1/6

Zinc Oxide Inhibits Axillary Colonization by Members of the Genus *Corynebacterium* and Attenuates Self-perceived Malodour: A Randomized, Double-blind, Placebo-controlled Trial

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Malodour from the axilla is commonly caused by specific microbes, and may be inhibited by zinc oxide. The aim of this study was to determine the effects of zinc oxide on the axillary microbiota, odour and pH in a randomized, double-blind, placebo-controlled trial in 30 healthy volunteers. In each participant one axilla was treated with zinc oxide and the other with a placebo for 13 days. The microbiota and pH were analysed before and during treatment. At the final visit, the participants judged their own axillary odour for comparison. With zinc oxide treatment total bacterial growth and, specifically, that of odour-producing Corynebacterium spp. and Staphylococcus hominis, decreased (p < 0.05), despite an increase (p < 0.0005) in skin-surface pH. Compared with the placebo, zinc oxide treatment reduced (p = 0.005) self-perceived malodour. In vitro, Corynebacterium spp. (19 isolated strains) survival was reduced (p < 0.0005) at pH 5.0 compared with pH 6.0; growth inhibition by zinc oxide occurred at ≤400 mg/l, and cell death occurred at ≤10,000 mg/l for 12 (63%) of the strains. In conclusion, application of zinc oxide reduced malodour and the counts of causative bacteria, but increased the pH of the axilla.

Key words: microbiota; malodour; topical antiseptics; susceptibility tests; skin pH.

Accepted Apr 28, 2020; Epub ahead of print May 4, 2020

Acta Derm Venereol 2020; 100: adv00145.

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B othersome odour from the axilla is commonly caused by decomposition of apocrine gland secretions by corynebacteria and staphylococci (1–5). There is a need for effective anti-odour treatments (6). Zinc oxide (ZnO) is a white, odour-free powder and a common ingredient in dermatological topical preparations due to its protective, antiphlogistic and antibacterial activities (7–11). The first commercial deodorant ("Mum") contained ZnO. Interestingly, topical ZnO reduces the occurrence of *Corynebacterium* spp. and malodour in open wounds (12). The release of odorants from *Corynebacterium* spp.

SIGNIFICANCE

Corynebacterium spp. thrive in the intertriginous axilla, and can cause malodour. Zinc oxide is a mild antiseptic compound effective against Gram-positive organisms. In healthy volunteers, this study found that, compared with a placebo, zinc oxide formulated in an oil-in-water emulsion reduced the counts of aerobic microbiota, including corynebacteria, and decreased self-evaluated axillary malodour. The isolated *Corynebacterium* spp. showed reduced survival at pH 5.0 and susceptibility to zinc oxide *in vitro*. This study provides novel data that may prove useful for basic microbiome research and the development of efficient deodorant products for the axilla.

mediated by zinc-dependent bacterial proteinases may counteract this anti-odour effect (13).

The generation of zinc ions (Zn^{2+}) is the major mechanism for the antibacterial activity of ZnO (9). The solubility of ZnO in pure water is low, but increases many-fold in the presence of zinc-binding ligands, such as proteins (9, 14). pH is another important factor for the ionization of ZnO (15, 16), and the solubility of ZnO in an aqueous solution is ~0.25 g/l at pH 7 and ~25 g/l at pH 6 (14). Thus, pH is a determinant of the antibacterial efficacy of ZnO. Furthermore, the effect of ZnO treatment on skin-surface pH is unknown.

The primary aim of this randomized, double-blind, placebo-controlled trial was to investigate whether applications of ZnO reduces colonization by *Corynebacterium* spp. in the axillae compared with that of an oil-in-water emulsion placebo in healthy volunteers.

MATERIALS AND METHODS

The trial was approved by the Committees of Health Research Ethics in the Capital Region of Denmark (H-16045754) and registered at ClinicalTrials.gov (NCT03221699). The trial was conducted at the Digestive Disease Center, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark.

Participants

Healthy non-smoking volunteers between 18 and 65 years of age were recruited via www.forsoegsperson.dk and were included after providing written informed consent. Individuals with skin disorders; those who were pregnant, breastfeeding or receiving immunosuppressive treatment; and/or those who were hypersensitive to zinc and/or other ingredients in the formulations were excluded.

Study design, bacterial sampling, pH measurements, randomization, application of formulations and odour evaluation

Participants discontinued their habitual application of deodorants, antiperspirants or other cosmetic products to the axillae within 8 h before their first visit to hospital (day 0) and throughout the entire trial period. At baseline, day 0, the participants' axilla vault was swabbed by rotating (1 s/rotation) the tip of a dry nylon-flocked swab (eSwab, Copan, Brescia, Italy) 3 times clockwise and then 3 times counter-clockwise. This process was repeated using the side of the swab. The recovery of corvnebacteria was not increased by moistening the swab with sterile saline (0.9% (w/v) NaCl(5). The pH of the non-hairy axillary fossa was measured with an InLab® surface electrode connected to a pH meter 1140 (Mettler-Toledo, Greifensee, Switzerland). Before each measurement, the calibrated electrode was rinsed in distilled water, dabbed dry and moistened with a non-woven swab saturated in distilled water. The electrode was positioned vertically on the skin surface and the pH was recorded when the signal had been stable for 7 s (17). The ZnO and placebo formulations were compared in each participant and were randomly assigned to the right or left axilla (with a different formulation in the contralateral axilla). The allocation (1:1 ratio) was computer-generated in blocks of 10 and concealed. Starting on day 0, participants applied 0.5 ml of the indistinguishable oil-in-water emulsion containing 1.1% (w/w) ZnO and an oil-in-water emulsion placebo from masked 1-ml syringes labelled with the participant's consecutive number and right or left axilla, from the vault to 5 cm beneath the hairline, using a powder-free, disposable latex finger cot. Participants entered the time of application as morning (06:00-11:00 h), noon (11:00-14:00 h) or evening (15:00-22:00 h) in their log chart. Washing of the treatment areas within 8 h of application was not allowed. Participants were requested to reapply the assigned formulations after showers. On days 8 and 13, bacterial swabs were again obtained, and the pH was measured at the hospital; on day 13, the participants were asked: (i) whether they had observed a difference in the odour from the left and right axillae and, if so, (ii) which axilla they considered to be less odorous. Pain was reported by the participants using the short version of the validated McGill questionnaire (18).

Microbiology

Culture of swabs, semi-quantification and bacteria identification. The swab was inserted into a tube containing 1 ml Amies transport medium and kept at 4° C until processing, which was performed within 24 h. Samples (10 µl) were applied to blue agar plates with modified Conradi-Drigalski diagnostic substrate selective Bacterial growth was estimated semiquantitatively as 0 (no growth), 1×10^2 , 1×10^3 , 1×10^4 and $\ge 1 \times 10^5$ CFU/ml.

Isolated bacteria were identified by light microscopy and Microflex matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Billerica, MA, USA) with FlexAnalysisTM software. Scores ≥ 2.0 were required for identification at the species level and ≥ 1.7 at the genus level (20–22). The individual isolated strains were cryopreserved in 10% glycerol at –80°C.

In vitro investigations on corynebacteria. The strains of *Corynebacterium* spp. isolated from the axillae at baseline were used for these investigations.

The survival of each bacterial strain was investigated in 1 ml saline (0.5 McFarland standard) buffered at pH 5.0, pH 6.0 and pH 7.0 with 0.20 M 4-morpholineethanesulfonic acid (16). The turbidity was measured at λ =580 nm by spectrophotometry (DensiCHEK Plus; BioMérieux, Durham, NC, USA). The bacterial suspensions were incubated at 35°C, and aliquots (100 µl) were taken after 0 (only saline), 4 and 24 h of incubation. Serial 10-fold dilutions were made in saline, and 10 µl was seeded on 5% horse blood agar plates, which were incubated in 5% CO₂/95% ambient air for 48 h at 35°C. Colonies were counted, and the concentrations (CFU/ml) were calculated (23).

The antibacterial effect of ZnO was investigated using a broth dilution method. Initial tests showed that Müller-Hinton broth did not support the growth of the fastidious corynebacteria; therefore, another broth (0.5% Lab-Lemco beef extract powder, 1.0% special peptone, 0.3% NaCl, 0.2% Na₂HPO₄ · 2H₂O, pH 7.4) that was supplemented with 5% (v/v) horse serum was used for serial 5-fold dilutions of 5.5% ZnO with vigorous vortex mixing in sterile 2-ml screw-capped microcentrifuge polypropylene tubes (Sarstedt, Nümbrecht, Germany). The primary particle size of ZnO (EMSURE®, Ph Eur; Merck KGaA, Darmstadt, Germany) was ~5 µm. The 200-µl ZnO suspensions and control (200 µl broth with 5% horse serum alone) were inoculated with 20 µl of the respective strain in sterile saline (1.0 McFarland standard corresponding to $\sim 1 \times 10^5$ CFU/ml) resulting in final ZnO concentrations of 50,000, 10,000, 2,000, 400, 80, 16 and 3.2 mg/l. Protein precipitation occurred at Zn^{2+} concentration > 400 mg/l. The same tests were performed in parallel on a reference strain (S. aureus ATCC® 25923). The tubes were incubated with continuous shaking (500 rpm) in humidified ambient air for 24 h at 35°C. Aliquots (10 µl) were seeded on 5% horse blood agar plates, which were incubated in ambient air for 48 h at 35°C. The minimum bactericidal concentration (MBC) was determined by the lowest ZnO concentration causing no growth. Sterile resazurin (R7017; Sigma-Aldrich) solution at 0.015% in phosphate-buffered saline (pH 7.4) was then added (60 μ l) to the tubes, which were incubated in ambient air for another 24 h





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at 35°C (24). The minimum inhibitory concentration (MIC) was determined from the shift of pink to violet/blue colour (Fig. 1). pH and zinc ion measurements of the formulations. One millilitre of the ZnO formulation or 1 ml placebo formulation was centrifuged at 17,800 rpm at 4°C for 3 h in 1.5-ml Eppendorf tubes. The pH of the almost clear top layer (200-300 µl) was determined using Hamilton® MiniTrode pH electrode (Merck) connected to a PHM 95 pH/Ion meter (Radiometer, Copenhagen, Denmark). For zinc determination, the top layer after the first centrifugation (200-300 μl) was transferred to a 0.22-μm centrifuge filter unit (Ultra free-MC, Millipore) that was centrifuged for 1.5 h at 17,800 rpm at 4°C. The clear filtrate (~15 μ l/ml formulation) was diluted with 2% ultrapure grade HNO, (69%, Sigma-Aldrich), and the zinc content was determined by inductively coupled plasma-optical emission spectrometry (Avio 200 ICP-OES; PerkinElmer, Waltham, MA, USA) equipped with a Meinhard concentric glass nebulizer (type K1 LDV). Calibration was performed on samples containing 0.000, 0.050, 0.100, 0.500, 1.000 and 5.000 ppm free Zn²⁺ in 2% HNO₂. The zinc emission lines used in this study were 206.200 nm, 213.857 nm and 202.548 nm. Milli-Q[®] water was used for sample dilution. Two syringes of each formulation were analysed.

Blinding

All investigators and participants were blinded. The identities of the formulations were decoded after the analyses had been completed, and conclusions were drawn.

Sample size calculation and statistical analyses

It was expected that *Corynebacterium* spp. would dominate in the axillae of 40% of the participants, and that ZnO treatment would reduce this value to 8% (5, 12). Based on these assumptions along with a 1-tailed McNemar's test (α =5%, β =20%), we calculated that a sample size of *n*=27 was necessary. Thirty participants were included to account for dropouts.

The log-transformed microbiota data and pH data were analysed by analysis of variance (ANOVA). Self-evaluated odour rating was analysed by the Pearson χ^2 test. The effect of different pH values on *Corynebacterium* spp. survival was analysed by the *t*-test. *p*-values < 0.05 were considered significant.

Fig. 2. Corynebacterium tuberculostearicum. The bacteria were flame-fixed, stained, cover-slipped with Pertex[®] mounting medium and examined with oil immersion 100× objective. Bar: 5 µm. Gram stain.

perspirants alone, one woman used both deodorants and antiperspirants, and one man used neither deodorants nor antiperspirants.

One woman withdrew from the study after the baseline visit. The ZnO treatment was allocated to the right axilla in 13 volunteers. The ZnO and placebo formulations were applied once daily for 13 days in 16 participants and once

able I. Prevalence of bacteria isolated from the axillae of 30 healthy
olunteers before the initiation of treatment (baseline) and after treatment
vith zinc oxide (ZnO) or a placebo for 8 and 13 days ^a

RESULTS

pH and zinc ion concentrations of the formulations

The pH of the aqueous phase of the ZnO formulation was 7.77, and the pH of the placebo formulation was 5.70. The Zn^{2+} concentration in the aqueous phase of the ZnO formulation, obtained after centrifugation and elimination of solid ZnO particles, was 1,450 mg/l. The placebo formulation contained 0.6 mg/l Zn²⁺.

Participants, product application/acceptance and timing of bacterial swabs/pH measurements

Thirty non-smoking Caucasians (age 21-39 years, mean \pm standard deviation [SD] 25.6 ± 4.7 years), 15 females and 15 males, were included from 2 February 2017 to 9 March 2017. All female and no male participants had shaved axillae. Eighteen participants used deodorants alone, 10 used anti-

	Baseline	•	Day 8		Day 13	
Bacteria	ZnO (<i>n</i> = 30)	Placebo $(n = 30)$	ZnO (<i>n</i> = 29)	Placebo (<i>n</i> = 29)	ZnO (<i>n</i> = 29)	Placebo (<i>n</i> = 29)
C. jeikeium	0	0	1	0	0	0
C. kroppenstedtii ^b	2	2	0	0	0	0
C. tuberculostearicum ^b	4	6	0	7	2	7
C. ureicelerivorans	8	6	1	1	0	0
C. spp.	3	3	0	2	1	1
Enterobacter aerogenes	1	1	1	1	1	0
Enterobacter cloacae	0	0	0	0	1	0
Micrococcus luteus	4	1	6	3	7	5
Moraxella atlantae	0	1	0	1	0	1
Moraxella spp.	0	0	1	2	0	0
Propionibacterium acnes	2	3	1	1	4	2
Pseudomonas spp.	0	0	1	0	0	0
Sphingomonas paucimobilis	0	0	0	0	1	0
S. aureus	0	1	0	0	0	0
S. capitis	1	1	3	0	5	2
S. caprae	1	1	0	0	0	0
S. epidermidis	27	27	21	23	22	23
S. haemolyticus	1	0	1	1	1	0
S. hominis	5	6	5	17	8	16
S. lugdunensis	3	4	4	0	4	3
S. pasteuri	0	0	1	0	0	0
S. warneri	1	2	0	0	1	0
S. spp.	1	1	0	2	0	2

^aThe numbers of axillae with the indicated bacteria are presented. ^b*C. kroppenstedtii* and *C. tuberculostearicum* were isolated from both axillae of one female participant at baseline.

4/6 M. S. Ågren et al.



Fig. 3. Effect of zinc oxide (ZnO) on the growth of: (a the mean (SEM).

for 9–12 days and twice for 1–4 days in 13 participants, distributed into 153 applications in the morning, 105 at noon and 150 in the evening. None of the participants experienced discomfort with any formulation, except for one female who reported itching from the 5th application onwards of the placebo formulation.

Bacteria were sampled, and pH was measured in the morning in 75 of the 88 measurement sessions at the hospital.

Microbiology

At least 3 different species of the genus *Corynebacterium* were isolated and identified at baseline from 19 participants (63%), namely, 9 women and 10 men. The typical morphology of an isolated *Corynebacterium* strain is shown in **Fig. 2**. *Staphylococcus hominis* inhabited the naive axillae of 4 women and 4 men. The most prevalent bacterium in the axilla was *Staphylococcus epidermidis*, isolated from 28 (93%) of the participants. Gram-negative bacteria occurred only sporadically. The baseline composition of the microbiota was similar for the ZnO and placebo groups (**Table I**).

ZnO treatment significantly reduced the overall bacterial growth (**Fig. 3**a) and, specifically, the growth of *Corynebacterium* spp. (Fig. 3b) and *S. hominis* (Fig. 3c).

Odour evaluation

Thirteen participants reported differences in odour between the 2 treated axillae, 11 (5 women and 6 men) of whom (10 with *Corynebacterium* spp.) rated the axilla treated with ZnO as having less odour than the other axilla, which was treated with placebo (p=0.005).

Skin-surface pH

The mean \pm SD baseline axillary pH of women was 5.44 \pm 0.47, and that of men was 5.38 \pm 0.41 (p=0.60). Compared with the placebo, the ZnO treatment increased (p<0.0005) the skin surface pH by ~0.5 on days 8 and 13 (**Fig. 4**).

In vitro investigations

These investigations were carried out on the 19 isolated strains (*C. kroppenstedtii*, n=2; *C. tuberculostearicum*, n=7; *C. ureicelerivorans*, n=8; *C.* spp., n=2).

The survival of the corynebacteria decreased (p < 0.0005) from 4 h onwards at pH 5.0 compared with pH 6.0 and pH 7.0, while survival did not differ between pH 6.0 and pH 7.0 at 4 h (p=0.69) or 24 h (p=0.39) (Fig. 5).

In vitro, ZnO inhibited the growth of *Corynebacterium* spp. isolates with MIC values ranging from 3.2 to 400

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Fig. 4. Effect of zinc oxide (ZnO) on the axillary skin surface pH. Mean \pm standard error of the mean (SEM).



Fig. 5. Effect of pH on the survival of the 19 isolates of Corynebacterium spp. after incubation at 35°C for 0, 4 and 24 at pH 5.0, 6.0 and 7.0. Mean \pm standard error of the mean (SEM).

Table II. Susceptibility of Corynebacterium spp. isolates (19 strains) to zinc oxide (ZnO) in vitro

	Strains growth-inhibited (MIC) or dead (MBC) at each ZnO concentration, n									
	≤3.2 mg/l	16 mg/l	80 mg/l	400 mg/l	2,000 mg/l	10,000 mg/l	≥50,000 mg/l			
Minimum inhibitory concentration (MIC) Minimum bactericidal concentration (MBC)	2 ^a	5 ^b	10 ^c	2 ^d 1 ^e	3 ^f	8 ^g	7 ^h			

^a1 C. kroppenstedtii and 1 C. ureicelerivorans. ^b1 C. kroppenstedtii and 4 C. tuberculostearicum. ^c2 C. tuberculostearicum, 7 C. ureicelerivorans and 1 C. spp. ^d1 C. tuberculostearicum and 1 C. spp. ^e1 C. kroppenstedtii, 4 C. tuberculostearicum, 2 C. ureicelerivorans and 1 C. spp. ^h1 C. tuberculostearicum, 5 C. ureicelerivorans and 1 C. spp.

mg/l and MBC of \leq 10,000 mg/l for 12 (63%) of the 19 tested strains (**Table II**). The MIC for ZnO against *S. aureus* ATCC[®] 25923 was determined as 2,000 mg/l.

DISCUSSION

In the present randomized double-blind trial, the topical ZnO treatment decreased the levels of *Corynebacterium* spp. in the axillae of healthy volunteers. The levels of *Corynebacterium* spp. correlate strongly with axillary malodour (1–4) and may explain the reduced self-perceived axilla malodour with ZnO compared with the placebo formulation. *S. hominis* was recently identified as a malodour producer (4, 25) and its growth in the axillae was also reduced by ZnO exposure.

The anti-odour effect of ZnO was obvious for 11 of 13 participants. Exclusion of subjects with low axillary odour and right-left asymmetry would probably have increased the power of the current trial (26). Furthermore, a panel of judges trained in malodour assessment may have detected more subtle differences than the participants could, as recommended by the cosmetic industry (26). Finally, we cannot exclude the possibility that the placebo formulation possessed anti-odour activity.

Corynebacterium spp. were isolated from 63% of the participants' axillae. Earlier studies reported a comparatively higher (85–93%) prevalence of *Corynebacterium* spp. in the axillae (1, 3). The discrepancy may be explained by their use of the more vigorous sampling scrub cup technique with buffer solutions, which presumably also recover bacteria inhabiting hair follicles (27). Environmental factors, such as climate and season, also influence the microbiota (5).

Culture-independent molecular methods may yield different results (3–5), although the MALDI-TOF technique correctly identifies most members of the genus *Corynebacterium* (20). On the other hand, the culture method allowed us to semi-quantify the growth of all bacteria.

All women had shaved their axillae, but the presence of hair in the axilla does not appear to appreciably alter the microbiota (1).

Skin pH is important for skin integrity and microbial colonization (28). It is thought that lowering the pH in the axilla mitigates malodour via inhibition of the growth of corynebacteria (29). To our knowledge, the effect of pH on growth of *Corynebacterium* spp. has not been investigated previously. The current study found that

the survival of the *Corynebacterium* spp. drastically decreased at pH 5.0 compared with pH 6.0. Notably, the pH in the hairy axillary vault is approximately 6.0, which is 0.5 units higher than that in the non-hairy axillary fossa (30). Interestingly, the malodour from cultured corynebacteria is maximal at pH 6.0 (2).

The increased pH of the formulation and of the skin surface with the addition of ZnO was attributed to the generation of alkaline hydroxide ions together with zinc ions $(ZnO(s) + H_2O \neq Zn^{2+}(aq) + 2OH^{-}(aq))$ (14). The current study found that approximately 16% of the solid ZnO was solubilized in the deodorant formulation. The pH-modulating effect of ZnO may counteract its antibacterial activity due to increased survival of *Corynebacterium* spp. organisms with increasing pH. Nonetheless, low skin pH promotes the dissolution of ZnO via acid-catalysed hydrolysis (ZnO(s) + 2H⁺(aq) \neq $Zn^{2+}(aq) + H_2O$, thereby increasing the zinc ion (Zn^{2+}) concentration and the antibacterial activity of ZnO (9, 31). In vitro tests in the current study indicated that ZnO is bacteriostatic at \leq 400 mg/l and bactericidal at \leq 10,000 mg/l for 63% of the clinical isolates of corynebacteria. Furthermore, the corynebacteria appeared to be more sensitive to ZnO than did S. aureus (9, 10).

Apart from its antibacterial activity (7–10), ZnO quenches volatile malodorous short-chain fatty acids (32). This mechanism may contribute to less malodorous axillae.

In conclusion, ZnO treatment effectively reduced self-perceived malodour originating from the axillae of healthy individuals, and this effect was most likely mediated by the antibacterial action of ZnO, primarily against *Corynebacterium* spp.

ACKNOWLEDGEMENTS

The authors thank Dr Chris Callewaert for commenting on the manuscript. The underarm formulations and some funding were provided by Colgate-Palmolive Company, Piscataway, NJ, USA.

The authors have no conflicts of interest to declare.

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