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TEST REPORT
Effects of Analemma water on organ-specific cell cultures

1 Question of the study

According to the manufacturer, coherent Analemma water is a structured water with the properties of natural water and improves the energy production in cells. Moreover, it is not merely a transport medium, but can influence the functioning of biomolecules, the communication between cells and the balance of complex biological systems. The aim of this study was to use animal-free cell biological test methods to investigate the effects of Analemma water on cellular level in comparison to the initial local tap water.

2 Organ-specific cell cultures

The investigations were conducted by using (1) porcine intestinal epithelial cells (IPEC-J2; DSMZ ACC 701) and (2) human promyelocytes (HL-60; DSMZ ACC 3). The promyelocytes were differentiated into functional neutrophils able to produce reactive oxygen radicals by an oxidative burst in the tissue, thereby further triggering an inflammatory process. Both cell types were routinely grown in their specific culture media (DMEM/Ham's F12 1:1 for IPEC-J2 and RPMI 1640 for HL-60) supplemented with 10 % growth mixture & 0.5 % antibiotics in an incubator at 37 °C in a humid atmosphere of 95 % air and 5 % CO_2.

3 Preparation of Analemma water and test concentrations

1.000 ml of fresh local tap water (temperature 10-12 °C) was structured by stirring clockwise for 1 minute with the Analemma water wand with stainless steel holder. The wand was provided by Water and Light B.V. for the current experiments. The initial local tap water and the structured Analemma water were added to cell culture media or reaction mixtures at a volume fraction ranging from 0 to 40 vol%.

4 Cell metabolism (Intestinal epithelial cells)

Cellular metabolism in the intestine is central to maintaining gut health, as intestinal epithelial cells rely on tightly regulated energy processes to support barrier function, nutrient absorption and immune signaling. For the experiments, cells were seeded at three different cell densities (200,000, 100,000, and 50,000 cells/well) in 96-well culture plates and were incubated for 48 hours until complete adhesion and recovery of cellular metabolic was achieved. Thereafter, a reaction mixture consisting of phosphate buffered saline with calcium and magnesium, 15 mM glucose as an energy source, the test concentrations of both water samples and a water-soluble tetrazolium dye (WST-1) was added to the cells. Through the activity of the enzymes responsible for energy production in the mitochondria, the dye was cleaved and the resulting color change was measured quantitatively as an indicator of cellular metabolism. Five independent experiments with replicates were conducted (n = 5).

Results: Analemma water resulted in an average stimulation of 22.8 ± 6.5 % at all test concentrations (mean value ± standard deviation) in direct comparison to the initial tap water. The difference of Analemma water to the initial tap water was always statistically significant ($p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test). The maximum stimulation of Analemma water by more than 30 % was observed at a volume concentration of 30 vol%.

5 Regeneration (Intestinal epithelial cells)

Intestinal regeneration refers to the processes by which the gut repairs its tissues during normal turn-over or after injury. Intestinal epithelial cells were seeded at a density of 200,000 cells/ml into the four compartments of silicone frames. The individual compartments are separated from each other by a 500 µm thick silicone bar. Due to the special adhesive surface of the silicone frames, these adhere firmly to the bottom of a culture dish, thus forming a cell-free space. After removal of the frames, the cells can colonize the cell-free space by division and migration. Immediately after removal of the silicone frames, the cell cultures were incubated with 25 vol% of both water samples in the culture medium for another 12 hours. The cells were then fixed and stained. The width of the residual cell-free space was determined using micrographs at 4 locations per cell culture and evaluated by a specialized software. Four independent experiments with replicates were conducted (n = 4).

Results: Based on the residual width at the end of the experiment, the medium increase in regeneration achieved by Analemma water compared to the initial tap water was 47.2 ± 20.5 % (mean value ± standard deviation), demonstrating a better restoration of intestinal barrier integrity and functionality (Figs. 1 and 2). Despite the large standard deviation, the difference of Analemma water to the initial tap water was statistically significant ($p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test).

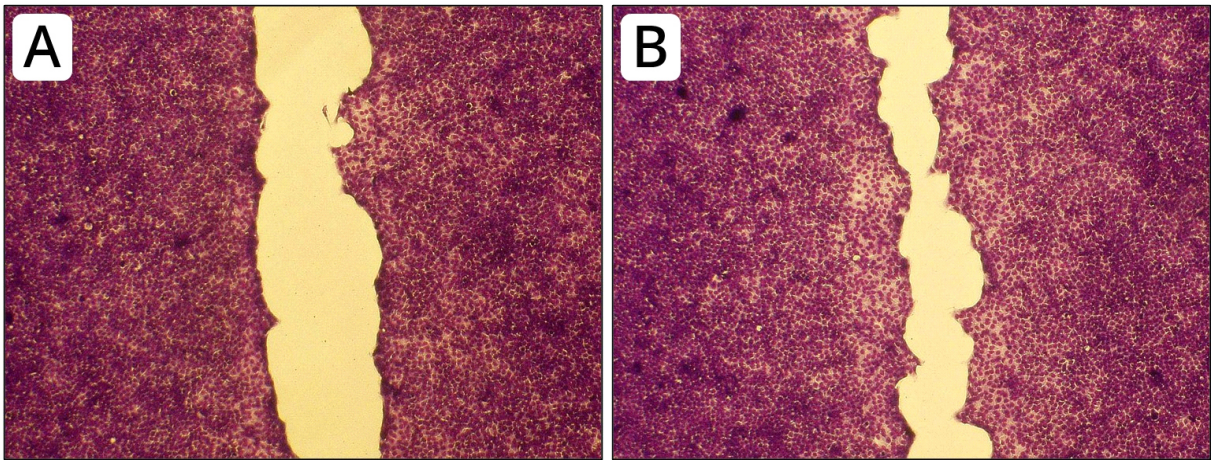

Fig. 1: Micrographs documenting the regeneration of intestinal epithelial cells after 12 hours with 25 vol% initial local tap water (A) and Analemma water (B). Fixed and stained cell cultures at brightfield illumination. Olympus IX 50 inverted microscope with a planachromat 4x lens and an Olympus E-20 digital camera at 5 megapixels.

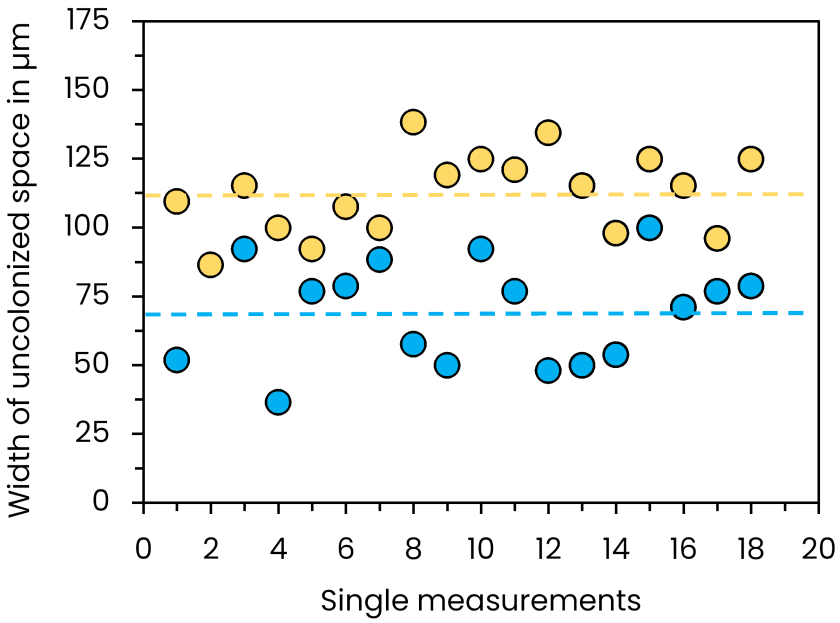

Fig. 2: Graphical presentation of the single measurement data for both water samples for one experiment. The blue circles represent the width of the residual cell-free area for 25 vol% Analemma water and the yellow circles for initial local tap water. The colored dashed lines give the mean values for both water samples in this experiment.

6 Oxidative stress (Intestinal epithelial cells)

Intestinal oxidative stress occurs when reactive oxygen overwhelm the intestinal antioxidant defenses and damage epithelial cells, lipids, proteins and DNA. Common triggers include food intolerances or diets, (chronic) inflammatory processes or injury by certain drugs or toxins. The resulting damage can impair the epithelial barrier, promote mucosal inflammation, disrupt nutrient absorption and contribute to conditions such as inflammatory bowel disease, intestinal permeability ("leaky gut") or even colorectal cancer. For the experiments, cells were seeded at a density of 100,000 cells/well in 96-well culture plates and were incubated for 48 hours until complete adhesion and resumption of cellular metabolic was achieved. Thereafter, 25 vol% of both water samples as well as hydrogen peroxide as a donor of reactive oxygen species (concentrations ranging from 0.5 to 2.5 mM) was added. After 24 hours of continuous exposure, cell viability was examined by using the enzymatic XTT test. Four independent experiments with replicates were conducted (n = 4).

Results: Intestinal epithelial cells cultured with Analemma water had a 25.8 ± 13.0 % better cell viability (mean value ± standard deviation) compared with cells cultured with initial tap water. The difference of Analemma water to the initial tap water was statistically significant ($p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test). The result indicates a better resistance of intestinal cells against oxidative stress and an increased cell survival after application of Analemma water.

7 Endogenous radical formation (Functional neutrophils)

Neutrophils are primarily responsible for the innate defense against foreign pathogens in the blood. Once they recognize these pathogens, they enclose them, kill them through the formation of reactive oxygen species via a process known as oxidative burst, and then eliminate them by phagocytosis. Here, the functional neutrophils were stimulated to form radicals by the addition of a phorbol ester in the presence of the test concentrations of both water samples (0 to 40 vol%). This led to the cleavage of the added water-soluble tetrazolium dye and a change in its color, which was measured as an indicator of the amount of radicals still present. Four independent experiments with replicates were conducted (n = 4).

Results: Analemma water stimulated the basal cell metabolism of functional neutrophils by 10.2 ± 9.6 % compared with initial tap water. However, this difference was statistically not significant. The formation of reactive superoxide anion radicals by the functional neutrophils occured in a dose-dependent manner with an average increase of 23,0 ± 4,2 % for all concentrations between 10 and 40 vol% (mean value ±

standard deviation). This difference of Analemma water to the initial tap water was statistically significant ($p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test). The results indicate that the coherent Analemma water might increase the primary innate immune defense of neutrophils in the blood.

8 Conclusions

In this study the coherent Analemma water has shown its beneficial health effects on the intestine, especially for the protection and maintenance of the intestinal epithelial barrier and, additionally, the primary innate immune defense by the neutrophils floating in the blood. Since the intestine plays an essential part for the systemic health situation, the observed effects on cellular level might also promote an overall well-being.

Responsible for the scientific accuracy of the test assays performed here and the content of the test report.

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