immunochem”, USA) and circulating immune complexes (with polyethylene glycol precipitation method) [19]. About phagocytic function of neutrophils judged by activity (percentage of neutrophils, in which found microbes - Phagocytic Index), intensity (number of microbes absorbed one phagocytes - Microbial Count) and completeness (percentage of dead microbes among absorbed - Killing Index) phagocytosis of museum cultures *Staphylococcus aureus* (ATCC N 25423 F49) and *Escherichia coli* (O55 K59) obtained from chemical-bacteriological laboratory of Hydrogeological Regime-Operational Station spa Truskavets' (Ukraine) [7,25]. The integral state of phagocytic function of neutrophils evaluated as amount of microbes killed by neutrophils containing in 1L of blood, named as Bacterocidity and calculated using formula [31]:

$$\text{Bacterocidity} = \text{Leukocytes}(10^9/l) \cdot \text{Neutroph.}(\%) \cdot \text{Phagocyt. Ind}(\%) \cdot \text{Microb. Count} \cdot \text{Killing Ind.}(\%)$$

For each immune and HRV parameter each volunteer we calculated Z-score using formula:

$$Z = (x - X) / \sigma = (x - X) / X \cdot C_v, \text{ where}$$

x is individual level, X is norm level, C_v is coefficient of variation at norm.

The integral state of immunity evaluated as mean Z-score for 11 immune parameters.

After immune tests we recorded ECG (seated) for 7 min and simultaneously performed with four 25-second recording EEG. ECG recorded hardware-software complex "CardioLab+HRV" produced "KhAI-MEDICA" (Kharkiv, Ukraine) in standard lead II. EEG recorded a hardware-software complex "NeuroCom Standard" (KhAI Medica, Kharkiv, Ukraine) monopolar in 16 loci by 10-20 international system, with the reference electrodes A and Ref tassels on the ears [33,34].

For further analysis the following parameters heart rate variability (HRV) were selected. Temporal parameters (Time Domain Methods): the standart deviation of all NN intervals (SDNN), the square root of the mean of the sum of the squares of differences between adjacent NN intervals (RMSSD), the percent of interval differences of successive NN intervals greater then 50 ms (pNN_{50}) [9]; heart rate (HR), moda (Mo), the amplitude of moda (AMo), variational sweep (MxDMn) [1]. Spectral parameters (Frequency Domain Methods): absolute (in ms^2) and relative (% of total) power spectrum (PS) components of HRV - high-frequency (HF, range $0,4\div 0,15$ Hz), low frequency (LF, range $0,15\div 0,04$ Hz), very low frequency (VLF, range $0,04\div 0,015$ Hz) and ultra low frequency (ULF, range $0,015\div 0,003$ Hz). Expectant classical indexes: LF/HF, $LFnu=100\% \cdot LF/(LF+HF)$ as well as Centralization Index ($CI=(VLF+LF)/HF$), Baevskiy's Stress Index ($SI=AMo/2 \cdot Mo \cdot MxDMn$) and Activity Ragulatory Systems Index [1]. We calculated Z-score for each HRV parameter each volunteer too. Among the options EEG considered the average amplitude (μV), average frequency (Hz), frequency deviation (Hz), index (%) and coefficient of asymmetry (%) of basic rhythms: β ($35\div 13$ Hz), α ($13\div 8$ Hz), θ ($8\div 4$ Hz) and δ ($4\div 0,5$ Hz).

After first testing 20 volunteers drank bioactive water Naftussya (BAWN) while 7 others volunteers simultaneously drank Ozokerite extract during 7 days and then all tests repeated. The calculation demonstrate that daily entering of organic substances (as C organic) with bioactive water Naftussya makes 0,3 mg/kg (bolus of BAWN $3,2$ mL/kg $\cdot 3$ time $\cdot 0,03$ mg/mL). The entering equal amount organic substances with Ozokerite extract achieved by usage 2 mL of it (contained 21,6 mg) dissolved in sweet water (2,5-2,6 mL/kg) one time daily. Early we infused in stomach of rats aqueous solution of organic substances extracted from BAWN in dose 0,4 mg/kg equally to obtained by drink ad libitum natural BAWN in dose 35 mL/kg [29].

For statistical analysis using the software package "Statistica 5.5".

RESULTS AND DISCUSSION

In order to identify the parameter which changes are almost equal in both groups we applied discriminant method (forward stepwise [16]). Among all registered parameter we selected 7 immune and 3 neural variables (Table 1 and 2).

Z-scores testify that maximal favourable changes takes place for initial reduced levels Bacterocidity against Staph. aureus and Circulating Immune Complexes which increased to norm while initial more reduced Killing Index against E. coli do not reached zone of norm. Entropy of Immunocytogram and Immunity Integral Index moderately increased in limits of norm. The increase Asymmetry of δ -Rhythm probably reflects increase HRV markers of vagal tone because we early detected relationships between its and SDNN ($r=0,43$) as well as HF ($r=0,38$) [34]. On the other hand decreased initial raised levels Sympathetic tone and Centralization Index HRV, thus take place favourable changes in autonomous nervous system.

Table 1. Discriminant Function Analysis Summary. Variables currently in the model
Grouping: 2 grps. Wilks' Lambda: 0,480; approx. $F_{(10,4)}=4,65$; $p=0,0002$

Variables currently in the model	Wilks' Λ	Partial Λ	F remove	p-level	Tolerance	Z-scores for Variables		
						Before treatment	After 7 days	Change
Bacterocidity against Staph. aur.	,481	,999	0,04	,84	,393	-1,23±0,39	+0,23±0,33	+1,46±0,41
Killing Index against E. coli	,482	,997	0,12	,73	,630	-4,71±0,31	-3,74±0,26	+0,97±0,37
Asymmetry of δ -Rhythm	,558	,861	6,95	,01	,774	0,00±0,19	+0,67±0,24	+0,67±0,28
Circulating Immune Complexes	,496	,968	1,44	,24	,673	-0,78±0,13	-0,21±0,13	+0,57±0,12
Immunity Integral Index-11	,546	,880	5,85	,02	,146	-0,10±0,13	+0,33±0,12	+0,43±0,08
Entropy of Immunocytogram	,533	,901	4,73	,03	,323	+0,35±0,18	+0,58±0,21	+0,23±0,14
LFnorm HRV	,518	,927	3,37	,07	,354	+1,01±0,21	+0,70±0,22	-0,31±0,19
IgA of serum	,526	,913	4,11	,05	,363	+0,55±0,21	+0,15±0,22	-0,40±0,09
IgM of serum	,559	,859	7,08	,01	,766	+1,52±0,17	+1,10±0,13	-0,42±0,16
(VLF+LF)/HF Ratio HRV	,494	,973	1,21	,28	,336	+4,63±1,15	+3,39±1,03	-1,24±0,89

Table 2. Summary of Stepwise Analysis

Variables currently in the model	F to enter	p-level	Λ	F value	p-level	Means for Variables		
						Before	After 7 day	Change
Circulating Imm. Comp., un.	9,44	,003	,846	9,4	,003	31±2	41±2	+10±2
Bacterocid. vs St. aur, 10^9 B/L	9,35	,004	,715	10,2	10^{-3}	89±4	104±3	+15±4
Asymmetry of δ -Rhythm, %	5,17	,027	,648	9,0	10^{-4}	40±5	57±6	+17±7
IgM of serum, g/L	3,45	,069	,606	8,0	10^{-4}	1,57±0,05	1,45±0,04	-0,12±0,04
Killing Ind. against E. coli, %	1,96	,168	,582	6,9	10^{-4}	39,2±1,5	43,9±1,2	+4,7±1,8
Entropy of Immunocytogram	1,54	,220	,563	6,1	10^{-4}	0,971±0,006	0,979±0,007	0,008±0,005
Immunity Integral Index, Z	1,53	,222	,545	5,5	10^{-3}	-0,10±0,13	+0,33±0,12	+0,43±0,08
IgA of serum, g/L	2,32	,134	,519	5,2	10^{-3}	2,05±0,06	1,92±0,07	-0,12±0,03
LFnorm HRV, %	2,21	,144	,494	5,0	10^{-3}	76,9±2,7	73,0±2,8	-4,0±2,4
(VLF+LF)/HF Ratio HRV	1,21	,278	,480	4,7	10^{-3}	18,9±3,4	15,3±3,0	-3,6±2,6
Squared Mahalanobis Distance between initial and final state:								
4,33; $F_{(10,4)}=4,65$; $p=0,0002$								
Chi-Square Tests with Successive Roots Removed	Eigenvalue	r^*	Wilks Λ	χ^2	df	p-level		
	1,082	0,72	0,480	34,5	10	10^{-4}		

Decrease of serum IgM is also favourable because reflects its trend to normalization while decrease of serum IgA is at least neutral because occurs in limits of norm.

Our data concorded with conception about antiinflammatory effects of vagal innervation [21,36-39].

Among variables currently not in the model (Table 3) it is necessary to note increasing reduced levels Bacterocidity against E. coli and Vagal tone (according to increase Asymmetry of δ -Rhythm!) as well as increasing normal levels Microbial Count for Staph. aureus and "Active" T-Lymphocytes while decreasing raised levels Bayevskiy's Stress Index and Activity Regulatory Systems Index HRV.

The interpretation increase Deviation α -Rhythm Frequency is still difficult for us.

Table 3. Discriminant Function Analysis Summary. Variables currently not in the model

Variables currently not in the model (Df for all F-tests: 1,42)	Wilks' Λ	Partial Λ	F-enter	p-level	Tolerance	Z-scores for Variables		
						Before treatment	After 7 days	Change
Bacterocidity against E. coli	,480	,999	,014	,90	,229	-1,28±0,52	+0,47±0,39	+1,75±0,40
Microbial Count for Staph. aur	,480	1,00	,002	,96	,610	-0,17±0,28	+0,69±0,32	+0,85±0,27
PS HF HRV Relative	,480	,999	,009	,93	,260	-1,78±0,53	-0,97±0,66	+0,81±0,43
Deviation α -Rhythm Frequency	,480	,999	,030	,86	,739	0,00±0,19	+0,58±0,28	+0,58±0,29
“Active” T-Lymphocytes level	,479	,996	,143	,71	,567	-0,21±0,19	+0,21±0,16	+0,41±0,12
Bayevskiy's Stress Index HRV	,473	,984	,674	,42	,689	+0,71±0,61	+0,15±0,35	-0,56±0,37
						Mean for Variable		
Activity Regul Syst Ind (N: 0÷3)	,479	,998	,070	,79	,881	3,7±0,6 un.	2,5±0,4 un.	-1,2±0,5 un.

The discriminant Neuroimmune information is condensed in one Canonical Root that correlated negatively with variables which increased while positively with decreased variables. The calculation of values of Unstandardized Canonical Scores of Neuroimmune Root by summation the multiplications of individual variables on the Raw Coefficients for Canonical Variables plus Constant (Table 4) allows visualization state of volunteers on the plane (Fig. 1).

Table 4. Standardized, Structural and Raw Coefficients and Constant for Canonical Variables

Variables currently in the model	Coefficients for Canonical Variables		
	Standardized	Structural	Raw
Circulating Immune Complexes	-0,304	-0,410	-0,0255
Bacterocidity against Staph. aureus	-0,068	-0,380	-0,0036
Immunity Integral Index-11	-1,255	-0,330	-1,960
Killing Index against E. coli	-0,093	-0,324	-0,0131
Asymmetry of δ -Rhythm	-0,588	-0,291	-0,0205
Entropy of Immunocytogram	0,769	-0,109	21,94
IgM of serum	0,596	0,263	2,767
IgA of serum	0,680	0,176	1,965
LFnorm HRV	0,629	0,135	0,0438
Centralization Index of HRV	-0,395	0,107	-0,0238
		Constant	-29,31

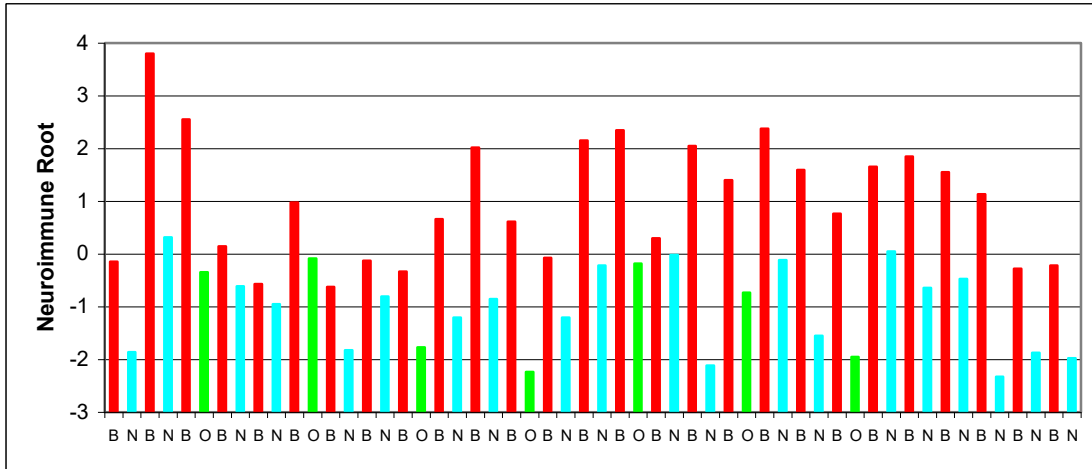


Figure 1. Unstandardized Individual Canonical Scores of Neuroimmune Root before (B) and after treatment by Naftussya (N) or Extract of Ozokerite (O) (see also Table 4)

Means of Unstandardized Canonical Scores of Neuroimmune Root testifies that relatively at least 10 Neuroimmune parameters effects both bioactive water Naftussya and Extract of Ozokerite are equal (Fig. 2).

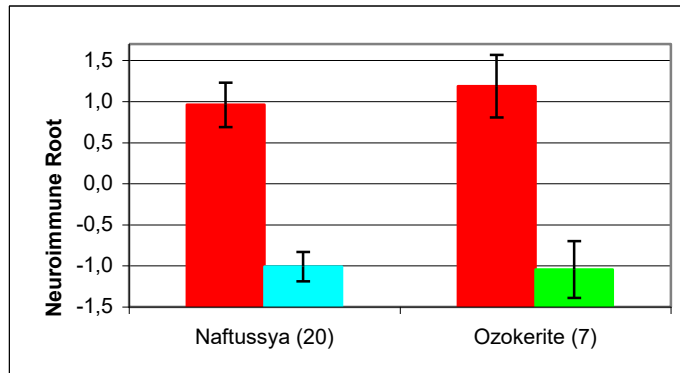


Figure 2. Means of Unstandardized Canonical Scores of Neuroimmune Root before and after treatment by Water Naftussya or Extract of Ozokerite (see also Table 4)

Table 4. Unstandardized Individual Canonical Scores of Neuroimmune Root

Volunteer	Group	Root 1
1	Baseline	-0,14
	Naftussya	-1,86
2	Baseline	3,80
	Naftussya	0,32
3	Baseline	2,55
	Ozokerite	-0,34
4	Baseline	0,15
	Naftussya	-0,61
5	Baseline	-0,57
	Naftussya	-0,95
6	Baseline	0,97
	Ozokerite	-0,08
7	Baseline	-0,63
	Naftussya	-1,82
8	Baseline	-0,12
	Naftussya	-0,80
9	Baseline	-0,34
	Ozokerite	-1,77
10	Baseline	0,66
	Naftussya	-1,20
11	Baseline	2,02
	Naftussya	-0,85
12	Baseline	0,61
	Ozokerite	-2,24
13	Baseline	-0,07
	Naftussya	-1,21
14	Baseline	2,15
	Naftussya	-0,22
15	Baseline	2,35
	Ozokerite	-0,18
16	Baseline	0,30
	Naftussya	-0,02
17	Baseline	2,05
	Naftussya	-2,12
18	Baseline	1,40
	Ozokerite	-0,73
19	Baseline	2,37
	Naftussya	-0,12
20	Baseline	1,60
	Naftussya	-1,55
21	Baseline	0,76
	Ozokerite	-1,95
22	Baseline	1,66
	Naftussya	0,05
23	Baseline	1,85
	Naftussya	-0,64
24	Baseline	1,55
	Naftussya	-0,47
25	Baseline	1,13
	Naftussya	-2,33
26	Baseline	0,28
	Naftussya	-1,87
27	Baseline	-0,22
	Naftussya	-1,97

Thus there are all bases to assume that the received original preparation will allow to arrange the industrial production of a bottle stable imitation of the bioactive water Naftussya and will give the chance to use its unique properties to the people who are far from the spa Truskavets.

This study was approved by the local ethical committee of Truskavets' Scientists Assotiation and was accordance with the declaration of Helsinki. Written informed consent was obtained from all volunteers. For all authors any conflict of interests is absent.

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