Research Article

Dead sea salt solution: composition, lack of cytotoxicity and in vitro efficacy against oral leukotoxins, endotoxins and glucan sucrose

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Abstract

Introduction: Dead Sea Salt, rich in minerals and ionic compositions and low in Sodium Chloride (NaCl) has many reported unique properties that set it apart from other salts.

Objectives: To evaluate the composition of Dead Sea Salt and assess its in vitro cytotoxicity, and efficacy against oral bacterial leukotoxins, oral endotoxins and oral glucan sucrase.

Methods: The cytotoxicity was evaluated in an established cell line (solution at 5000 μg/mL of culture medium) using positive and negative control groups. The effect on oral bacterial leukotoxin (LtxA) and different concentrations of lipopolysaccharide and glucan sucrase was established at 24, 36, 48, 60, 72, 84, and 96 hours using the H PLC method (high-performance liquid chromatography).

Results: The most predominant elements detected were the water of crystallization (H2O, water that is found in the crystalline framework of salt and which is not directly bonded), magnesium chloride (MgCl2), potassium chloride (KCl), sodium chloride (NaCl), calcium chloride (CaCl2), bromide (Br-) and sulfates (SO4). In vitro, Dead Sea Salt presented no cytotoxicity and was highly effective against leukotoxin, endotoxin, and glucan sucrase enzyme.

Conclusion and clinical significance: We believe that rinsing with Dead Sea Salt has the potential to contribute to the prevention of periodontal, peri-implant and dental disease and merits clinical research.

Background

Dead Sea Salt is a potent salt rich in minerals and ionic compositions. Dead Sea Salt has significantly less sodium chloride (NaCl %4.5) vs. Ocean Salt (80% - 85% NaCl) vs. table salt (98% NaCl) [1]. NaCl is associated with high blood pressure [2].

In ancient Egypt, a crucial component of the mumification process was borate-containing salt which was also used to cleanse the body, minor cuts, and wounds [3]. In the 1st century AD, the Greek physician Pedanio Dioskurides wrote that salt was an effective remedy for suppurring wounds [4]. In 2004, White introduced salt water as an archaic yet effective way of killing oral bacteria [5]. Sea salt is a clear mineral that contains the elements of sodium and chlorine, iodine, magnesium, sulfur, calcium, potassium, phosphorus, fluorine, titanium, beryllium, germanium, and zinc [6].

Sukenik, et al. evaluated the efficacy of Dead Sea balneotherapy in patients suffering from osteoarthritis of the knees in a randomized controlled study and provided evidence of significant improvement as measured by the Lequesne index of severity of osteoarthritis. The improvement lasted up to 3 months of follow-up. The authors concluded that balneotherapy in the Dead Sea area has a beneficial effect on patients with osteoarthritis of the knees, an effect that lasts at least 3 months [7].
Katz, et al. in a systematic review assessed the level of evidence for the claims of therapeutic effects of Dead Sea treatments in several rheumatologic diseases and psoriasis as well as reviewed these treatments’ safety. Dead Sea salt was found to be beneficial in several rheumatologic diseases and psoriasis with a good safety profile [8].

As early as 1905, Carlsbad salt, consisting of a mixture of potassium sulfate, sodium chloride, sodium bicarbonate, and sodium sulfate, was recommended for use in gingival pockets after calculus removal [9]. In 2013, Michel, et al. In a study entitled “The street children of Manila are affected by early-in-life periodontal infection: description of a treatment modality: sea salt” examined the effect of Sea Salt in 617 abandoned children who were living in the streets of Manila in the Philippines and provided evidence of the effectiveness of sea salt in the reduction or elimination of periodontal bacterial pathogens [10].

In 2017, Rodriguez, et al. Ajdaharian evaluated the in vivo effects of a Dead Sea Salt mouthwash (Oral Essentials, Beverly Hills, CA 90210) to reduce dental plaque and improve gingival health in an in vivo prospective, randomized, controlled, double-blinded study and reported significant levels of plaque control and reduction in gingival inflammation comparable to a commonly used chlorhexidine-based mouthwash [11]. Ajdaharian, et al. also provided evidence of in vivo enamel remineralization measured by standard Knoop microhardness testing [12].

Although there are many different kinds of salts, Dead Sea Salt has many unique properties that set it apart from all other salts. The purpose of the present investigations was to evaluate the composition of Dead Sea Salt and assess its cytotoxicity and in vitro efficacy against leukotoxins, endotoxins, and glucan sucrose.

Material and methods

Dead Sea Salt composition determination

Dead Sea Salt used in the present investigations was an unrefined, solar evaporated salt from the southern Dead Sea in Israel. Crystals were naturally white, low in sodium levels with high in mineral content. The Dead Sea Salt solution used in the present investigations was negative for Salmonella, Listeria, Staphylococcus aureus, and E. coli. The following lists the most predominant elements detected (due to rounding, values do not add up to 100%): Water of Crystallization (H2O, water that is found in the crystalline framework of salt and which is not directly bonded) %39.280, Magnesium Chloride (MgCl2) %32.670, Potassium Chloride (KCI) %23.090, Sodium Chloride (NaCl) %4.580, Calcium Chloride (CaCl2) %0.390, Bromide (Br -) %0.350, and Sulphates (SO4) %0.030. (Gas Chromatography-Mass Spectrometry, Thermo Scientific™). (Table 1).

In vitro lack of cytotoxicity

The cytotoxicity of the Dead Sea Salt solution (solution at 5000 μg/mL of culture medium) was evaluated in an established cell line. The study was designed to determine the in vitro biological response of mammalian cells using appropriate biological parameters.

The solution at 5000 μg/mL of culture medium (DMED, Gibco, 31966 - 1772990) was prepared from Dead Sea Salt according to ISO 10993 - 12 and ISO 10993 - 5 (Biological evaluation - Evaluation and testing within a risk management process) to evaluate its ability to induce a cytotoxic effect. Controls and (negative) were run in parallel. Complete culture medium served as the negative control and phenol 0.64 mg/mL as the positive control (positive: phenol, SIGMA, p5566 - BCBR0509V) (Table 2).

A monolayer of Balb/c 3T3 clones A31 cells (mouse embryonic fibroblasts) (ATCC, The American Type Culture Collection, CCL 163) was exposed to Dead Sea Salt solution at 5000 μg/mL and its dilutions for 24 hours. At the end of the incubation period, the cytotoxicity was evaluated by counting the living cells (Trypan blue exclusion test). Cells were seeded in multi-well plates (24 wells, 15.5 mm in diameter) at the starting density of 30,000 cells/cm; culture medium is DMEM supplemented with 10% (v/v) FCS. No antibiotics were used. Cultures were incubated at 37° humidified atmosphere containing 5%(v/v) CO2 for 24 hours. Cultures were examined with a microscope (AmScope 40X-2000X LED Biological Trinocular Compound Microscope with 5MP Camera) to verify that cells constituted a sub-confluent monolayer and that their morphology was not altered. Culture medium was withdrawn and replaced with the solution at 5000 μg/mL and its dilutions (1500, 500 and 150μg/mL), negative and positive controls supplemented with 10% (v/v) FCS and 1% antibiotics (v/v).

Table 1: Dead Sea Salt composition.

<table>
<thead>
<tr>
<th>Material</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water of crystallization</td>
<td>39.28</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl2)</td>
<td>32.67</td>
</tr>
<tr>
<td>Potassium Chloride (KCI)</td>
<td>23.09</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>4.58</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl2)</td>
<td>0.39</td>
</tr>
<tr>
<td>Bromide (Br)</td>
<td>0.35</td>
</tr>
<tr>
<td>Sulfate (SO4)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 2: Cytotoxicity test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5000 μg/mL</th>
<th>1500 μg/mL</th>
<th>500 μg/mL</th>
<th>150 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead Sea Salt Solution</td>
<td>89065 ± 4935</td>
<td>101720 ± 4795</td>
<td>109845 ± 2245</td>
<td>106405 ± 1645</td>
</tr>
<tr>
<td>Negative Control (Complete Culture Medium)</td>
<td>106405 ± 8905</td>
<td>46875 ± 3500</td>
<td>103 NS</td>
<td>106405 ± 1645</td>
</tr>
<tr>
<td>Positive Control (Phenol 0.64 mg/mL)</td>
<td>8905 ± 4935</td>
<td>101720 ± 4795</td>
<td>109845 ± 2245</td>
<td>106405 ± 1645</td>
</tr>
</tbody>
</table>

*Versus negative control
NS: Not Statistically Significant
Dead sea salt: composition, lack of cytotoxicity and in vitro efficacy against oral leukotoxins, endotoxins and glucan sucrose

Wells were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂ for a 24-hour period. Photos were made (x 320) showing the cell layer in contact with negative control, positive control, and the solution at 5 000 μg/mL, to observe the morphology and cell density of the cultures. At the end of the incubation period, the culture medium was removed. Cells were detached (2 minutes) using 250μL trypsin (LEMI, Technopôle Montesquieu, 33650 Martillac, France, OC290616 - 2) (0.05% (w/v) in Hank’s balanced solution Ca++ and Mg•• free supplemented with 1mM EDTA). Then 250 μL of a Trypan blue (XS080816 - l) solution at 0.2% (w/v) in 0.15 M NaCl + 10% (v/v) FCS were added (incubation for 2 minutes). Thereafter living cells (uncolored) were counted using a hemocytometer (Malassez cell) (LEMI, SOP n° MB08/23, Technopôle Montesquieu, 33650 Martillac, France). Results are expressed as a number of cells per cm² (cell density). Statistically significant inhibition of cell growth superior to 30% compared to the negative control had to be observed. Morphology and cell density of the cell layer in contact with the solution at 5000 μg/mL were compared to the negative control, and the positive control. The inhibition of cell growth was used to evaluate the cytotoxic effect of the test item.

Statistical analysis was performed using the Student’s t-test. p - values less than 0.05 were considered statistically significant. (Statistical software package, SAS Institute Inc., Cary, NC. The USA) However, the biological relevance of the results was considered first. A test item was considered cytotoxic if the inhibition of the cell growth was superior to %30.

The solution at 5000 μg/mL (pH 7.3 - osmolality 340 mOsm/kg H₂O) and dilutions, negative control (pH 7.5 - osmolality 337 mOsm/kg H₂O), and dilutions did not show any inhibition of cell growth statistically significantly superior to %30 and the solution at 5000 μg/mL prepared from the test material was not cytotoxic. Positive control induces a 56% (p < 0.001) inhibition of cell growth which validated the study. In vitro effectiveness against oral bacterial leukotoxin

Leukotoxin (LtxA) is a protein toxin that is secreted from the oral bacterium Aggregatibacter actinomycetemcomitans and targets phagocytes, natural killer cells, dendritic cells, and T lymphocytes and negatively affects both innate and adaptive immune responses. The effect of Dead Sea Salt solution on bacterial Leukotoxin (LtxA) was measured by exposing A 3T3 clone A31 Cell Line from mouse to a 5000 μg/ml Dead Sea Salt solution for 24, 36, 48, 60, 72, 84, and 96 hours. The leukotoxin concentration was decreased by %84 after 84 hours of exposure (Table 3).

### Table 3: Dead Sea Salt effect on leukotoxin. The toxin concentration was %84 decreased after 84 hours of exposure.

<table>
<thead>
<tr>
<th>Exposure Time (μg/ml)</th>
<th>Dead Sea Salt (μg/ml)</th>
<th>Water (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>36 hours</td>
<td>0.10</td>
<td>0.19</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.08</td>
<td>0.18</td>
</tr>
<tr>
<td>60 hours</td>
<td>0.07</td>
<td>0.18</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>84 hours</td>
<td>0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>96 hours</td>
<td>0.03</td>
<td>0.19</td>
</tr>
</tbody>
</table>

### Table 4: Lipopolysaccharide (endotoxin) concentration was %40 decreased after 72 hours of exposure.

<table>
<thead>
<tr>
<th>Exposure Time (μg/ml)</th>
<th>Dead Sea Salt (μg/ml)</th>
<th>Water (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.19</td>
<td>0.2</td>
</tr>
<tr>
<td>36 hours</td>
<td>0.19</td>
<td>0.2</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.18</td>
<td>0.2</td>
</tr>
<tr>
<td>60 hours</td>
<td>0.17</td>
<td>0.2</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.12</td>
<td>0.2</td>
</tr>
<tr>
<td>84 hours</td>
<td>0.12</td>
<td>0.2</td>
</tr>
<tr>
<td>96 hours</td>
<td>0.12</td>
<td>0.2</td>
</tr>
</tbody>
</table>
In vitro effectiveness against glucan sucrose also known as glucosyltransferase

Glucansucrase also known as glucosyltransferase (the glycoside hydrolase family GH70) is an enzyme that lactic acid bacteria use to break sucrose and the resulting glucose molecules from biofilm chains and the adhesion of more acid-producing bacteria [13]. Streptococcus mutans metabolizes sucrose into lactic acid and lower the pH around teeth and dissolves enamel calcium phosphate leading to dental decay [14]. A determination of Glucansucrase concentration was performed using the HPLC method. The standard reaction for enzyme assay was performed in the mixture containing 50 mM sodium acetate (pH 5.2), 100 mM sucrose, and an appropriately diluted enzyme. The reaction mixture was incubated at 30 °C for 30 min. The resulting fructose was measured by the dinitrosalicylic acid (DNS) method (Miller 1959). One unit was defined as the amount of enzyme that catalyzes the release of 1 μmol fructose from sucrose per min at 30 °C. For the glycosylation of l-ascorbic acid to ascorbic acid 2-glucoside, the glycosylation reaction mixture contained 50 mM sodium acetate (pH 5.2), 1 mM MgCl₂, 0.1% l-ascorbic acid, 100 mM sucrose, and an appropriately diluted enzyme. Then, the reaction mixture was incubated at 30 °C for 3 h. The products were analyzed by using thin-layer chromatography (TLC). The samples were spotted onto the silica gel 60 F254 TLC plate (Merck, Germany) and developed with a solvent (TLC). The samples were spotted onto the silica gel 60 F254 TLC plate (Merck, Germany) and developed with a solvent (TLC) system consisting of nitromethane/1-propanol/water (2:5:1.5, v/v/v). The plate was then visualized by spraying with 10% H2SO4 in methanol. The products were then analyzed by high-performance liquid chromatography (HPLC) (Thermo Scientific™, USA), using a Gemini 5 μm C18 110A column (4.6 mm × 250 mm, Phenomenex, USA) with 0.1 M KH2PO4 buffer (pH 2.0) as a mobile phase. Sample Calculation:

\[
\% \text{ of 2-glucose in Products} = \left( \frac{\text{Areaspl}}{\text{Mean Areastd}} \right) × \left( \frac{\text{Cstd}}{\text{Wspl}} \right) × 100 \text{ mL} × 100
\]

\[
\text{Areaspl} = \text{Area of 2-glucose in Sample preparation}
\]

\[
\text{Mean Areastd} = \text{Mean Area of Arginine in working standard preparation}
\]

\[
\text{Cstd} = \text{Concentration of standard in working standard (mg/mL)}
\]

The Glucansucrase concentration decreased by 90% after 84 hours of exposure (Table 5).

Discussion

The Dead Sea is bordered by Jordan, Israel, and the West Bank. It lies in the Jordan Rift Valley and its main tributary is the Jordan River. The Dead Sea is 430.5 meters below sea level, the lowest land-based elevation on Earth, and the deepest hypersaline lake in the world (304 meters). Due to its salinity, plants and animals cannot flourish there, hence its name [1].

Dead Sea Salt contains a significant number of minerals and ions. The most predominant elements detected in the present investigations were the water of crystalization (H₂O), magnesium chloride (MgCl₂), potassium chloride (KCl), sodium chloride (NaCl), calcium chloride (CaCl₂), bromide (Br⁻), and sulphates (SO₄). Calcium oxide, silicon dioxide, potassium oxide, and magnesium could reduce tooth sensitivity by closing the dentinal tubules and remineralization. Bromide and chloride have a natural cleaning and whitening effect.

Magnesium salts, the prevalent minerals in Dead Sea water, exhibit favorable effects on inflammatory diseases. Proksch, et al. examined the efficacy of bathing atopic subjects in salt rich in magnesium chloride from deep layers of the Dead Sea [15]. Volunteers with atopic dry skin submerged one forearm for 15 min in a bath solution containing 5% Dead Sea salt. The second arm was submerged in tap water as control. The authors reported that bathing in the salt solution was well tolerated, improved skin barrier function, the enhanced stratum corneum hydration, and reduced skin roughness and inflammation. Magnesium salts are known to bind water, influence epidermal proliferation, and differentiation, and enhance permeability barrier repair [15].

In 2019, Portugal-Cohen, et al. elucidated the effect of topically applied Dead Sea water via the expression of different skin biomarkers related to barrier function, homeostasis, inflammation, and irritation [16]. In vitro skin equivalents and ex vivo human skin organ culture were used. β-endorphin secretion was tested on human skin organ culture. The capability of Dead Sea water to protect against skin inflammation and irritation was tested on ex vivo human skin organ culture by lipopolysaccharides and sodium dodecyl sulphate addition, respectively. The topical application of Dead Sea water encouraged the expression of the barrier-related proteins: filaggrin, involucrin, and transglutaminase. Additionally, Dead Sea water application had increased skin secretion of β-endorphin and attenuated the expression of inflammatory and irritation-related cytokines [16].

Cytotoxicity is defined as living cell damage or cell death caused by the action of chemotherapeutic agents. Cytotoxicity tests are important in the determination of the proposed biomedical use [17,18]. In the present investigations, cytotoxicity tests assessed mouse embryonic fibroblast cells before and after exposure, and control materials were well defined to facilitate comparisons. The solution at 5000 μg/mL (pH 7.3 - osmolality 340 mOsm/kg H₂O) and dilutions did not
show inhibition of cell growth and were not cytotoxic. Positive control induced a 56% ($p < 0.001$) inhibition on cell growth.

Aggregatibacter actinomycetemcomitans bacterium is associated with severe periodontal bone loss as well as other systemic diseases [19,20]. Clinical isolates from patients frequently produce significantly higher amounts of leukotoxin (LtxA) that play a key role in A. actinomycetemcomitans pathogenicity. Over the past 30 years research has been focused on understanding the mechanisms by which LtxA can be neutralized by anti-LtxA strategies to prevent and treat disease [21].

Human microbiota plays an important role in human health. For example, there may be a relationship between oral bacteria and oral squamous cell carcinoma. Zhang, et al. using the 16S rDNA sequencing provided evidence that the richness and diversity of bacteria were significantly higher in tumor sites than in the control tissues [22]. At the species level, the abundances of Fusobacterium nucleatum, Prevotella intermedia, Aggregatibacter segnis, Capnocytophaga leadbetteri, Peptostreptococcus stomatis, and genes involved in bacterial chemotaxis, flagellar assembly and lipopolysaccharide (LPS) biosynthesis which are associated with various pathological processes, were significantly increased. Bacteria release LPS fragments into the human body, while this layer is constantly renewed to maintain bacterial integrity. LPS activates cells of the innate immune system, such as macrophages and neutrophils, which synthesize pro-inflammatory factors, IL-1β and TNF, MMPs, and free radicals that lead to significant secondary tissue inflammation [19,20,22].

Glucosyltransferase is an extracellular or cell-associated enzyme synthesized by the Streptococcus mutans as well as the S. sanguis in the initiation of glycan chains and the adhesion of more acid-producing bacteria [13]. The metabolic acids demineralize enamel and dentine. The glucosyltransferase enzyme is the key factor in dental decay. In the present investigations, the glucosyltransferase concentration decreased by 90% after 84 hours of exposure to Dead Sea Salt solution.

**Conclusion**

Although there are many different kinds of salts, the composition of Dead Sea Salt sets it apart from other salts. In vitro, Dead Sea Salt, rich in magnesium and potassium, presented no cytotoxicity and was effective against bacterial leukotoxin, bacterial endotoxin, and bacterial glucansucrase enzyme. We believe that rinsing with Dead Sea Salt has the potential to contribute towards the prevention of periodontal, peri-implant, and dental disease and merits clinical research.

**References**

1. Long-Term changes in the Dead Sea. Israel Oceanographic and Limnological Research - Israel Marine Data Center (ISRAMAR).


