IJMCM
Spring 2020, Vol 9, No 2
DOI: 10.22088/IJMCM.BUMS.9.2.165

Original Article

Comprehensive Analysis of Zinc Derivatives Pro-proliferative, Anti-Apoptotic and Antimicrobial Effect on Human Fibroblasts and Keratinocytes in a Simulated, Nutrient-deficient Environment *In Vitro*

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Submmited 16 March 2020; Accepted 7 July 2020; Published 10 August 2020

Zinc as therapeutic agent in skin and wound care has been known for centuries, but its role is controversial and comprehensive investigations in nutrient-deficient environments are lacking. We aimed to provide a broad analysis of different zinc derivatives on proliferation, apoptosis and antimicrobial properties in a simulated nutrient-deficient environment *in vitro*. Human fibroblasts (CRL2522) and keratinocytes (HaCaT) were treated with a broad concentration range (10 – 0.0001 µg/mL) of zinc-sulfate ($ZnSO_4$), -gluconate (ZnGluc) and -histidine (ZnHis) for 1-6 days under nutrient-deficient media conditions. Cell proliferation was investigated by XTT assay. Targeted analyzes in proliferation (*E2F1*, *PCNA*) and apoptosis (*TP53*) associated genes were performed via qRT-PCR and apoptosis was determined via FACS (annexin V/7-AAD staining). Antimicrobial efficacy was investigated using a quantitative suspension method against *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. The results indicated that 0.1 to 0.001 µg/mL Zn increased cell proliferation in both cell lines. Fibroblasts were more susceptible with significant proliferation peaks on days 2 & 6, and days 1 & 4 for keratinocytes. No relevant changes in gene expression were detected for *E2F1* and *PCNA* nor for *TP53*. Annexin-V/7-AAD-staining of fibroblasts revealed a small, yet insignificant reduction of apoptosis induction for ZnGluc and $ZnSO_4$. ZnGluc and $ZnSO_4$ (0.1%) achieved high microbial reductions (4-5 $\log_{10}$ reductions) against tested pathogens. ZnGluc and $ZnSO_4$ showed relevant pro-proliferative and antimicrobial, as well as tendential anti-apoptotic features in a simulated nutrient-deficient microenvironment *in vitro*. This further validates a potential benefit of local zinc treatment in deficient wound microenvironments.

Key words: Wound healing, zinc, fibroblasts, keratinocytes, antimicrobial efficacy, cell proliferation, apoptosis

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[DOI: 10.22088/IJMCM.BUMS.9.2.165]

Zinc (Zn) is an essential trace element with important functions in tissue repair, regeneration (1) and re-epithelialization (2). With a total concentration of 2-4 g Zn in the human body (3), the epidermis contains about 50-70 µg/g, the dermis about 10-50 µg/g (approximately 6 % of total body Zn) (4, 5). Zn deficiency is associated with several skin diseases such as eczematous dermatitis or serious forms of epidermolysis bullosa (6, 7) as well as general immune dysfunction leading to impaired responses in inflammation and infection on a systemic level (3). Mainly diagnosed in elderly patients (due to malnutrition) it also facilitates disturbed and prolonged wound healing, demonstrating the prominent role of Zn in human skin regeneration (8, 9).

During reparative processes, Zn concentrations increase up to 20% compared to intact skin (10). Zn is an essential component of protein complexes such as metallothioneins (MT), matrix metalloproteinases (MMPs), integrins and Zn finger transcription factor, all of which are involved in wound healing and dermal regeneration (11, 12). MMPs are Zn-dependent enzymes, containing a catalytic Zn-binding domain, for proteolytic cleavage of extracellular matrix (ECM) components to modulate cell migration and ECM reconstruction for wound closure (13, 14). Supplemented Zn ions increase the upregulation of MTs, accelerating biochemical and molecular processes in hemostasis, inflammation and cell proliferation (15), while also influencing integrin subunit induction and keratinocyte motility (16).

Even though Zn as topical agent in skin disease and wound therapy has been around for centuries and is still widely applied today, evidence for Zn as topical agent in wound healing remains poor (17). While widely investigated on a systemic level and in different dermatologic conditions (18-20), studies aiming for the benefits of topical Zn treatment and supplementation, especially regarding cell proliferation and protection in a stressful, nutrient-deficient microenvironment as generally encountered in chronic wounds in elderly patients with arterial, venous, diabetic ulcers or pressure injuries, are certainly rare (21).

Nonetheless, application of topical Zn proved partially effective in varying chronic wounds and Zn-deficient patients (19, 22). Also, to some extent, antimicrobial activity has been reported for Zn (23, 24), which is highly relevant considering that all chronic wounds are at least contaminated, and infectious exacerbation needs to be prevented.

Consequently, this *in vitro* study aimed to investigate the potential benefits of topical Zn supplementation in a nutrient-deficient setting (as encountered in chronic wound environments), using different Zn derivatives (Zn-sulfate, Zn-gluconate and Zn-histidine) in a broad concentration range regarding proliferation, apoptosis and targeted, suspected associated gene expression in human fibroblasts and keratinocytes. To monitor a certain long-term dynamic, a 6-days' time period was studied. Additionally, the antimicrobial efficacy of the tested derivatives within 48 h was investigated on four microbial wound pathogens.

Materials and methods

Zn derivatives, cell cultivation and test organisms

$ZnSO_4$, ZnGluc (both Dr. Paul Lohmann GmbH, Emmerthal, Germany) and ZnHis (Dr. Falk Pharma GmbH, Freiburg, Germany) were obtained as salts and dissolved in double-distilled water. Dilutions ranging from 0.0001 µg/mL to 10 µg/mL were prepared for investigations to cover a broad range of concentrations.

Human fibroblasts (BJ ATCC® CRL-2522™; LGC Standards GmbH, Wesel, Germany) and HaCaT keratinocytes (DKFZ, Heidelberg, Germany) were cultured and maintained in DMEM (Biochrom, Berlin, Germany) with phenol red and 10% FBS (PAN Biotech, Aidenbach, Germany). The medium was supplemented with 1% kanam-

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ycin, 1 mM pyruvate, 2 mM glutamine, and 1 ng/mL EGF/bFGF (all PAN Biotech, Aidenbach, Germany). Cells were maintained at 37 °C in a humidified (90%) incubator with 5% CO_2 (ThermoFisher, Marietta, USA). Cells were subcultured by trypsinization. Prior to test assays, cells were freshly cultured under described ideal conditions for 24 h.

Pseudomonas aeruginosa (DSM-939), *Staphylococcus aureus* (DSM-799), *Escherichia coli* (DSM-11250), and *Candida albicans* (DSM-1386; all DSMZ, Braunschweig, Germany) were used as microbial test strains for antimicrobial efficacy testing. Bacteria and fungi were cultivated in sterile casein/soy peptone or malt/soy peptone broth (CSB/MEB) and on casein/soy peptone or malt/soy peptone agar plates (CSA/MEA). Microbial test solutions were prepared by colony picking from the third subculture and adjusted to an initial colony-forming unit (CFU) counts of 1.5 – 3.0 x 10^8 mL^{-1} (0.5 McFarland standard). Microbial growth controls (untreated) were conducted over the test-period for proliferation, stasis or reduction calculations.

Nutrient-deficient *in vitro* cell culture set up

A nutrient-deficient set up was established to approximately mimic the potentially nutrient-deprived, stressful microenvironment for cells within a chronic wound. Therefore, cells were cultured in DMEM, supplemented with 3% FBS only and without phenol red during test periods. Respective dilutions of Zn derivatives were added in the beginning and medium was changed on the third day, again containing appropriate dilutions of the Zn derivatives. For cell proliferation, fibroblasts were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 500 cells/well and HaCaTs at a density of 1500 cells/well. For gene expression analysis, fibroblasts and HaCaTs were seeded at a density of 70% confluency in 6-well plates (Sarstedt, Nümbrecht, Germany).

For proliferation and apoptosis assays, cells were treated for 1-6 days after initial cultivation for 24 h. Proliferative behavior was observed in both cell lines for a broad range of Zn concentrations from 0.0001 to 10 µg/mL. Subsequent analyzes of gene expression and apoptosis induction were conducted more focusely, targeting concentrations and time-points that proved most promising in proliferation assays. Gene expression analysis was performed for 0.01 and 0.001 µg/Ml Zn after 48 h in fibroblasts and 0.1 and 0.01 µg/Ml Zn after 24 h in HaCaTs, based on the first “proliferation peak” observed for both cell lines, targeting the anticipated highest mRNA transcription. Additionally, 10 µg/mL of each derivative was used as a control, impairing proliferation. Culture medium was controlled for basal Zn concentrations and proven to contain no Zn of its own (as declared by manufacturer).

Cell proliferation assay

To measure proliferation and viability of fibroblasts and keratinocytes, an XTT assay (Trevigen via Biozol Diagnostica GmbH, Eching, Germany) was used according to manufacturer's instruction. Cell proliferation was measured as difference in absorbance at 430 and 690 nm using a spectrophotometer (EON™; BioTek Instruments, Bad Friedrichshall, Germany) compared to the untreated control. All experiments were performed as three biologic replicates, and results were calculated as percentage relative to the untreated control.

Gene expression analysis via quantitative real-time PCR (qRT-PCR)

Changes in the proliferation-associated genes *PCNA* (proliferating cell nuclear antigen) and *E2F1* (E2F transcription factor-1) as well as the apoptosis-associated gene *TP53* (tumor protein P53) were investigated by quantitative real-time PCR (qRT-PCR) using Brilliant II SYBR® Green QPCR Master Mix and the Stratagene Mx3000P qPCR System (Aligent Technologies, Ratingen, Germany). RNA-isolation was conducted using

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RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Cells were washed with refrigerated PBS and lysed with 350 µL RLT-Buffer (containing ß-mercaptoethanol). RNA samples were eluted in 30 µL RNAse-free water and nucleic acid was quantified via spectrophotometer (EON™; BioTek, Bad Friedrichshall, Germany). cDNA was synthesized using RevertAid RT Kit (Thermo Fisher Scientific, Marietta, USA). In general, 1 µg of total RNA was reverse transcribed, and subsequently stored at -20 °C. The synthesis was controlled by performing a semi-quantitative PCR with glyeraldehyde-3-phosphate dehydrogenase (*GAPDH*) primer. Relative gene expression quantification was calculated by applying the $\Delta\Delta C_t$ method (32,33) with *GAPDH* as calibrator. The levels of target gene expression were calculated by using $2^{-\Delta\Delta Ct}$. Exon-overlapping primers (Invitrogen, Karlsruhe, Germany) were designed for PCR reactions.

FACS analysis of apoptosis induction

Induction of early and late apoptosis or necrosis were assessed via fluorescence-activated cell sorting (FACS) using an AnnexinV/7-AAD staining. Briefly, cells were washed with PBS, dissociated with accutase (Gibco, Dramstadt, Germany), resuspended in PBS and subsequently centrifuged for 10 min at 300 × g. Supernatant was discarded. Cell pellets were incubated for 20 min at room temperature in a dark place with 5 µL annexin V-APC and 5 µL 7-amino-actinomycin D (7-AAD), a DNA intercalating dye. Subsequently, 400 µL annexin V binding buffer was added to each sample and mixed gently. Cells were analyzed using the BD Accuri™ C6 flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). For annexin V/7-AAD staining; 10.000 cells per cell gate were analyzed. All used components for FACS analysis were obtained from BD Pharmingen (Becton Dickinson GmbH, Heidelberg, Germany). Viable cells were tracked as annexin V and 7-AAD negative, cells in early phase of apoptosis as annexin V-positive and 7-AAD-negative, and cells at the end stage of apoptosis or necrosis respectively as annexin V and 7-AAD positive.

Antimicrobial efficacy evaluation

The antimicrobial efficacy of the Zn derivatives was evaluated in a concentration range of 0.0001% to 0.1% after 2, 4, 24 and 48 h of treatment using a modified quantitative suspension method essentially based on DIN EN 13727:2012 (25). Briefly, 1 mL of freshly prepared microbial test solution was added to 9 mL of prepared Zn derivative dilutions and incubated under agitation. After the designated exposure time, an aliquot of 0.5 mL was extracted, serially 10-fold diluted and 25 µL of each dilution step was plated onto CSA or MEA and incubated under aerobic conditions at 37 °C over night. Surviving microorganisms (in CFU ml^{-1}) were determined via Colony Counter Pen (eCount™, VWR Leicestershire, UK). Assays were performed in triplicates for each tested Zn derivative and microorganism.

Statistical analysis

For all experiments mean values ± standard error of the mean (SEM) were calculated from three experimental biological replicates and differences were considered statistically significant at $P < 0.05$. Statistical evaluations and graphical design were conducted with FCS Express Cytometry 7 (DeNovo Software, Pasadena, USA) and GraphPad Prism 8.1.0 statistical software (GraphPad Software Inc., La Jolla, USA), using a two-way repeated measures ANOVA with Dunnett's test as post-hoc analysis for multiple comparison. For gene expression changes, one-way ANOVA with Dunnett's test was used.

Results

While a broad range of concentrations (10, 1, 0.1, 0.01, 0.001 and 0.0001 µg/mL) was tested for all three Zn derivatives over the course of 6 days, results are being displayed condensed with focus on

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results with relevant or significant impact.

Cell proliferation of short-term and long-term Zn treatment

For all Zn derivatives ($ZnSO_4$, ZnGluc, and ZnHis), 0.01 and 0.001 µg/mL overall demonstrated the most significant cell proliferation enhancement under simulated stress conditions (Figure 1). Within 24 h, 0.01 µg/mL of nearly any tested derivative (except $ZnSO_4$ in keratinocytes) significantly increased the proliferation of fibroblasts and keratinocytes ($P < 0.05$; Figure 1a and c). After 48 h, proliferation rates had dropped for keratinocytes, yet still showing about 10-20% more growth than the untreated control (Figure 1d). Fibroblasts on the other hand were highly stimulated resulting in significant growth increase of 30-50% for 0.1, 0.01, and 0.001 µg/mL ZnHis (29.9%, 45.3%, and 49.4%; $P < 0.05$), 0.01 µg/mL $ZnSO_4$ (45.9%; $P < 0.05$), 0.01 µg/mL ZnGluc (50.5%; $P < 0.001$; Figure 1b). A long-term Zn application showed a remarkable and significant amelioration of fibroblast proliferation within six days (Figure 2), especially for 0.001 µg/mL ZnGluc (65.1%; $P < 0.001$), and 0.01 and 0.001 µg/Ml ZnHis (83.2% and 74.4%; $P < 0.05$) on day 6. Overall, fibroblasts demonstrated proliferation peaks on days 2 and 6, while keratinocyte proliferation peaked on days 1 and 4 (Figure 2).

Fig. 1. Proliferation of human fibroblasts and keratinocytes in a simulated nutrient-deficient microenvironment *in vitro* under treatment with varying Zn derivative concentrations after 24 and 48 h. Fibroblasts (a, b) and keratinocytes (c, d) treated with effective Zn derivatives in concentrations of 0.001 up to 10 µg/mL for 24 and 48 h compared to an untreated control. Each experiment was performed three times, values were determined in triplicates. Data represent mean values ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control

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Fig. 2. Course of fibroblast and keratinocyte proliferation over 6 days under repeated zinc supplementation in a simulated nutrient-deficient microenvironment *in vitro*. Fibroblasts (left column) and keratinocytes (right column) treated with zinc derivatives over the course of 6 days compared to an untreated control. Arrows indicate time-point of medium change (nutrient-deficient) and repeated zinc supplementation. Each experiment was performed three times, values were determined in triplicates. Data represent mean values ± SEM. Symbols represent significant changes in proliferation compared to the untreated control (# for 10 µg/mL; + for 0.1 µg/mL; * for 0.01 µg/mL, and § for 0.001 µg/mL). One symbol equals P < 0.05, two P < 0.01 and three P < 0.001 vs. control

Keratinocytes on the other hand, demonstrated a lower susceptibility to Zn derivatives. A long-term application of Zn resulted in a proliferation peak at day 4, especially for 0.1 and 0.01 µg/mL for all three derivatives, yet significant results were observed on days 5 and 6 with lower growth increase. ZnHis thereby proved significant in concentrations of 0.1 µg/mL on days 4 (28.1%; P<0.05) and 5 (42.4%; P < 0.05) as well as in 0.001 µg/mL on day 6 (20.9%; P < 0.05). ZnGluc significantly improved proliferation at 0.01 µg/mL on day 4 (34.4%; P < 0.05) and at 0.1% on day 6 (28.0%; P < 0.05), while $ZnSO_4$ showed significant inductions only at 0.01 µg/mL on days 5 (20.2%; P < 0.05) and 6 (15.6%; P <0.05) with lower increase. Overall, Zn effects on keratinocyte proliferation

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were less pronounced and exerted a variegated pattern (Figure 2).

Overall, after 24 h, a significant positive effect was observed in comparison with untreated control, especially for ZnGluc, which achieved the most short-term proliferation induction (48 h; Figure 1a) and the most sustained long-term effect over a concentration range of 0.1 to 0.001 µg/mL (up to 6 days; Figure 2). In terms of absolute numbers, ZnHis showed the highest proliferation rates in fibroblasts in the long-term, while $ZnSO_4$ demonstrated the lowest overall effect. HaCaT cells were generally less affected by Zn, both positively (lower proliferation induction) but also negatively, proving more resilient to higher Zn doses (10 µg/mL), which proved to be toxic for fibroblasts (Figures 1 and 2).

Proliferation and apoptosis-associated genes expression

Statistically significant changes were only

Fig. 3. Gene expression of proliferation- and apoptosis-associated genes of human fibroblasts and keratinocytes. Fibroblasts and keratinocytes cultured in nutrient-deficient media and treated with different concentrations of Zn derivatives for 48 and 24 h, respectively. qRT-PCR measurements of proliferation-associated genes *E2F1*, *PCNA* and apoptosis gene *TP53* mRNA levels were performed in cells treated with Zn derivatives as well as untreated cells and normalized to *GAPDH* levels. Each experiment was performed three times, values were determined in triplicates. Data represent means ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control.

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Fig. 4. Apoptosis in human fibroblasts after treatment with Zn derivatives on days 1 and 6. Cells were treated with effective concentrations of (a) ZnGlu and (b) $ZnSO_4$ (0.001 up to 0.1 µg/mL) for an incubation time of 6 days total. Apoptosis was evaluated using staining with annexin V FITC and 7-AAD and FACS analysis. Upper row demonstrates results on day 1, lower row on day 6, of Zn treatment. Summary of vital, early- and late-apoptotic/necrotic cells on day 1 or 6, dependent on concentration (0.001 – 0.1 µg/mL) and calculated in relation to an untreated control, is shown on the far right. Additionally, exemplary FACS results from controls (far left) and treatments with 0.01 µg/mL ZnGlu or $ZnSO_4$ (middle) on day 1 (upper row) and day 6 (lower row) are depicted, showing absolute levels of late-apoptotic/necrotic (gate 1; G1), early-apoptotic (gate 2; G2) and vital cells. Each experiment was performed three times, values were determined in triplicates. Data represent means ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control.

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Fig. 5. Antimicrobial efficacy of Zn derivatives against common wound pathogens. S. aureus (a), E. coli (b), P. aeruginosa (c) and C. albicans (d) were treated with 0.1 (dark grey bar) and 0.01% (light grey bar) ZnGluc, ZnSO4 or ZnHis for a time-period of 48 h compared to an untreated control (black line). Evaluated time-points were 2, 4, 24 and 48 h and data is presented as means ± SEM of bacterial reductions compared to initial bacterial counts (in Δlog10 CFU ml-1). * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. control.

observed in treated fibroblasts in comparison with untreated control (Figure 3). 10 µg/mL $ZnSO_4$ induced a 2-fold increase in *E2F1* expression ($P < 0.05$) and a 1.5-fold increase in *PCNA* expression ($P < 0.05$). Additionally, 0.01 µg/mL ZnHis induced a 1.6-fold increase in *TP53* expression ($P < 0.05$). Generally, the mRNA expression of *E2F1*, *PCNA* and *TP53* was slightly increased, whereby a tendentially higher increase in expression for all three genes could be observed for lower Zn concentrations. However, no clear pattern was observable for either addressed gene in both cell lines (Figure 3).

Apoptosis induction by Zn derivatives

ZnGluc demonstrated continuously balanced pro- and anti-apoptotic effects, without detectable statistically significant changes. Overall, ZnGluc appeared to act tendentially protective especially in concentrations of 0.01 and 0.001 µg/mL yielding slightly less apoptotic and more viable cells in the nutrient-deficient microenvironment. At short treatment periods, ZnGluc led to the reduction of early and late apoptotic/necrotic cells, while after longer periods (days 5 and 6) the anti-apoptotic effect seemed to wear off resulting in rising percentages of apoptotic/necrotic cells primarily in higher Zn concentrations (Figure 4). Exposure to $ZnSO_4$ led to a certain anti-apoptotic effect within the first 24 h, especially in concentrations of 0.1 and 0.01 µg/ml (Figure 4). After 48 h however, an increase in early and late apoptotic/necrotic cells was observed indicating a short-term anti-apoptotic effect. This pattern was also observed on days 4 and 5, after repeated supplementation of Zn. The annexin V/7-AAD staining for ZnHis on the contrary, demonstrated mainly an increase in early

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and late apoptotic cells (Figure 4), with some non-homogeneous distribution of increase and decrease of apoptotic cells over the course of 6 days. However, the results regarding apoptosis only demonstrated small and tendential changes with no statistical significance.

Antimicrobial efficacy of Zn derivatives

Overall, 0.1% Zn showed the highest efficacy against the tested pathogens. Against *S. aureus*, 0.1% ZnGluc reached a significant growth reduction rate of 5.91±0.72 (P = 0.017) after 24 h in comparison with the untreated control. While ZnGluc failed to completely eradicate *S. aureus* within 48 h (maximum growth reduction rate: 7.25±1.15; P = 0.045), $ZnSO_4$ managed a full reduction of the pathogen after 48 h treatment (Figure 5a). 0.01% ZnGluc and $ZnSO_4$ also showed reductive tendencies yet didn't exceed 3 log_{10} reductions. After 4 h 0.1% ZnGluc achieved a significant and relevant reduction of *E. coli* (5.85±0.79; P = 0.037) and a full eradication after 24 h (Figure 5b). 0.01% ZnGluc needed 24 h to reach a significant reduction of *E. coli* (5.29±0.89; P = 0.040) and 48 h for complete eradication. $ZnSO_4$ showed a similar reduction pattern on *E. coli*, except for a higher reduction rate of 0.01% $ZnSO_4$ after 24 h (7.57±1.00; P = 0.022). In contrast, ZnHis demonstrated no relevant reductive potential in any concentration for any tested pathogen. While neither 0.001% ZnGluc nor $ZnSO_4$ demonstrated relevant efficacy against *P. aeruginosa* or *C. albicans*, 0.01% ZnGluc achieved significant reduction of *P. aeruginosa* after 24 h (4.34±0.33; P = 0.006), as did $ZnSO_4$ (7.12±1.27; P = 0.017). Interestingly, *P. aeruginosa* showed regrowth after 48 h for both derivatives (Figure 5c). In case of *C. albicans*, only 0.1% ZnGluc showed a significant reduction with a maximum of 3.54±0.66 after 48 h treatment (Figure 5d).

Discussion

The trace element Zn is ubiquitously present in the human body and is involved in numerous metabolic processes and an important component of countless enzymes and transcription factors during cellular proliferation, differentiation and regeneration (9, 12, 17, 26). Therefore, it is not surprising that Zn deficiency leads to impaired wound healing and an increased susceptibility to infection (3). Especially, zinc-oxide (ZnO) is a well-known therapeutic for various skin conditions such as dermatitis or diaper rash, and has been used in wound care for decades, even though regularly disputed. Also, antimicrobial properties have been reported *in vitro* as well as *in vivo* for ZnO (23, 24). However, the role and capability of different Zn derivatives in wound healing, especially in chronic deficient wound conditions are still poorly understood and only selectively investigated.

This study showed that some Zn derivatives have a significant proliferative impact on human fibroblasts and keratinocytes in a stressful, nutrient-deficient environment. The results thereby comply with the biologic availability of the individual Zn derivatives. It is well-documented that organic Zn salts, such as ZnHis (27) and ZnGluc (28) have a higher bioavailability than the inorganic $ZnSO_4$. Under physiological conditions, Zn undergoes stable bonding with amino acids, mainly histidine or cysteine (29). Aiken et al. showed that apart from the well-known membrane-bound Zn transporter of the ZIP family responsible for the Zn^{2+} uptake in cells, Zn efflux in red blood cells in rats occurred via ZnHis complexes (30). Also, in human skin and serum, Zn^{2+}-ions are histidine-bound and cellular intake is L-histidine dependent (27-30). Therefore, ZnHis was expected to be specifically suitable for an application in wound therapy, which was only partially confirmed. Overall, fibroblasts proved more susceptible to Zn stimulation.

Regarding antimicrobial efficacy ZnGluc and $ZnSO_4$ demonstrated a significant antimicrobial effect on common wound pathogens. However, higher concentrations of Zn derivatives (0.1 and 1

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mg/mL) than tested in cell culture assays (0.0001 – 10 µg/mL) were necessary to achieve a significant reduction, while lower concentrations showed no reductive impact on tested microorganisms (data not shown). This is in line with previously reported results and visible in daily clinical practice (9, 18, 19, 23), yet the efficacy needs to be interpreted in relation to possible cytotoxic damage if repeatedly used in a chronic wound setting: concentrations of 10 µg/mL already demonstrated a relevant amount of cytotoxicity, especially on fibroblasts after 48 h, without relevant antimicrobial efficacy observed. While no simultaneously exerted pro-proliferative and antimicrobial properties were detected, at least no bacterial growth was promoted alongside the proliferation induction of fibroblasts and keratinocytes, which is crucial in phases of granulation-tissue formation and re-epithelization to maintain a clean wound-bed.

To investigate possible mechanisms of the demonstrated proliferation induction targeted analyzes of gene expression for proliferation-associated genes *E2F1* and *PCNA* as well as apoptosis-associated gene *TP53* was performed at time-points with the highest proliferation peak. During the S-phase of cell cycle, *PCNA* is expressed in dividing cells, and its expression corresponds with cell proliferation and DNA replication (31, 32). *E2F1* acts in the G1/S transition phase by regulating cyclin A and cyclin E, crucial for cell cycle progression and DNA synthesis, thereby serving as proliferation marker (33-36). In previous studies, the inhibition of cell proliferation in murine embryonic fibroblasts caused by treatment with chelating agents could be negated by the administration of $ZnSO_4$ (37, 38). Li and Maret (2009) showed that Zn^{2+} was most effective in the G1 and the late G1/S-phase (39) by detection of enhanced expression of the phase associated genes *E2F1* and *PCNA*. On the other hand, Deters et al. (2003) showed very similar data by BrdU incorporation of primary human keratinocytes after ZnHis and $ZnSO_4$ application, basing the positive effect on the influence of Zn during the S-phase (40). Unfortunately, despite the highly positive effects in the proliferation assay, the gene expression data for *PCNA* and *E2F1* could not adequately match and therefore explain the described findings. Even though an overall trend of mRNA expression increase could be detected in the most effective pro-proliferative concentrations and derivatives, the results showed no statistical significance. A possible explanation might be, that the actual expression induction preceded the chosen time-points of gene expression analysis, yielding already declining mRNA expression changes missing the actual expression peaks. The significant expression increase upon 10 µg/mL $ZnSO_4$ stimulation, might reflect the reaction to the combined stress of the nutrient-deficient environment and the high Zn concentration triggering a reflective upregulation of mRNA expression. Therefore, the addressed genes of *PCNA* and *E2F1* cannot be postulated as the underlying cause of the pro-proliferative and protective features observed.

Another reason for the increased proliferation could be a prolonged cell viability due to a reduction in cell apoptosis/necrosis. The *TP53* gene is upregulated by cellular stress as exerted in a chronic wound environment, e.g. oxidative or metabolic stress, causing DNA damage, which was intended to be simulated in the nutrient-deficient cell culture model. This leads to cell cycle arrest and either reparative processes or apoptosis induction. In the presented data, *TP53* expression somewhat correlated with the strong cell stress induced by the nutrient deficiency, but the data presents heterogeneously with no clear tendency regarding suppression or induction of gene expression. ZnHis demonstrated the highest upregulation of *TP53* levels in fibroblasts after 48 h of application with the only significant upregulation for 0.01 µg/mL ZnHis.

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To address the possibility of a gene-independent apoptosis-related mechanism of action, the overall influence of Zn derivatives on apoptosis, induction of early and late apoptosis or necrosis respectively, was assessed in a next step. Fluorescence-activated cell sorting (FACS) with an annexin V/7-AAD staining was used to do so. These evaluations demonstrated a slight, yet insignificant reduction of early- and late-apoptotic/necrotic and an increase in viable cells for ZnGluc. The inorganic Zn salt $ZnSO_4$ only displayed some anti-apoptotic effects within 24 h following Zn application on days 1 and 4. Subsequently, the effect wears off and a pro-apoptotic impact with less viable cells can be observed. This indicates only a potential small, short-term protective effect of $ZnSO_4$ (24 h) compared to the more sustained, however still marginal effect of ZnGluc. Unexpectedly, ZnHis showed non-homogeneous results, so no sure conclusion regarding its protective or non-protective effect on human fibroblasts can be given. Despite missing statistical significance, especially the results for ZnGluc coincide with the results of Poiraud et al. (2012), who demonstrated an anti-apoptotic effect of ZnGluc by upregulating the anti-inflammatory peroxisome proliferator-activated receptors-α (*PPAR-α*) in skin explants infected with bacterial toxins (41). It is well known, that Zn has an antioxidative role in protecting cells against oxidative damage by free radicals (17). Reactive oxygen species can enhance the mitochondrial membrane permeability which leads to destruction of mitochondria followed by cell death (42, 43). Overall, the results regarding apoptosis and related gene expression (*TP53*) are missing statistical significance and relevant changes in expression and apoptosis induction under Zn treatment. Nonetheless, the observed pro-proliferative effects could still be driven on a gene-regulative level, however not by the here investigated genes. Also, even though not significant, the results for apoptosis induction still demonstrate tendencies, that fit the proliferation results. This could possibly point to the transcription-independent mitochondrial apoptotic pathway as partially influenced mechanism. The mitochondrial apoptotic pathway includes p53 acting in cytosol and mitochondria to advance apoptosis through transcription-independent mechanisms (44). Hereby, apoptosis is associated with mitochondrial breakdown and subsequent loss of cell respiration and metabolism. Cytoplasmatic p53, in response to cellular stress signals like cytotoxic agents and DNA damage, interacts with several members of the Bcl-2 family and promotes an increase in permeability of the mitochondrial outer membrane (45), leading to a collapse of cellular respiration and viability. For the measurement of proliferation and viability, cells require an intact mitochondrial succinate-tetrazolium reductase system; therefore, a possibility is that the significant increase in cell proliferation in lower Zn concentrations could be explained by stabilization of mitochondrial membrane, by functioning as an antioxidant in the highly stressful microenvironment, resulting in ameliorated cell viability of fibroblasts. With increasing concentration of Zn however, a threshold to cytotoxic levels of Zn is passed resulting in Zn itself becoming a stress-inducing cytotoxic agent inducing the mitochondrial p53 pathway. These results coincide with Rudolf et al. (2008) stating that the exposure of cells to high $ZnSO_4$ concentration induced cell injury and death by activation of extracellular-signal-regulated kinase (ERK)- and p53-dependent apoptotic pathway (46).

The presented work and study naturally need to be interpreted with caution, due to the limitations arising from its experimental *in vitro* nature. Further investigations are necessary and need to be encouraged addressing possible alternative mechanisms mentioned, such as the influence of Zn derivatives on p53 changes on the protein level, oxidative damage or possible alternative

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transcriptional pathways. Nonetheless, the necessity to further investigate Zn derivatives as local therapeutic agent in wound care is highly relevant, given the fact that Zn is still widely used in daily practice and specific knowledge regarding mechanisms of action, clear indications and limitations based on fundamental data is scarce. The fact, that no exact mechanism of action could be identified in this work, does not diminish the highly relevant and significant results regarding the proliferation induction in fibroblasts and keratinocytes and the width of proliferation and antimicrobial efficacy data it adds to the field, for future investigations and study designs.

In conclusion, the tested derivatives $ZnSO_4$, ZnGluc and ZnHis displayed concentration-dependent, highly positive effects on cell proliferation and viability as well as tendential anti-apoptotic influence for human fibroblasts and keratinocytes in a stressful, nutrient-deficient microenvironment. In contrast to keratinocytes, the extracellular matrix-forming fibroblasts showed a more profound susceptibility to local Zn supplementation. Especially ZnGluc induced the highest and most sustained proliferation and additionally seemed to exert a certain inhibitory effect on apoptosis in human fibroblasts. Regarding antimicrobial efficacy, ZnGluc and $ZnSO_4$, only achieved microbial reduction in considerably higher, potentially cytotoxic, concentrations. Carefully translated to clinical practice, the use of Zn derivatives, especially ZnGluc in a concentration-dependent manner could be beneficial in chronic wound management as a local supportive additive. However, careful consideration would be necessary regarding the appropriate concentration based on a wounds current healing phase (higher concentration in infected, inflammatory settings for antimicrobial and anti-inflammatory purposes compared to lower concentrations for proliferation induction in granulation and re-epithelization). Further investigations into the mechanism of action as well as dose-response relationship in models more closely resembling the chronic nutrient-deprived wound microenvironment as well as clinical studies will be necessary to add clarity to the long-debated case of Zn in wound management.

Acknowledgments

The authors are grateful to the colleagues V. Koester and A. Richard for their technical assistance. The complete data set for proliferation, apoptosis and antimicrobial efficacy results is available from the authors upon reasonable request. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

The authors declare no potential conflicts of interest

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