THE ROLE OF ZINC PLUS OCTENIDINE IN THE REGULATION OF GENE EXPRESSION: AN *IN VITRO* STUDY

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Zinc was known in ancient times, and is diffused in the environment. The potential benefits offered by zinc supplementary therapy have been demonstrated in numerous clinical trials using oral or topical zinc products. The benefit of zinc can be in principle increased through association with other actives. The aim of this study is to evaluate the effect on primary human gingival fibroblast cell of a new formulation containing zinc and octenidine cations. Human gingival fibroblast cells were obtained from three healthy patients (14-year-old man, 15-year-old woman and 20-year-old man) during extraction of teeth. The gene expression of 14 genes (ELANE, FN1, FBN, ITGA1, HAS1, ELN, DSP, ITGB1, HYAL1,TGFB1, TGFB2, TGFB3, TGFBR1 and TGFBR2) was investigated in HGF cell culture treated with 80µM of Octenidine, 1000µM of Zinc, 80µM Octenidine + Zinc solution and the medium alone at 30 min. PrestoBlue™ data showed that as the active concentration increases (Octenidine, Zinc and Octenidine + Zinc) the percentage of cell vitality compared to that of untreated cells decrease. In this study, no statistically significant gene expression was observed between cells, treated with difference substances, and control cells. Our results points out that zinc plus octenidine shows a positive potential in periodontal disease treatment.

The human mouth is colonised by microbioma, composed by hundreds of species selected from several thousand species found across human populations. The oral microbioma is in relationship with specific oral habitats (1-5). In spite of different genetic patterns, the oral microbioma remains broadly similar across human populations. This stability is the result from the interaction of the entire oral microbioma and the metabolic interactions between bacteria. Environmental changes often allow the increase of opportunistic microorganism, resulting in conditions such as oral candidiasis (6-12). With these exceptions, the microbioma usually exists in a benign and stable relationship with the host. However, the oral microbioma is also the cause of the most common chronic diseases affecting

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humans, such as dental caries, gingivitis, periodontal disease and peri-implantitis (13-20).

The specific microbioma causing gingivitis remains unclear, however it is known that excessive accumulation of dental plaque produces gingivitis. Periodontitis while started by bacteria also appears to be linked to immune-inflammatory.

Gingivitis is prevented or treated by domestic oral hygiene. The effect of tooth brushing rests on the mechanical removal of dental plaque, with this process assisted by toothpaste formulations containing excipients such as abrasives and surfactants. However, mechanical removal is not sufficient, as a consequence the incorporation of additional antimicrobial actives in toothpastes as an adjunct to mechanical plaque control has been proposed (21).

Zinc (Zn) has been advocated based on a number of beneficial antimicrobial effects. In *in vitro* studies, Zn inhibits a range of microbial enzyme systems, including a range of glycolytic enzymes in streptococci, with concomitant increased sensitivity of the organisms to acid (22). In two typical oral anaerobes, *Fusobacterium nucleatum* and *Prevotella intermedia*, a range of enzymatic processes are inhibited by Zn, including catabolism of amino acids, sugars and peptides, as well as oxidative metabolism (23). Zn can also deliver beneficial effects for bad breath: reducing breath concentrations of volatile sulphur compounds (VSCs) associated with halitosis (24, 25) as well as have direct (26) and indirect inhibitory effects on anaerobe mediated- VSC production.

In this paper we describe the effect on primary human gingival fibroblast cell (HGF) of a new liquid formulation composed by zinc and octenidine (ZO).

MATERIALS AND METHODS

Primary human gingival fibroblast cell (HGF) culture

Human gingival fibroblast cells were obtained from three healthy patients (a 14-year-old man, a 15-year-old woman and a 20-year-old man) during extraction of teeth. The gingival pieces were fragmented with a scalpel and transferred in culture dishes containing Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Inc., St. Louis, Mo) supplemented with 20% fetal calf serum (FBS) and antibiotics, i.e. penicillin 100U/ml and streptomycin 100mg/ml (Sigma Aldrich, Inc.).

Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of gingival tissue were removed from the culture dishes. Cells were harvested after additional 24h of incubation.

Cell viability test

Stock solutions of Octenidine, Zinc and Octenidine + Zinc 0.2% were prepared. Further dilutions were made with the culture medium to the desired concentrations just before use. HGF cells were seeded into 96-well plates at a density of 10^4 cells per well, in 100µl of cell culture medium, and incubated for 24h to allow cell adherence.

Serial diluted solutions containing Octenidine, Zinc and Octenidine + Zinc (10µM, 50µM, 150µM, 200µM, 3000µM, 500µM, 1000µM, 1500µM) were added (three wells for each concentration). The cell culture medium alone was used as negative control. After 30 minutes, 3h and 24h of incubation, cell viability was measured using PrestoBlue™ Reagent Protocol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the PrestoBlue™ solution (10µl) was added into each well containing 90µl of the treatment solution.

Plates were then placed back into the incubator for 1h, after that absorbance was measured at the wavelength of 570 nm by an automated microplate reader (Sunrise™, Tecan Trading AG, Switzerland). The percentage of viable cells was determined by comparing the average absorbance in treated wells with the average absorbance in control wells. The results are presented as the mean ± standard deviation of three measures only for 30 minutes of treatment because at 3h and 24h of treatment the percentage of living cell was less than 60% for all the different tracts.

Cells treatment

Once determined the most appropriate concentration for the treatments, HGF cells were seeded at a density of 1.0 x 10^5 cells/ml into 9cm^2 (3ml) wells. Cells were washed two times with PBS and incubated for 18h at 37 °C with serum-free DMEM.

After serum starvation, cells were treated with the following solution: 80µM of Octenidine, 1000µM of

Zinc, 80μM Octenidine + Zinc solution and medium alone for 30 min.

All solutions were obtained in DMEM supplemented with 2% FBS, antibiotics and aminoacids. For each treatment three biological replicates were performed. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. At the end of the exposure time cells were trypsinized and processed for RNA extraction.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, Inc., St. Louis, Mo), following the manufacturer's instructions. Total RNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary cDNA was obtained using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) starting from 500 ng of total RNA.

Real Time Polymerase Chain Reaction (Real Time PCR) and statistical analysis

The cDNA was amplified by Real Time PCR. The amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), and the specific assay was designed for the investigated genes. SYBR assay reactions were performed in a 20μl volume using the ABI PRISM 7500 (Applied Biosystems). Each reaction contained 10μl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems), 400nM concentration of each primer and cDNA.

All experiments performed included non-template controls to exclude contamination of reagents. PCR was performed with two biological replicates.

Expression was quantified using Real Time PCR. The

Table I. *Primers sequences for SYBR® Green assay.*

Gene symbol	Gene name	Primer sequence (5' 3') →
DSP	*Homo sapiens* desmoplakin	F - ATGACCTGAGGAGAGGACGAA R - AGGCTCTCTCTTTCCTGTACCAC
ELN	*Homo sapiens* elastin	F - CTAAATACGGTGCTGCTGGC R - CATGGGATGGGGTTACAAAG
HAS1	*Homo sapiens* hyaluronan synthase 1	F - CTCGGAGATTCGGTGGACTA R - CGCTGATGCAGGATACACAG
ELANE	*Homo sapiens* elastase, neutrophil expressed	F - CTACGACCCCGTAAACTTGCT R - CCTCACGAGAGTGCAGACGTT
HYAL1	*Homo sapiens* hyaluronoglucosaminidase 1	F - ACAGATGTATGTGCAACACCG R - AAGGGCCCCAGTGTAGTGTC
FN1	*Homo sapiens* fibronectin 1	F - CCATAGCTGGAAGTGTTTTG R - CCAGTAAATCTACCATCATCC
FBN1	*Homo sapiens* fibrillin 1	F - AAAGATTTGATGAGTGTGC R - GTATGGTGTTGGGTAAATCC
ITGA1	*Homo sapiens* integrin subunit alpha 1	F - CAGGTTGGAATTGTACAGTATG R - TGTCTATTCCAAGAGCTGTC
ITGB1	*Homo sapiens* integrin subunit beta 1	F - ATTCCCTTTCCTCAGAAGTC R - TTTTCTCCATTTTCCCCTG
TGFB1	*Homo sapiens* transforming growth factor beta 1	F - AACCCACAACGAAATCTATG R - CTTTTAACTTGAGCCTCAGC
TGFB2	*Homo sapiens* transforming growth factor beta 2	F - AGATTTGCAGGTATTGATGG R - ATTTCTAAAGCAATAGGCCG
TGFB3	*Homo sapiens* transforming growth factor beta 3	F - TGTTGAGAAGAGAGTCCAAC R - ATCACCTCGTGAATGTTTTC
TGFBR1	*Homo sapiens* transforming growth factor beta receptor 1	F - AGACAATGGTACTTGGACTC R - GTACCAACAATCTCCATGTG
TGFBR2	*Homo sapiens* transforming growth factor beta receptor 2	F - GGAGAAAGAATGACGAGAAC R - AGATGATGTTGTCATTGCAC
RPL13	*Homo sapiens* ribosomal protein L13	F - ATTCACAAGAAGGGAGACAG R - GAAATTCTTCTCTTCCTCAGTG

F: forward; R: reverse.

gene expression levels were normalized to the expression of the housekeeping gene Homo sapiens ribosomal protein L13 (RPL13). Mean expression levels of treated cells were calculated as fold changes relative to the expression of untreated cells with the delta/delta calculation method (27-31). Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems), and are listed in Table I.

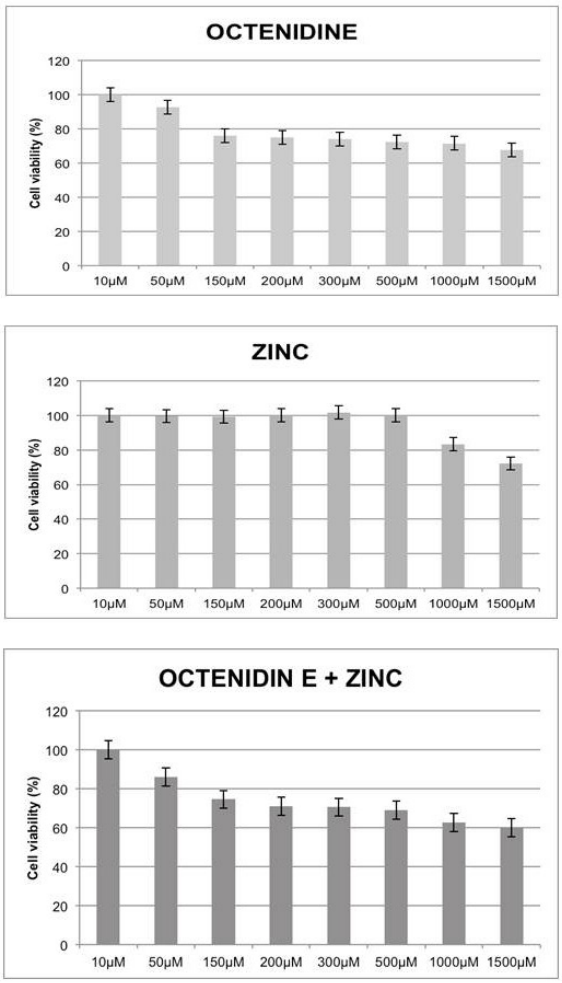

Fig. 1. *Cell viability of Octenidine (**a**); Zinc (**b**); Octenidine + Zinc (**c**) solution in HGF cell line assessed by PrestoBlue™ Reagent Protocol.*
Cells were treated with different concentrations of Octenidine, Zinc and Octenidine + Zinc solution (10 µM, 50 µM, 150 µM, 200 µM, 3000 µM, 500 µM, 1000 µM, 1500 µM) for 30 min.

RESULTS

PrestoBlue™ data showed that as the concentration of actives increases (Octenidine, Zinc and Octenidine + Zinc 0.2%) the percentage of cell vitality compared to that of untreated cells decreases (Fig. 1).

The gene expression of 14 genes (ELANE, FN1, FBN, ITGA1, HAS1, ELN, DSP, ITGB1, HYAL1,TGFB1, TGFB2, TGFB3, TGFBR1 and TGFBR2) was investigated in HGF cell culture treated with 80µM of Octenidine, 1000µM of Zinc, 80µM Octenidine + Zinc solution and medium alone at 30 minutes (Fig. 2-4).

In this study, no statistically significant gene expression was observed between cells treated with different substances, and control cells.

DISCUSSION

Zn is known from ancient times, and is diffused in the environment. Zn is second only to iron in being the most abundant trace element in the human body, and its importance in metabolic processes was evident only in the 1960s, following reports of zinc-responsive growth failure in infants in rural Egypt and Iran (32).

The potential benefits offered by Zn supplementary therapy have been demonstrated in numerous clinical trials using oral or topical zinc therapy. In keeping with the early studies on supplementary Zn therapy, evidence is now available to show that not only Zn is beneficial in the healing profile but that it provides an effective level of anti-infective action.

These roles of Zn are of importance for maintaining biological homoeostasis, at both the whole organism and cellular levels. Zn has demonstrated a central role in a number of important disease processes, in fact human genome association studies reveal a strong association of a specific allele of the LC30A8 (solute carrier 30A8) gene with Type 2 diabetes mellitus (33, 34), and results of recent functional studies corroborate this association (35, 36). A role for Zn in the pathology of Alzheimer's disease has been longer-established, supported by many lines of evidence, including the accumulation of Zn in Alzheimer's disease cortex (37) and the

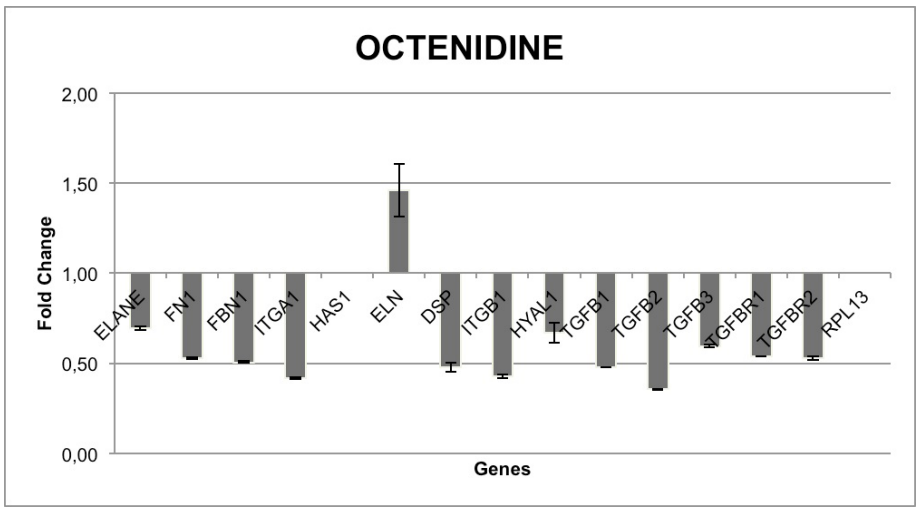

Fig. 2. *Gene expression profile of HGF treated cells (80μM of Octenidine) at 30 min. SYBR® Green assay.* ***ELANE*** *= 0.69;* ***FN1*** *= 0.53;* ***FBN1*** *= 0.51;* ***ITGA1*** *=0.42;* ***HAS1*** *= 1.00;* ***ELN*** *= 1.46;* ***DSP*** *= 0.48;* ***ITGB1*** *= 0.43;* ***HYAL1*** *= 0.67;* ***TGFB1*** *= 0.48;* ***TGFB2*** *= 0.35;* ***TGFB3*** *= 0.59;* ***TGFBR1*** *= 0.54;* ***TGFBR2*** *= 0.53;* ***RPL13*** *= 1.00.*

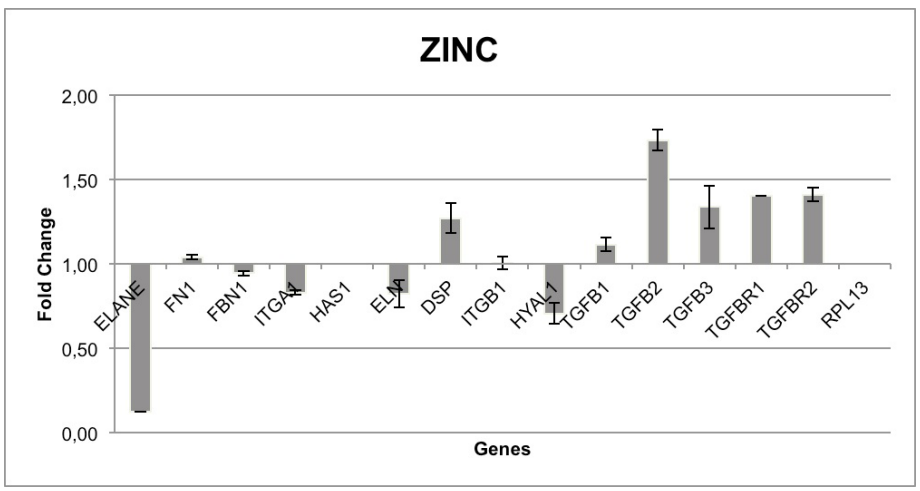

Fig. 3. *Gene expression profile of HGF treated cells (1000μM of Zinc) at 30 min. SYBR® Green assay.* ***ELANE*** *= 0.12;* ***FN1*** *= 1.04;* ***FBN1*** *= 0.95;* ***ITGA1*** *=0.83;* ***HAS1*** *= 1.00;* ***ELN*** *= 0.82;* ***DSP*** *=1.27;* ***ITGB1*** *=1.00;* ***HYAL1*** *= 0.71;* ***TGFB1*** *= 1.12;* ***TGFB2*** *= 1.73;* ***TGFB3*** *= 1.34;* ***TGFBR1*** *= 1.41;* ***TGFBR2*** *= 1.41;* ***RPL13*** *= 1.00.*

observation that perturbation of Zn homoeostasis, either pharmacologically or through diet (38), affects disease symptoms in mouse models. Toothpastes or solutions formulated to contain zinc were tested for antibacterial activity. Previous studies tested the efficacy of ZO as an antimicrobial composition. The results obtained demonstrated that ZO exerts a substantial and beneficial chemotactic effect on the macrophages and neutrophils (39).

In this work, we evaluated the *in vitro* effect of ZO on fibroblasts behaviour in term of cell viability, survival, growth and collagen production.

Our PrestoBlue™ data showed that as the concentration of substances, Octenidine, Zinc and Octenidine + Zinc 0.2%, increases the percentage of cell vitality compared to that of untreated cells

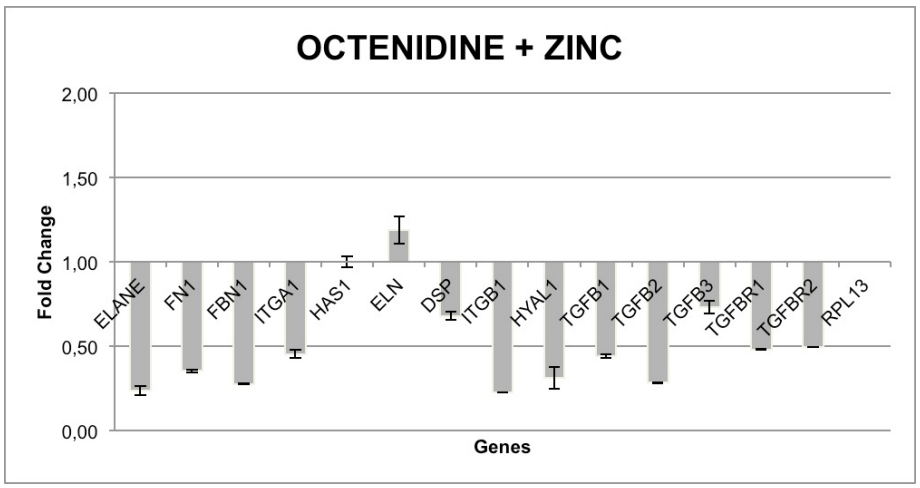

Fig. 4. *Gene expression profile of HGF treated cells (80µM of Octenidine + Zinc) at 30 min. SYBR® Green assay.* ***ELANE*** *= 0.23;* ***FN1*** *= 0.35;* ***FBN1*** *= 0.28;* ***ITGA1*** *=0.45;* ***HAS1*** *= 1.00;* ***ELN*** *= 1.19;* ***DSP*** *=0.68;* ***ITGB1*** *=0.23;* ***HYAL1*** *= 0.31;* ***TGFB1*** *= 0.44;* ***TGFB2*** *= 0.28;* ***TGFB3*** *= 0.73;* ***TGFBR1*** *=0.48;* ***TGFBR2*** *= 0.50;* ***RPL13*** *= 1.00.*

decreases.

ZO inhibits the growth of several bacterial species. Gram-positive organisms appear to be more sensitive to ZO than Gram-negative bacteria. In particular it is effective against *Streptococcus* groups A, *Staphylococcus aureus*, *Streptococcus* group B. *Escherichia coli*, *Klebsiella sp*., *Enterobacter sp*. *Proteus sp*., *Pseudomonas aeruginosa*, *Enterococcus sp*. ZO shows also antibacterial activity against aerobic and anaerobic pathogens. ZO inhibits attachment and growth of *S. aureus in vitro* and *in vivo*.

There is currently a trend in the increased use of antimicrobial agents like silver and iodine. The effectiveness of antimicrobial therapies depends on host defence mechanisms and virulence factors. Altogether, our results point out that the percentage of cell vitality compared to that of untreated cells decreases when in contact with ZO, as a consequence we can foresee an interesting positive potential for the new formula in periodontal disease treatment.

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