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## **Preliminary investigations on the cellular effects of water influenced by different Transition State Elements (TSE)**

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### **1 Question of the present study**

In this scientific study, current cell biological methods were used to investigate whether local tap water, after being activated by TSE samples provided by Orynoco Ltd, UK, has received and stored informations that possess beneficial health effects. The effects of the influenced water on cultured cells were directly compared with the initial tap water without any TSE sample influence.

### **2 Transition State Elements (TSE) and water test concentrations**

For the present study, we have used 200 ml of tap water which was influenced during a 24 hour period at room temperature without agitation by the four following TSE samples: Sunshine TSE, CO<sub>2</sub> TSE, Zinc TSE and Copper TSE. The initial tap water of the region at the location of Dartsch Scientific without any influence was used as corresponding control. Although the daily intake of 1.5 liter of water is equivalent to a percentage of only 3 % of the whole body fluid, the continuous daily intake of influenced water might result in an accumulation within the body. This explains the use of higher test concentrations of water in the culture medium or reaction mixture of this study. The water test concentrations ranged from 0 (= control) to 70 vol% in the cell-free test assay (maximum 40 vol% for bioassays).

### **3 Effect of TSE water samples on cell regeneration**

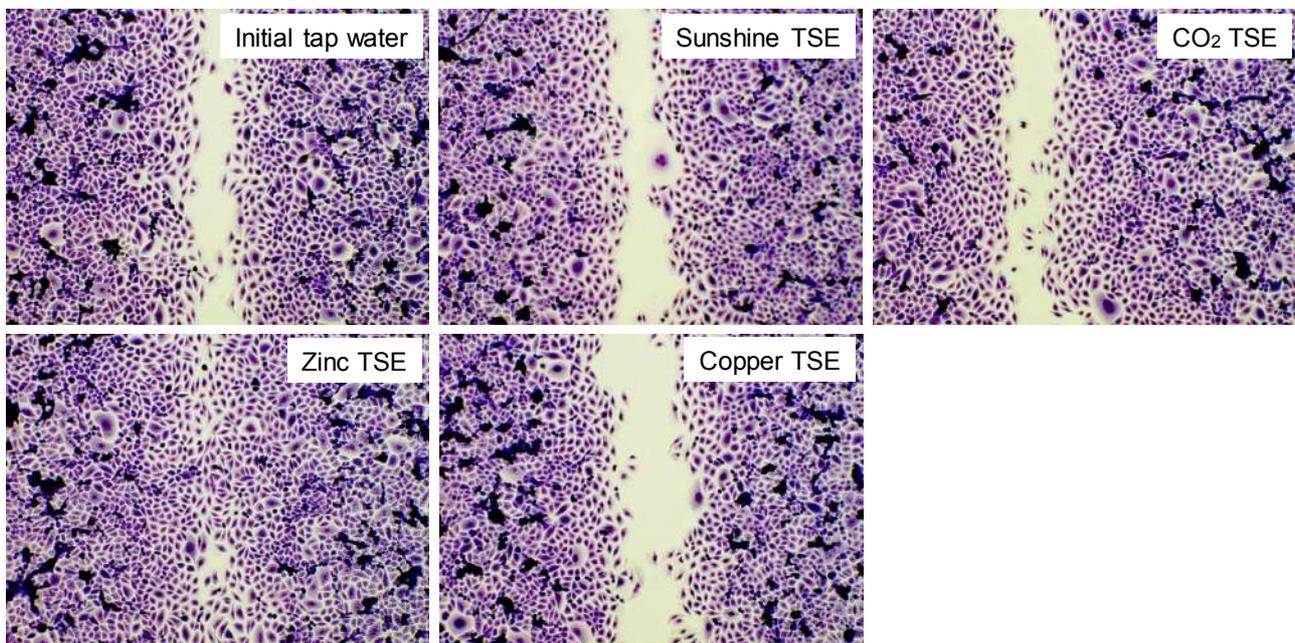
Connective tissue is the tissue that connects, separates and supports all other types of tissues in the body. It consists of mainly fibroblasts, which are embedded in a viscous ground substance, the extracellular matrix, which forms the structural framework of the tissue. The fibroblasts play an essential role by producing the extracellular matrix and are also involved in cell regeneration/wound healing. The test conducted here simulates the granulation phase which is especially characterised by an increased proliferation and migration of the cells to close the cell-free space.

The investigations were conducted with connective tissue fibroblasts (cell line L-929, ACC-2; Leibniz Institute, DSMZ, Braunschweig, Germany). Cells were routinely grown in RPMI 1640 medium with 10 % growth mixture and standard amounts of gentamycin and incubated at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air.

For the test, cells were seeded at a density of 50,000 cells/ml into the three cell culture compartments of silicone Culture-Insert 3 Wells (ibidi, Gräfelfing, Germany) which have been carefully placed on each well surface of a 12-well plate. The three compartments are separated by a 500 µm thick wall. Due to their especially designed bottom, the inserts stick to the surface firmly and completely prevent any cell growth under their silicone walls.

After a dense cell layer was achieved within 48 hours after cell seeding, the inserts were removed to leave a sharp cell-free space (artificial wound) without any cells. Fresh culture medium containing 25 vol% of initial tap water or TSE influenced water was added. After 24 hours of continuous incubation, cells were washed, fixed with methanol, stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) and air-dried. Finally, the width of the remaining cell-free space was measured morphometrically.

**Results:** As depicted in Figure 1, cell regeneration was increased significantly by Zinc TSE sample water in comparison to the control water without any influence. The other water samples influenced by Sunshine TSE, CO<sub>2</sub> TSE and Copper TSE were not effective in stimulating cell regeneration. Especially Copper TSE even showed a significant reduction in cell migratory and proliferative activity.



**Figure 1:** Micrographs of the cell regeneration/wound healing process of cultured connective tissue fibroblasts after 24 hours of cultivation with 25 vol% water sample in the culture medium. Note the different closure of the cell-free space. Olympus IX 50 with Planachromate 10x and Olympus E-10 digital camera at bright field illumination.

#### 4 Antioxidative effect of TSE water samples

With this cell-free assay it was tested whether the water samples with and without TSE influence were able to inactivate free exogenous oxygen radicals and, thus, prevent oxidative stress. For the test, different concentrations up to 70 vol% of the various water samples were pipetted to a potassium peroxide solution and a water-soluble tetrazolium dye WST-1 (Roche Diagnostics, Mannheim, Germany) was added. The reactive superoxide anion radicals still present in the reaction mixture caused a change in the optical density (= color) of the dye. The change of optical density was recorded at definite time points as a differential measurement  $\Delta OD = 450 - 690 \text{ nm}$  by an Elisa reader (BioTek SLx808 with software Gen 5 version 3.00) for the time interval up to 30 minutes.

**Results:** The results showed the best antioxidative effect for Zinc TSE water sample with a reduction of approximately 20 % in comparison to the control water without any influence. The water samples influenced by Sunshine TSE and  $\text{CO}_2$  had an antioxidative effect of 12 to 13 %, whereas the water sample influenced by Copper TSE was similar to the control water and was not effective.

#### 5 Anti-inflammatory effect of TSE water samples

Neutrophils are the most abundant type of granulocytes and make up about 60 % of all white blood cells in humans and are normally found in the bloodstream. They form an essential part of the innate immune system and, therefore, play a key role in the front-line defense against invading microbial pathogens. They are also one of the first responders of inflammatory cells to migrate from the bloodstream towards the site of inflammation. They are able to generate superoxide anion radicals upon stimulation.

*In vitro*, HL-60 cells can be used as a routine cell line and cells can be differentiated to become functional neutrophils. These cells are capable of generating reactive superoxide anion radicals upon stimulation by a phorbol ester, which is called an oxidative or respiratory burst. This reflects the situation when neutrophils have migrated into the inflamed tissue and generate superoxide anion radicals which might cause a prolongation or even chronification of an inflammatory process.

The investigations presented here were conducted with human promyelocytes (HL-60 cell line; ACC-3; ECACC 98070106; Leibniz Institut, DSMZ, Braunschweig, Germany). The cells were routinely grown in RPMI 1640 medium with 10 % growth mixture and 0.5 % gentamycin and cultivated in an incubator at 37 °C in an atmosphere of 5 %  $\text{CO}_2$  and 95 % air at 98% humidity. The non-adherent cells were routinely cultivated as suspended mass cultures and were regularly subcultured in fresh culture medium.

By adding 1.5 % dimethyl sulfoxide to the culture medium, the cells were differentiated over a period of 5 to 6 days into functional neutrophils. The differentiated cells were exposed to the water samples at different concentrations up to 40 vol% and were induced to generate reactive oxygen radicals by adding a phorbol ester to the reaction mixture. The co-

lor change (= optical density) of the reaction was measured as a differential measurement  $\Delta OD = 450 - 690$  nm at definite time points by an Elisareader (BioTek SLx 808 with software Gen 5/3.00) up to 30 min and evaluated with Microsoft Excel.

**Results:** The anti-inflammatory effect of the influenced water samples due to an inactivation and/or release of superoxide anion radicals generated by functional neutrophils was one of the most prominent characteristic features of the water influenced by the TSE samples in comparison to the untreated initial tap water. We measured that the water influenced by Zinc TSE had the strongest effect by inactivating the radicals by nearly 60 %, followed by Copper TSE with a value of 48 % and Sunshine TSE by approximately 34 %. CO<sub>2</sub> TSE showed only a 14 % inactivation.

## 6 Conclusions

Taken together, the water which was influenced by the TSE samples partially showed other characteristics than the initial tap water without any influence. In terms of a beneficial effect on the cellular level, Zinc TSE seemed to be the best all-purpose sample with a special emphasis on regeneration/wound healing processes as well as the inactivation of exogenous or endogenous reactive oxygen radicals. From my point of view this is not surprising, because zinc in various chemical compounds is known to promote the processes mentioned above.

Interestingly, Copper TSE was only effective in the inactivation of endogenously generated radicals. All other tests conducted with Copper TSE were not really successful. This might be related to the fact that copper compounds are often cytotoxic, but possess antimicrobial activity (which was not checked in this study). The other two TSE samples were more or less inconspicuously.

However, primary attention should be also focussed on the fact that TSE samples were able to influence or inform the water within a period of 24 hours. Moreover, it should be considered that the presented results are preliminary results of one experiment, i.e. they need some more independent replications to allow a scientifically relevant statement.



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