Research article

Mineral waters from Spring 1 and Spring 1 bis from Slănic Moldova - molecular mechanisms responsible for triggering the prophylactic and therapeutic effects

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Abstract: In this study, we investigated mineral water’s therapeutic and prophylactic effects from springs 1 and 1 bis in Slănic Moldova, focusing on identifying the molecular mechanisms responsible for these effects. We collected water samples from these springs and analyzed their chemical composition using various analytical techniques. In addition, we tested the effects of water on cell viability using primary fibroblasts in culture and performed MTT assays to assess the metabolic activity of the cells. Our results indicate that water from both springs has beneficial properties on cells, including improving cell viability and stimulating metabolic activity. This suggests that the mineral water from springs 1 and 1 bis could have therapeutic and prophylactic potential due to its unique chemical composition. Our study could contribute to developing new mineral water-based therapies for various health conditions.

Keywords: fibroblasts, aqueous sulfurous mineral waters, cytokine, factor of necrosis tumor (TNF)-α and interleukine (IL)-1β and IL-6, chemokine, cyclooxygenase (COX)-2

1. Introduction

Biological investigations conducted at the cellular and molecular levels provide insights that support existing concepts regarding the action mechanisms of natural therapeutic factors (1–5). These studies will be carried out through electrophoresis, ELISA, and Western blotting on primary fibroblast cultures derived from Wistar rats. Specifically, these investigations target two primary physiological mechanisms: inflammatory processes and oxidative stress balance (6–9). These are hypothesized to be influenced by therapeutic mud and natural sulfurous mineral waters, constituting the fundamental biological level from which scientific reasoning can be built for the therapeutic effects of mud therapy and sulfurous mineral waters (10–16).

Mineral water is a valuable natural resource, renowned for its healing properties for centuries (17–19). Furthermore, the mineral water from Slănic Moldova has been
particularly admired for its unique properties, boasting a rich chemical composition that includes various ions and minerals beneficial to health (20–23).

This study aims to address this gap in our knowledge by investigating how the water from springs 1 and 1 bis in Slanic Moldova affects health at the molecular level. More specifically, we focused on assessing the effects of these waters on cellular viability and metabolic activity, using primary fibroblasts as a cellular model. The findings from this study could have significant implications, not only for understanding the healing properties of mineral water but also for developing new therapeutic strategies based on these natural resources.

2. Materials and methods

Biochemical analysis spectroscopic methods measure the concentrations or activities of particular chemical substances within biological samples (24).

Determination of Creatinine: The Jaffé method measures creatinine levels based on the reaction between creatinine and sodium picrate in an alkaline environment to form a colored complex. The color intensity, measured spectroscopically at 492 nm, is proportional to the sample's creatinine concentration.

Determination of Alkaline Phosphatase Activity: This method uses a specific substrate hydrolyzed by alkaline phosphatase in the presence of magnesium or zinc ions. The hydrolysis reaction produces a colored compound, with color intensity measured spectroscopically at 405 nm, reflecting the enzyme's activity.

Determination of Urea: This method utilizes the urea and urease enzyme reaction, producing ammonia and carbon dioxide. Subsequent reactions yield glutamate and NAD, with a decrease in NADH concentration, measured spectroscopically at 340 nm, reflecting the urea concentration.

Determination of Cholesterol: An enzymatic reaction catalyzed by cholesterol oxidase is used. The reaction involves converting cholesterol into an intermediate compound, which further reacts with a specific reagent to produce a colored compound.

Determination of Triglycerides: This method uses an enzymatic reaction catalyzed by lipase to decompose triglycerides. The resulting glycerol reacts with a specific reagent to produce a colored or fluorescent compound.

Determination of TGP Activity: This standard method involves measuring the conversion of alanine and alpha-ketoglutarate to pyruvate and glutamate, catalyzed by TGP. The formation of pyruvate is detected spectroscopically.

Determination of LDH Activity: LDH catalyzes the conversion of lactate into pyruvate, generating NADH. The amount of NADH is measured spectroscopically at 340 nm.

Determination of Magnesium: This common method uses the reaction between magnesium and specific organic compounds, forming a colored complex.

Determination of Calcium: This involves forming a complex between calcium ions and cresol phthalein, yielding a distinctive color measured spectroscopically at 550 and 570 nm wavelengths.
Determination of Iron: A colorimetric ferrozine method measures iron concentration. The intense violet-colored complex with divalent iron (Fe2+) is estimated at 546 nm.

ELISA (Enzyme-Linked Immunosorbent Assay): This well-known method is based on binding antigens or antibodies to a solid surface and detecting the antigen-antibody complex using an enzyme-labeled secondary antibody. The detection is done by adding an enzyme substrate, producing a visible signal.

Determination of IL-6 by ELISA: Utilizing 96-well plates coated with anti-IL-6 antibody, the study employs biotin-conjugated anti-IL-6 as a detection antibody. The structure of the sandwich, including various components, is analyzed. The absorption of samples and standards is read at 450 nm, with color intensity reflecting IL-6 concentration.

Determination of TNF-α by ELISA: Similar to the IL-6 determination, this method uses 96-well plates coated with anti-TNF-α antibody and a corresponding detection antibody conjugated with biotin. The entire process culminates in a sandwich structure that allows the TNF-α concentration to be measured.

3. Results

Table 1 Organic and inorganic compounds determined in the mineral waters of Spring 1 and 1 bis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ca (mg/dL)</th>
<th>Mg (mg/dL)</th>
<th>Fe (ug/dL)</th>
<th>Proteins (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Urea (mg/dL)</th>
<th>Creatinin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>0.211</td>
<td>0.006</td>
<td>28</td>
<td>0.002</td>
<td>0.006</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Spring 1</td>
<td>3.892</td>
<td>0.035</td>
<td>6.4</td>
<td>0.002</td>
<td>0.003</td>
<td>0.002</td>
<td>0.004</td>
</tr>
<tr>
<td>Spring 1 bis</td>
<td>3.628</td>
<td>0.042</td>
<td>63.2</td>
<td>0.001</td>
<td>0.005</td>
<td>0.002</td>
<td>0.003</td>
</tr>
</tbody>
</table>
As seen in Table 1 and Figure 3, the values of organic compounds determined from the mineral waters of the two springs do not differ from those of the distilled water used as a Control. They fall between 0.001 and 0.006 mg/dL.

From the data presented in Table 1 and Figure 3, the calcium concentration is between 3,628 and 3,892 mg/dL (36.28 and 38.92 mg/L) in the case of the two springs compared to 0.211 mg/dL (2.11 mg/L) in the case of distilled water.

From the data presented in Table 1 and Figure 4, the concentration of magnesium is between 0.035 and 0.042 mg/dL (0.35 and 0.42 mg/L) in the case of the two springs compared to 0.006 mg/dL (0.06 mg/L) in the case of distilled water.

In the case of iron, it can be observed, from the data presented in Table 1 and Figure 5, that its concentration is lower in the mineral water from Spring 1 than in the case of distilled water, and the concentration of iron corresponding to Spring 1 bis is double that of distilled water and approximately 10 times higher than in the case of Spring 1 (63.2 vs. 6.4 ug/dL). This result can probably be explained by iron binding in various chemical combinations, including iron sulfide.

Conclusions:

1. The presence of organic peptide, lipid, and nitrogen compounds was found in the mineral water samples from Spring 1 and 1 bis.
2. Calcium and magnesium concentrations in both mineral waters were similar.
3. The concentration of iron is much higher in the case of Spring 1 bis compared to Spring 1.

**Table 2 Biochemical parameters of fibroblast culture media**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mineral water</th>
<th>Mg (mg/dL)</th>
<th>Creatinin (mg/dL)</th>
<th>Urea (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>TGP (U/L)</th>
<th>Alkaline phosphatase (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>13.10</td>
<td>0.12</td>
<td>3.4</td>
<td>2.2</td>
<td>5.8</td>
<td>49.3</td>
<td>26.1</td>
<td>62.01</td>
</tr>
<tr>
<td>2</td>
<td>Spring 1-10%</td>
<td>13.06</td>
<td>0.13</td>
<td>3.8</td>
<td>2.0</td>
<td>5.5</td>
<td>52.1</td>
<td>18.7</td>
<td>57.80</td>
</tr>
<tr>
<td>3</td>
<td>Spring 1-15%</td>
<td>13.08</td>
<td>0.12</td>
<td>3.5</td>
<td>3.0</td>
<td>6.4</td>
<td>49.9</td>
<td>22.1</td>
<td>61.66</td>
</tr>
<tr>
<td>4</td>
<td>Spring 1-18%</td>
<td>13.04</td>
<td>0.12</td>
<td>3.7</td>
<td>2.5</td>
<td>6.0</td>
<td>52.0</td>
<td>19.1</td>
<td>55.72</td>
</tr>
<tr>
<td>5</td>
<td>Spring 1-20%</td>
<td>12.98</td>
<td>0.11</td>
<td>3.6</td>
<td>2.3</td>
<td>6.1</td>
<td>50.8</td>
<td>17.9</td>
<td>55.54</td>
</tr>
<tr>
<td>6</td>
<td>Spring 1bis-10%</td>
<td>12.98</td>
<td>0.12</td>
<td>3.3</td>
<td>2.3</td>
<td>6.3</td>
<td>50.8</td>
<td>24.2</td>
<td>57.15</td>
</tr>
<tr>
<td>7</td>
<td>Spring 1bis-15%</td>
<td>13.08</td>
<td>0.13</td>
<td>3.6</td>
<td>2.4</td>
<td>6.5</td>
<td>51.6</td>
<td>17.9</td>
<td>57.65</td>
</tr>
<tr>
<td>8</td>
<td>Spring 1bis-20%</td>
<td>13.07</td>
<td>0.12</td>
<td>3.6</td>
<td>2.4</td>
<td>6.2</td>
<td>50.3</td>
<td>17.3</td>
<td>56.18</td>
</tr>
</tbody>
</table>
As can be seen from the data presented in Table 2 and Figure 7, the magnesium concentration in the culture media did not change significantly during treatment with different concentrations of mineral water from the 2 sources.

From the data presented in Table 2 and Figure 8, it can be seen that the nitrogenous substances (creatinine and urea) do not show significant variations compared to the Control when treated with different concentrations of mineral water from the 2 sources. Creatinine concentration varies slightly in the 0.11 – 0.13 mg/dL range while urea is between 3.3 and 3.6 mg/dL.

From the data presented in Table 2 and Figure 9, it can be observed that in the case of cholesterol and triglycerides, a slight increase occurs when treating the culture medium with concentrations of 15-20% mineral water from Spring 1, as well as when treating with concentrations of 10-20% mineral water from Spring 1 bis. In the case of treating the culture medium with 10% mineral water from Spring 1, no variations are observed compared to the Control.

The enzymatic activity of transaminases does not show variations compared to the Control, located in the range of 49.3 – 52.0 U/L as seen from the data presented in Table 2 and Figure 10. The enzymatic activity of alkaline phosphatase and LDH shows a slight decrease in the values compared to the witness, but it is not statistically significant.

**Conclusions:**

The biochemical data obtained from the culture medium of the muscle fibroblasts from the cultures on the 12th day do not show significant differences for the cases of mineral water administration from Spring 1 and 1 bis.

**Variation of IL-6 and TNF- concentration in fibroblast cultures**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mineral Water</th>
<th>IL-6 (ug/mL)</th>
<th>TNF-α (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>47.2</td>
<td>116.5</td>
</tr>
<tr>
<td>2</td>
<td>Spring 1- 10%</td>
<td>49.8</td>
<td>106.8</td>
</tr>
<tr>
<td>3</td>
<td>Spring 1- 15%</td>
<td>53.7</td>
<td>110.6</td>
</tr>
<tr>
<td>4</td>
<td>Spring 1- 18%</td>
<td>52.1</td>
<td>108.4</td>
</tr>
<tr>
<td>5</td>
<td>Spring 1- 20%</td>
<td>54.8</td>
<td>112.3</td>
</tr>
<tr>
<td>6</td>
<td>Spring 1bis- 10%</td>
<td>46.9</td>
<td>115.1</td>
</tr>
<tr>
<td>7</td>
<td>Spring 1bis- 15%</td>
<td>55.3</td>
<td>118</td>
</tr>
<tr>
<td>8</td>
<td>Spring 1bis- 20%</td>
<td>56.2</td>
<td>106.3</td>
</tr>
</tbody>
</table>
From the data presented in Table 3 and Figure 11, a slight tendency to increase IL-6 concentration values can be observed depending on the concentration of mineral water in the environment, regardless of the source.

Unlike IL-6, the expression of TNF-α is not significantly influenced by the concentration of the mineral waters used, showing a slight decrease compared to the Control, as shown by the data in Table 3 and Figure 12

Conclusion:
1. The administration of mineral waters to the culture of fibroblasts does not significantly change the expression of the investigated cytokines.
1. The treatment with a concentration of 10% water from Spring 1 determined a slight increase in the expression of several electrophoretically analyzed protein fractions compared to the control case;
2. In general, the treatment with 15% and 20% water from Spring 1 decreases very slightly, insignificant from a statistical point of view, the expression of several protein fractions compared to the control case;
3. Between the treatments with 15% and the one with 20% water from Spring 1, there are no differences regarding the level of the analyzed protein fractions;
4. The differences in the expression of the protein fractions analyzed in all cases are not significant from a statistical point of view, it being necessary to adjust the concentrations of the mineral water used experimentally;
5. From an experimental point of view, it is not justified to continue molecular analyses to determine the level of GSK-3β expression by Western blotting.

4. Discussion

4.1 Impact of Hypotonic Mineral Waters on Keratinocytes
A recent study by Zoller et al. (2015) utilized HaCaT keratinocyte cultures to investigate the effects of four types of hypotonic mineral waters. The research particularly examined DNA proliferation concerning cytotoxicity, interleukin-6 (IL-6) expression, and the formation of reactive oxygen species (ROS) following ultraviolet B (UVB) stimulation. The results indicated a significant reduction in fundamental parameters such as proliferation and cytotoxicity by thermal waters and decreased IL-6 levels post UVB irradiation, comparable to the levels observed with 17-valerate betamethasone, a reference anti-inflammatory (Positive Control). Additionally, ROS levels were notably decreased in comparison to non-irradiated UVB controls. This effect was attributed to the trace elements within the mineral waters (25,26).

4.2 Mineral Water and Immune Regulation
Natural mineral water suppressed the proliferation of Th1, Th2, and Th17 cells with anti-CD3 stimulation. Conversely, thermal water treatment promoted Treg cell proliferation and differentiation. When treated with poly (I: C), TLR3-stimulated APCs isolated from Balb/c mouse spleens showed decreased MHC class II expression and reduced TNF-α and IL-6 production (27–29).

4.3 Thermal Water Effects on Cellular Integrity
Boisnic et al. (2001) demonstrated that Avène thermal water protects cellular membranes, genomic DNA, and human keratinocyte proteins in a UVA-induced oxidative stress cellular model. It also evaluated its anti-allergic effect, where Avène thermal water inhibited histamine and prostaglandin D2 release by rat mast cells exposed to substance P or antigen-induced cell degranulation (30,31).

4.4 H2S and Sulfurous Thermal Water in Immune Modulation
In vitro studies revealed that H2S and sulfurous thermal water could inhibit normal lymphocyte proliferation and T cells in patients with chronic immune-mediated diseases. Moreover, hydrotherapy was shown to modulate peripheral leukocyte ratios and T lymphocyte proliferative response (32).
H2S's ability to interact with various ion channels, modifying their activity, results in the regulation of intracellular calcium levels and consequently affects different calcium-dependent signaling pathways and enzymes, depending on the cell type (33).

4.5 H2S and Cellular Signaling
H₂S was also reported to modulate the activity of several signaling molecules involved in biological processes, including phosphorylation, oxidation, and protein degradation. Mitogen-activated protein kinase (MAPK) activity regulation was notably demonstrated in human pulmonary endothelial cells, where exogenous H₂S prevented MAPK activation (34,35).

4.6 Therapeutic Potential of Sulfurous Thermal Waters

Characterized by high pH, low mineralization, and various ions and salts, sulfurous thermal waters are the most common. Indicated as an alternative for treating various respiratory diseases (e.g., allergic rhinitis, asthma, and chronic obstructive pulmonary disease), these waters contain abundant hydrogen sulfide (H₂S). Enzymes related to H₂S, expressed in human lungs, have mucolytic, antioxidant, anti-inflammatory, and antibacterial roles, contributing to respiratory epithelium homeostasis (15,36–39).

4.7 Implications in Chronic Obstructive Pulmonary Disease (COPD)

COPD, characterized by irreversible and persistent limited airflow, is associated with a chronic increased inflammatory response and emphysematous changes. It involves severe pulmonary and functional changes such as basal, goblet, and mucous cell hyperplasia and airway fibrosis. The chronic influx of inflammatory cells in the bronchial wall, including T lymphocytes, neutrophils, and alveolar macrophages, plays a crucial role in the pathology. The presence of sulfur compounds in thermal waters, specifically H₂S, may offer therapeutic potential in modulating oxidative stress responses, inflammatory cascades, and cellular proliferation within the respiratory tract (40–42).

5. Conclusions

The cumulative research findings suggest a multifaceted impact of hypotonic mineral waters and sulfurous thermal waters on various biological processes. The exploration of hypotonic mineral waters and sulfurous thermal waters has unveiled intriguing biological effects that transcend conventional understanding. The amalgamation of in vitro studies, cellular models, and limited clinical observations offers promising insights but simultaneously poses new questions. The complex interplay between mineral content, cellular response, and therapeutic potential demands a concerted effort across disciplines, ranging from biology and medicine to environmental science.

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