Research article

The effects of Mineral Waters from Slănic Moldova's Spring 1 and Spring 1 bis on Fibroblast activity: An In Vitro Study

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Citation: Munteanu C., Hoteteu M., and Onose G. - The effects of mineral waters from Spring 1 and Spring 1 bis from Slănic Moldova on fibroblasts, in vitro Balneo and PRM Research Journal 2023, 14(3): 591

Abstract: Primary fibroblast cultures obtained from Wistar rats were investigated with a focus on two vital physiological mechanisms: inflammatory processes and oxidative stress balance. These are believed to be affected by mud and sulfurous natural mineral waters, forming the fundamental biological basis for understanding the therapeutic effects of these substances. Existing scientific research highlights that various cell types, including fibroblasts, are recruited during inflammation. These cells respond to a wide array of intercellular and microenvironmental signals, leading to a regulated production of both pro- and anti-inflammatory mediators. Examples include cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, as well as chemokines and enzymes like cyclooxygenase (COX)-2. Together, these play vital roles in modulating the inflammatory response.

Keywords: Fibroblasts, Sulphurous Mineral Waters, Cytokines, Tumor Necrosis Factor (TNF)-α, Interleukins (IL)-1β and IL-6, Chemokines, Cyclooxygenase (COX)-2

1. Introduction

Research data show that different types of cells are recruited during the inflammatory process, including fibroblasts, which respond to different intercellular and microenvironmental signals (1). This leads to the regulated production of various pro- and anti-inflammatory mediators, including cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β and IL-6, chemokines and enzymes such as cyclooxygenase (COX)-2, which play critical roles in controlling the inflammatory process (2,3).

The concept of oxidative stress caused by free radicals argues for the consideration of biomarkers of oxidative stress (4–8). The oxidative and reductive activity of enzymes that act on glutathione, thioredoxin, and other substrates of interest in the oxidation-reduction process reflect the level of antioxidant protection and are also relevant biomarkers for rheumatic degenerative diseases (9–11).

In the case of natural sulfurous mineral waters, the protective effect of H₂S on neurons is
expressed against oxidative stress by increasing the substrate for producing the antioxidant GSH, including the cystine/glutamate antiporter and intracellular Cys concentrations (12,13). H₂S has vasculoprotective properties in endothelial cells and vascular smooth muscle cells, such as triggering vasorelaxation and decreasing platelet aggregation. H₂S possibly activates plasma membrane voltage-dependent channels (L-type and T-type Ca²⁺ channels) and mobilizes intracellular Ca²⁺ stores (14–16). In addition, endogenous H₂S has been found to activate chloride (Cl⁻) channels and potassium (K⁺) channels, providing neuroprotective effects. H₂S donors or H₂S-rich mineral waters target multiple pathophysiological mechanisms (17–21).

The skin, with an area of almost 2 m², has an essential role in defending the human body against microorganisms in the environment and also in maintaining water homeostasis (22,23). When the wound damages the integrity of the skin, the body immediately begins the healing process using a sequence of biochemical and biophysical events to restore the integrity of the skin at the site of the wound (24,25).

Cell culture has been and is used as a significant tool to investigate the healing process. This method allows the study of the biological behavior of cells, taking into account either the kinetics of cell proliferation or the biosynthesis of several components of the extracellular matrix (26–28).

Understanding wound healing at the cellular level is essential to prevent unwanted outcomes such as increased scarring or fibrosis. After dermal injury, regenerative processes begin immediately to close the wound and slowly restore tissue integrity rapidly (29). Different cell types tightly regulate wound healing and many cytokines and involve interactions with the extracellular matrix (ECM). After the initial closure of the wound by a fibrin clot, resident dermal fibroblasts, and putative progenitor cells are attracted to the wound site. They deposit ECM proteins and exert forces on the existing ECM, leading to tissue contraction. Thus, the surrounding ECM becomes stiffer, and the prestressed matrix transforms fibroblasts into pro-to-myofibroblasts containing actin stress fibers. Prestressed matrices require stronger traction forces to ensure wound closure (30–33).

For this reason, pro-to-myofibroblasts differentiate into myofibroblasts. These cells possess a pronounced cytoskeleton, enhanced production of ECM molecules (e.g., collagen I and III, fibronectin, and proteoglycans), and a strong capacity for tissue contraction, which is accomplished by incorporating alpha-smooth muscle actin (αSMA) in their actin stress fibers. αSMA incorporation is one of the most prominent markers of myofibroblast differentiation (34,35).

TGFβ1 is known to be a key player in wound healing, particularly in myofibroblast differentiation. This pro-inflammatory and heparin-binding cytokine is secreted by immune cells, proto-myofibroblasts, and myofibroblasts in a temporally defined paracrine and autocrine manner. But lack of resolution of TGFβ1 release, sustained inflammation, and disrupted Smad signaling (intracellular signal transducers of TGFβ1 signaling) causes myofibroblasts to contract and produce ECM over prolonged periods, leading to hypertrophic scarring, excess fibrous tissue, fibrosis, and associated loss of tissue function. Therefore, TGFβ1 is also used as a target for preventing fibrotic diseases (36,37).
2. Brief presentation of the mineral waters from Spring 1 and Spring 1 bis

Spring 1 from Slanic Moldova contains a combination of essential electrolytes and minerals, each offering distinctive therapeutic benefits for various medical conditions, especially those related to the digestive system. The key components include:

- **Bicarbonate (HCO3):** Vital for regulating the acid-alkaline balance in the body, bicarbonate may reduce gastric reflux symptoms and improve overall digestive function, potentially aiding in the treatment and prevention of kidney disease (38).

- **Sodium (Na+), Potassium (K+), and Calcium (Ca2+):** These essential electrolytes are crucial for regulating heart rate, muscle, and nerve function (39–43). They can enhance digestive function and alleviate symptoms of biliary dyskinesia and chronic cholecystitis.

- **Chloride (Cl):** An integral contributor to fluid and electrolyte balance, chloride supports the digestive system’s functions (44).

- **Sulfate (SO4):** This compound can aid in detoxification, helping to remove toxins and heavy metals (45).

- **Magnesium (Mg):** Involved in over 300 enzyme reactions in the body, magnesium plays roles in metabolizing food, synthesizing fatty acids and proteins, and transmitting nerve impulses (46).

- **Hydrogen Sulfide (H2S) and Carbon Dioxide (CO2):** These dissolved gases possess therapeutic potential, such as improving mitochondrial function and reducing inflammation (47).

The water’s therapeutic indications are wide-ranging, extending to the internal treatment of various gastric and hepatobiliary conditions and metabolic diseases like gout, obesity, and dyslipidemias.

Spring 1bis from Slanic Moldova, another significant spring, has a specific chemical composition that confers various potential health benefits. Its components include:

- **CO2 (Carbon Dioxide):** Promotes digestion and nutrient absorption by stimulating gastric juice production (48).

- **HCO3 (Bicarbonate):** Helps regulate pH levels and reduce stomach acidity symptoms.

- **Cl (Chloride), Na (Sodium):** Essential for maintaining electrolyte balance and proper cell and nerve functioning.

- **Br (Bromine):** A trace element potentially impacting thyroid function and nervous system health.

- **SO4 (Sulfate), H2S (Hydrogen Sulfide):** Possesses antimicrobial properties and may reduce inflammation (49).

- **K (Potassium), Ca (Calcium), Mg (Magnesium):** Vital for muscle function, bone health, and nerve function (50).

The therapeutic applications of these waters are diverse, ranging from internal treatments for gastric and hepatobiliary disorders (including chronic gastroduodenitis, unspecified enterocolitis, biliary dyskinesia, chronic a lithiasis cholecystitis, and post-cholecystectomy disorders) to utilization as sprays and aerosols for chronic respiratory and ENT diseases (51–53).
2. Material și Metode

Water Sample Details. The quality of natural factors is substantially determined by their physicochemical properties resulting from the composition of mineral or organic substances, dissolved gases, or suspended particles, and living organisms present.

Primary Fibroblast Cell Cultures and Their Relevance to Study

Fibroblasts have essential roles in various physiological processes, such as extracellular matrix (ECM) synthesis, epithelial differentiation, inflammation regulation, and wound healing. They are also responsible for the secretion of growth factors and act as a scaffold for several other cell types, mediating tissue fibrosis and scar formation (54,55).

Fibroblasts are easy to grow and maintain in vitro, and human fibroblast lines established from patient biopsies have been used in numerous studies to elucidate the pathogenesis of several diseases (56).

Fibroblast collection is done by explant, isolating the dermis from the epidermis with scalpels and scissors. Derm samples are then fragmented into 5.0 mm2 pieces and placed on 100 mm2 Petri dish surfaces in square areas marked by perpendicular lines made with scalpel blades. The Petri dishes are kept semi-open in laminar flow for 40 minutes to adhere the dermis specimens to the culture surface, ensuring the desiccation of dermis samples is avoided by instilling physiological solution over them to maintain cellular viability (57,58).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) Assay is a cellular viability assay based on the ability of living cells’ mitochondria to reduce tetrazolium salts into dark blue formazan compounds (59). This reaction occurs only in living cells, so the color intensity is directly proportional to the number of viable cells. To conduct this test in primary fibroblast culture, the following steps are taken:

- Cell Seeding: Seed the cells in 48-well plates, with 10,000 cells per well, in a complete DMEM F12/HAM medium.
- Cell Inoculation: Allow the cells to grow in an incubator until they reach approximately 80% confluence.
- MTT Addition: Add the MTT solution to the culture medium (usually at a final concentration of 0.5 mg/ml) and incubate the cells for 2-4 hours, during which the MTT will be reduced to formazan by the living cells.
- Formazan Dissolution: Remove the MTT solution from the cells and add a solvent (usually DMSO or ethanol) to dissolve the formazan crystals.
- Absorption Measurement: Use a spectrophotometer to measure the absorption at a wavelength of 570 nm. The color intensity will be directly proportional to the number of viable cells.

Electrophoresis is an analytical and preparative method for separating electrically charged particles and particle aggregates under the influence of a uniformly applied external electric field. The technique relies on the physicochemical phenomenon of differential movement or migration of different particles within an electric field (60).
3. Results

Primary fibroblasts, 48-well plates, 10,000 cells per well, complete DMEM F12/HAM medium

<table>
<thead>
<tr>
<th>Group</th>
<th>Viability at 96 Hours</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>5,195607</td>
</tr>
<tr>
<td>I - Spring 1 - 15 %</td>
<td>95,20078</td>
<td>3,365599</td>
</tr>
<tr>
<td>II - Spring 1 - 10%</td>
<td>97,45348</td>
<td>5,378499</td>
</tr>
<tr>
<td>III - Spring 1 - 20%</td>
<td>93,04603</td>
<td>4,696374</td>
</tr>
<tr>
<td>IV - Spring 1 bis - 10%</td>
<td>96,49363</td>
<td>2,328495</td>
</tr>
<tr>
<td>V - Spring 1 bis - 20%</td>
<td>91,96866</td>
<td>6,614756</td>
</tr>
</tbody>
</table>
Morphology results - experiment Spring 1

<table>
<thead>
<tr>
<th>Control culture of muscle fibroblasts of 6 days (X150)</th>
<th>6-day muscle fibroblast culture Spring 1 – 15% (X200)</th>
<th>6-day muscle fibroblast culture Spring 1 – 20% (X100)</th>
<th>6-day muscle fibroblast culture Spring 1 – 10% (X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control culture of muscle fibroblasts of 8 days (X200)</td>
<td>8-day muscle fibroblast culture Spring 1 – 20% (X150)</td>
<td>8-day muscle fibroblast culture Spring 1 – 15% (X100)</td>
<td>8-day muscle fibroblast culture Spring 1 – 10% (X100)</td>
</tr>
<tr>
<td>Control culture of muscle fibroblasts of 12 days (X100)</td>
<td>12-day muscle fibroblast culture Spring 1 – 20% (X100)</td>
<td>12-day muscle fibroblast culture Spring 1 – 15% (X100)</td>
<td>12-day muscle fibroblast culture Spring 1 – 10% (X100)</td>
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Conclusions:

1. Although during the experiment, areas appear where, due to the administered mineral waters, voids are formed in the monolayer, at the end of the investigation, the appearance of the cultures becomes uniform, being similar to that of the control case;
2. The culture of muscle fibroblasts obtained by seeding the cells at the density of 50,000 cells per plate reaches the level of confluence from the 5th day of cultivation;
Morphologically, no significant differences exist between the analyzed cases and the control.
Morphology results - experiment Spring 1 bis

Conclusions:
1. Although during the experiment, areas appear where, due to the administered mineral waters, voids are formed in the monolayer, at the end of the investigation, the morphology of the cultures becomes uniform, being similar to that of the control case;
2. The culture of muscle fibroblasts obtained by seeding the cells at the density of 50,000 cells per plate reaches the level of confluence from the 5th day of cultivation;
Morphologically, no significant differences exist between the analyzed cases and the control.
Discussion and Conclusion

Studies by Gambari et al. (2020) (61) have confirmed that in vitro stimulation with sulfurous thermal waters increases the intracellular concentration of H₂S in osteocytes. These waters act as an alternative type of H₂S donor. Tests revealed higher intracellular H₂S levels after treatment with sulfurous thermal waters containing 100 mg/L H₂S than those frequently used with 8 mg/L H₂S. Furthermore, the intracellular concentration of H₂S can be modulated through different dilutions of sulfurous thermal waters. The analysis demonstrated that these waters significantly increased the expression of osteogenic markers such as BSP, OC, RUNX-2, and OPN and had varying effects on cell viability depending on H₂S concentration (62).

Used for years in balneotherapy and dermatology, La Roche-Posay thermal water has shown protective effects against harmful oxidative species, e.g., UV light. Rich in elements like bicarbonate, silicate, and selenium (53 μg/L), it exhibits antioxidant, immunomodulatory, anti-inflammatory, and anti-irritant effects. It even acts as a probiotic due to its unique microbial composition. These findings justify its therapeutic use in inflammatory skin conditions (51).

Nitrodi water promotes the viability and migration of dermal fibroblasts, stimulating protein expression in skin health. It also reduces reactive oxygen species (ROS), enabling the anti-aging effect and skin protection (53).

A study (52) revealed that Avène mineral water modulates immune responses by affecting the differentiation and maturation of dendritic cells, leading to decreased production of inflammatory molecules. This implies an immunomodulatory potential that limits inflammatory responses.

Studies on human keratinocytes with waters like Comano, BJ1, and BG have shown anti-inflammatory and anti-angiogenic effects, depending on their specific chemical compositions. These findings provide insights into therapeutic benefits for skin diseases such as rosacea and psoriasis (63).

Influence on Endothelial Cells: Hydrogen sulfide affects the migration and survival of endothelial cells to form vascular networks through genes promoting the secretion of VEGF, which in turn inhibits apoptosis (64,65).

Involvement in Inflammatory Processes: Mitogen-activated protein kinases (MAPK) are central to inflammatory processes. Studies with inhibitors and H₂S donors have shown effects on IL-6 and IL-8 expression, indicating potential therapeutic applications (66,67).

Effects on Human Chondrocytes: Research (68) into human chondrocytes and mesenchymal progenitor cells’ ability to synthesize H₂S in response to pro-inflammatory mediators shed light on their reactions to exogenous sources of slow-releasing H₂S (69–72).

In conclusion, studies show their potential in modulating biological processes, enhancing skin health, and offering new avenues for treating inflammatory and autoimmune disorders. While some findings are still preliminary, they pave the way for further exploration and clinical applications.
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