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N-Chlorotaurine - A New Antiseptic for Root Canal Treatment

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Introduction

Injuries, inflammations, and operations on parts of the body that are strongly colonized by germs, such as the mouth, nose, nasopharyngeal zone, or external genitalia, represent an ever-larger problem in medicine. To avoid complications from germs penetrating into the tissues, the skin and mucosal areas involved are disinfected locally, and targeted or untargeted systematic antibiotics are also administered. Yet an increasing resistance to antibiotics is a growing problem in medicine. In order to be able to reduce the systematic use of antibiotics and thus also bacterial resistance to them, local defense against germs, in the form of local disinfection, must be strengthened. The current usual local disinfection agents in the clinic are active chlorine components, which have been used for over 180 years and have virtually no potential for the development of resistance [1]. The inhibition of bacterial growth in wound infections is often accompanied by undesirable side-effects such as tissue irritation and pain, due to the high oxidation activity of local disinfection agents. For example, active chlorine components for local disinfection are used in the oral cavity of patients undergoing dental root canal treatment. The aggressiveness of these substances often leads to severe inflammatory reactions with pain and redness in the jaw region. In order to avoid such undesirable side-effects without forgoing the microbicidal effect, researchers have for a long time been searching for new, locally applicable antimicrobial substances that are more tolerable.

N-Chlorotaurine (NCT), which is endogenous to the human body, plays an important role in the human immune system. In the case of an infection, the human immune system forms free radicals (so-called “Oxidants”), which are used by the phagocytes to attack and kill pathogens. Both neutrophil and eosinophil granulocytes as well as monocytes form the active agent Hypochlorous Acid (HOCl) during phagocytosis. This HOCl oxidizes immediately on NH compounds, whereby less reactive, longer-lasting oxidants are formed, which are identified as chloramines. The most frequently demonstrated representative of the human chloramines is N-chlorotaurine, which arises from the reaction of HOCl with the amino acid taurine. It serves to protect human cells from HOCl, and its cytotoxicity is significantly lower. Furthermore, NCT inhibits the production of tumor necrosis factor, nitrogen monoxide, and prostaglandins, and thus exerts an immune-regulating function [1,2].

The mechanism of attack against the bacteria takes place through chlorination of the active chlorine components on the external protein matrix of the bacterial surface [2]. NCT thereby achieves bactericidal, virucidal, and vermicidal effects. Additionally, a post-antibiotic effect through delay of a new incrustation of bacteria has been demonstrated [4-10].

For humans, attention for NCT is currently directed toward topical applications, both as a means of disinfection for surgical interventions and also for infectious diseases on the skin and mucous membranes. In smaller clinical studies, the application of NCT has shown good results, confirming the bactericidal effect and good tolerability in people [3-15], without causing allergic side-effects [11].

For diseases of the mucous membranes in the ear-nose-throat area, the focus of the side-effects directs itself toward, among others, the ciliary beat frequency in the nasal mucosa [12] and acute Otitis externa [13,14]. A trial with NCT in the external ear canal was conducted with the antibiotically effective Otosporin as a comparison group. In all 25 patients of the NCT study group, the infection was complete cured. Therapy with NCT was well tolerated and was more efficient than the application of the antibiotically effective Otosporin. In a study investigating the ciliary beat frequency of the nasal mucosa, samples were studied

from 10 adult patients, who had undergone a conchotomy. Contact with the nasal mucosa after 20 minutes of incubation time with 1% NCT solution led to a reduction of the beat frequency of only 10%; absolutely no reduction could be found for 0.1% and 0.01% NCT solution. By comparison, the usually applied 7% cocaine solution for the same incubation time led to a reduction of the ciliary beat frequency of 50%.

Due to the good tolerability and the lack of cytotoxic side-effects, a further possible topical application of NCT is dental root canal treatment. Root canal treatment is necessary when, due to the invasion of microorganisms, a tooth is irreversibly damaged on the nerve but is still vital or is completely dead (devital). An entry point frequently arises due to damage took the tooth by germs that normally exist in the oral flora [16]. Among those microorganisms that inhabit the oral cavity, there is *Staphylococcus aureus* [17], which as soon as it breaks through the mucosal barrier can lead to painful infections in the area of the periodontal membrane with bone-destroying periodontitis. If this infection shifts to the area of the root tip, further pyogenic agents, such as *staphylococcus spp and streptococcus spp*, *E. coli*, *Pneumococcus*, *Pseudomonas aeruginosa* and various anaerobes of the oral mucosa can invade the periapical tissues and install themselves there [18].

The aim of this study was to test the possible bactericidal effect of NCT topically applied for a dental root canal treatment. Since a pig model has already excelled in previous studies as a good animal model [19-22], dissected pig teeth of various sizes were chosen as the experimental model.

Methods

In order to demonstrate the bactericidal effect of NCT, a target bacterium (*Staphylococcus aureus*) existing in the oral mucosa was chosen. The substrate was pig teeth, upon which the target bacteria were studied, under the most realistic conditions possible, under the effect of NCT in four different preparations.

Thirty-eight pig teeth were mechanically removed from a pig jaw after a 24-hour water bath and placed for 7 days in a 3% solution of hydrogen peroxide. After this 7-day bleaching, the entire canals and the pulp chamber of the teeth were exposed with the aid of special dental drill. To fixate the dissected teeth, they were individually affixed with white silicon to small custom-made plastic pedestals (Metasys; Rum, Austria) (Figure 1). The silicon paste was draped around the pig teeth such that the entire tooth roots or their canals were sealed. In this way the subsequent filling with the bacterial suspension could be carried out without the suspension being able to leak through holes or permeable roots. The holes arising from the drilling were closed before the rinsing treatment in all teeth using a hybrid-composite paste that is also used for treatment of caries in humans and then, also as is usual in dentistry, hardened with UV light. In order to ensure a moist environment, the dissected teeth were always kept covered with a wet cloth.

All 38 teeth were inoculated with an overnight (23 h) culture of *Staphyloccocus aureus* ATCC 25923, which was prepared the previous night in 5 ml of 3% Casein Pepton solution at 37°C without stirring in the incubator. Before the inoculation, the bacteria suspension was centrifuged for 10 minutes at 3200 rpm (ca. 1800xg). The supernatant was discarded, and the Falcon conical tubes (Falcon; Corning; Corning, NY, USA) were filled to 14ml with 0.9% NaCl. Depending on their size, each tooth was filled with 100-300 µl of bacterial suspension and left to sit at room temperature for 12 hours. In order to simulate the dampness in the mouth, a damp cloth was laid again over the dissected teeth, which should have promoted the formation of a biofilm. Meanwhile, a 1% NCT solution was prepared in distilled water (250 ml).

Thereafter, the teeth were distributed into the 4 study groups presented in Table 1.

	I	II	III	IV
Tooth Diameter	NaCl 0.9%	NCT 1%	NCT 1% + dexamethasone	NCT 1% mech. Pretreat
A) ≤ 7 mm	3	3	3	3
B) 8-12 mm	2	2	2	2
C) >13 mm	3	3	3	3

Table 1: Presentation of the Group Distribution. NCT = N-Chlorotaurine; mech. pretreat = mechanically pretreated dissected teeth. The 4 groups were composed as follows: a control group treated with isotonic cooking salt solution; an NCT 1% group; a group treated with NCT 1% in combination with dexamethasone, and a group of dissected teeth that was mechanically pretreated and then rinsed with NCT 1%. The teeth were distributed to the groups such that each study group had the same number of teeth of any given size. NCT + dexamethasone was applied in an already mixed-together solution.

The study groups were numbered I-IV:

I. NaCl 0.9%

II. 1% NCT

III. 1% NCT + dexamethasone

IV. 1% NCT mechanically pretreated

Additionally, the intact teeth were distributed into three size grades according to their largest diameter measured with a caliper:

A: ≤ 7mm,

B: 8-12 mm,

C: > 13 mm.

Each of the four study groups was composed of 3 large (grade C) teeth, 2 medium (grade B) teeth, and 3 small (grade A) teeth (Table 1). An additional substitute group was composed of 6 teeth. These teeth were mechanically pretreated and inoculated with *S. aureus* exactly as were all the other teeth but without any kind of rinsing.

These teeth served as back-up substitute material, in case of any technical problems or experimental errors.

The study groups I-IV were each rinsed in four cycles. For each cycle, all teeth were rinsed with 7ml of the corresponding rinsing solution. Then this process was repeated three more times. Again, in order to work under the most realistic conditions possible, all teeth were covered with a damp cloth after each rinsing.

The mechanical pretreatment of group IV described, which was intended to simulate a root canal treatment, required various drillings with four different drill bits of various widths and torsions. The rinsing stages in this group consisted of the following. After the 1st to 3rd drillings, rinsing was done with 0.9% NaCl. Afterwards, there was an NCT rinsing, whereby the action of NCT was limited only to the rinsing process. After the 4th drilling, rinsing was done first with 0.9% NaCl, and then NCT was left in the pig tooth for a reaction time of 5 minutes.

Four hours after the rinsing procedures, the teeth were excavated from their silicon fixation and removed from their pedestals. Each tooth was briefly flushed 3x with sterile 0.9% NaCl via a 25ml Falcon conical tubes (Falcon; Corning; Corning, NY, USA). After flushing, the 0.9% NaCl was collected in the Falcon tubes. The pig teeth were placed into these tubes with the recaptured 0.9% NaCl rinse liquid, and then filled up to 15ml with sterile NaCl.

Then the Falcon tubes with the dissected teeth were briefly agitated 5x in the vortexer and then put in an ultrasound bath for 3 minutes. This procedure served, if necessary, to loosen or separate any bacteria still remaining.

Once again, the conical tubes with teeth were briefly agitated 3x in the vortexer, and then finally the 0.9% NaCl rinsing solution surrounding the pig teeth was plated out into agar dishes. For each tooth, the solution in the tubes was plated out onto 2 plates with the aid of an automatic spiral plater (Whitley Automated Spiral Plater (WASP); Don Whitley Scientific; West Yorkshire, UK). This yielded a sample series of twice each of I 1, II 1, III 1, IV 1, I 2, II 2, etc. up to IV 8 (Table 2).

Group I	Group II	Group III	Group IV
1	1	1	1
1	1	1	1
2	2	2	2
2	2	2	2
3	3	3	3
3	3	3	3
4	4	4	4
4	4	4	4
5	5	5	5
5	5	5	5
6	6	6	6
6	6	6	6
7	7	7	7
7	7	7	7
8	8	8	8
8	8	8	8

Table 2: Plating of the rinse solution of every tooth of all study groups. Two agar plates were used for each tooth's surrounding rinse solution. Thus, there were a total of 64 agar plates.

The plates were incubated in the warm container at 37°C for 48 hours, and then counted with the aid of a counting chamber.

Results

A total of 38 teeth were treated according to the methods described above. It was possible, without any problems, to fixate all of the already dissected pig teeth onto pedestals with white silicon, which was completely water-resistant after 24 hours (Figure 1).

Figure 1: Already dissected teeth during the drying of the silicon.

This photograph shows a pig tooth of the size grade C (> 13 mm). It has already been drilled open and the pulp chamber and all canals have been exposed. The tooth was fixated onto a specially prepared pedestal with white silicon that became entirely water-resistant and thus was sealed watertight.

The allocation to groups took place after fixation and inoculation with S. aureus, shortly before the rinsing procedures. The mechanical pretreatment of group IV also ran without any complications. The treatment was successfully carried out with special drill bits. The drill bits of various sizes were inserted into the root canals, one after another, beginning with the smallest diameter (Figures 2-4), without any splintering of the teeth occurring.

Figure 2: Simulated root treatment with a drill bit in the study group IV; (drill bit shown from Komet Dental / Gebr. Brasseler GmbH & Co. KG; Leipzig, Germany).

Figure 3: Simulated root treatment with a drill bit in group IV; (drill bit from Komet Dental / Gebr. Brasseler GmbH & Co. KG; Leipzig, Germany).

Figures 2 and 3 show the mechanical step of the root treatment with a drill bit on a small tooth in group IV. The pulp canals were drilled into with each of the bits as far as possible without applying pressure. Thus, the pulp canals were exposed and freed from any possibly existing necrotic tissue.

Figure 4: Simulated root treatment with a drill bit on a tooth in group IV; (drill bit from Komet Dental / Gebr. Brasseler GmbH & Co. KG; Leipzig, Germany).

Figure 4 (like figures 2 and 3) shows the mechanical step on a medium-sized pig tooth.

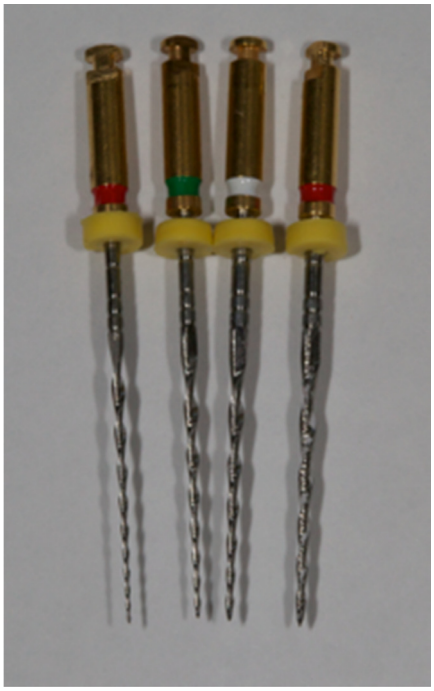

Figure 5: Photograph of the drill bits used; courteously made available by the company, Komet Dental (Komet Dental/Gebr. Brasseler GmbH & Co. KG; Leipzig, Germany).

Figure 5 shows the drill bits that were used for the drilling in study group IV. They are shown in the order of their usage - red, green, white, and red (broader). The drill bits differ from one another above all in their cross-sectional diameter.

The canals of the dissected teeth were flushed with the aid of the syringe shown in Figure 6. In the background of the figure, an already assembled group for approach I (with 0.9% NaCl) can be seen.

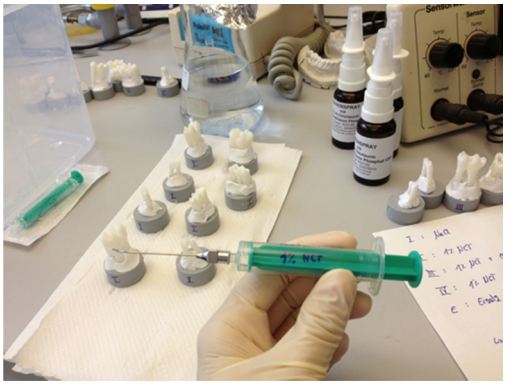

Figure 6: Photograph of the syringe used to flush the pig teeth in group II. In the background is a ready assembled group of teeth.

The NaCl solution from each dissected tooth was plated onto 2 agar plates, so there were two plates per tooth available for each of the groups (Figure 7, Table 2).

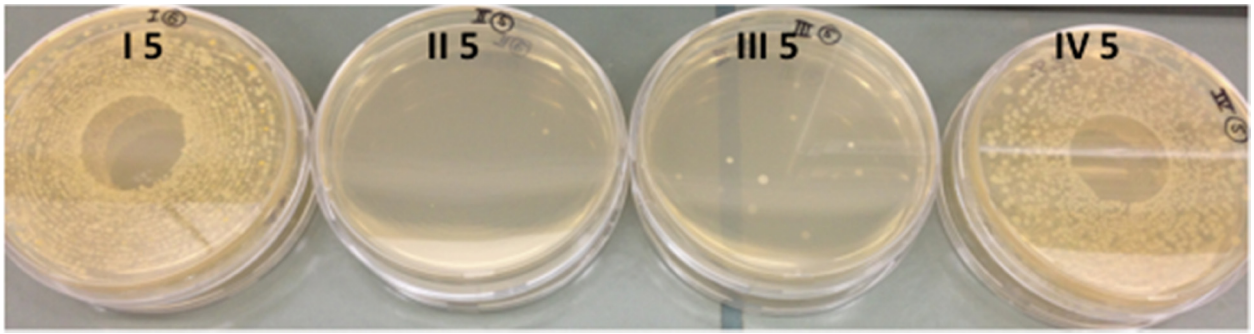

Figure 7: Arrangement of the approach in groups I-IV for the example preparation 5.

In Figure 7, the already incubated plates of the 5th experimental row can be seen. The diverging approach of group IV stands out clearly. The heavy foreign bacteria burden made an exact count difficult. The count of *S. aureus* on plate IV 1 came to ca. 60 microbes (Table 3).

Tooth Preparation	Group I	Group II	Group III	Group IV
1	1.3×10^5	0	20	1.7×10^2
2	8.3×10^4	0	$2,6\times10^2$	0
3	3.9×10^3	2.2×10^4	0	0
4	9.0×10^3	0	0	0
5	1.9×10^3	0	0	60
6	6.5×10^2	0	0	0
7	8.7×10^2	0	0	10
8	2.9×10^3	0	0	0

Table 3: Determination of the bacterial count of *Staphylococcus aureus.*

The step of counting the colonies to determine the number of bacteria took place with the aid of a counting chamber (Figure 8). In cases of especially heavy foreign bacteria burden, a separate *S. aureus* culture (same incubation time, defined culture provided by the Division of Hygiene and Medical Microbiology) was used to compare the colors of the colonies. With the aid of a comparison of the color of a known *S. aureus* culture, the experimental *S. aureus* colonies, whose number needed to be determined, could be better differentiated.

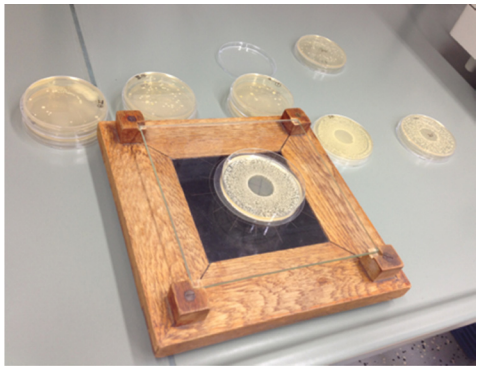

Figure 8: Counting chamber for the evaluation of the number of colonies.

The ascertained number of colonies of both plates was added together and evaluated by means of microbiological counting tables. The results of this enumeration are presented in Table 3.

The positive controls of group I, which were rinsed only with NaCl, showed growth of *S. aureus* for all preparations (1-8). Most of the dissected teeth were afflicted with foreign bacteria:

I. 1-8 - both plates heavily loaded

II. 1 and 3 - both plates, 6, 7, and 8 - one plate each with minimal loading of foreign bacteria (1-3 foreign colonies)

III. 1 - both plates minimally, 2 - both plates heavy, 4-6 and 8 - both plates minimally loaded with foreign bacteria

IV. 1 and 5 were loaded, 3 - one plate with 2 foreign colonies, 4 and 6 - one plate each with at most 2-3 foreign colonies, 7 - one plate with tiny unidentified colonies.

In preparation 3 of approach II, an enormous foreign bacterial burden was ascertainable and an unexpected growth of *S. aureus* was observed. Furthermore, in preparation 2 of approach III and in preparation 1 of approach IV, a small growth of *S. aureus* was seen. The bacteria reduction of *S. aureus* and other foreign bacteria was 100% in approach II 1, 2, 4-8; approach III 1-8; and approach IV 1-8. A muted reduction, which affected above all foreign bacteria, was found in the 1st preparation of approach IV. Notwithstanding, it was possible to reduce *S. aureus* in this approach to a contamination of a maximum of 12 colonies per plate.

Tooth Preparation	Group I	Group II	Group III	Group IV
1	1.3×10^5	0	20	1.7×10^2
2	8.3×10^4	0	2.6×10^2	0
3	3.9×10^3	2.2×10^4	0	0
4	9.0×10^3	0	0	0
5	1.9×10^3	0	0	60
6	6.5×10^2	0	0	0
7	8.7×10^2	0	0	10
8	2.9×10^3	0	0	0
Mean	29027.5	2750	35	30
Standard Deviation	49508.5	7778.17	91.18	60.24
Outlier test		2.4749	2.4676	2.3241
according to Grubbs		for prep. 3	for prep. 2	for prep. 1
Mean without outliers	**29027.5**	**0**	**2.86**	**10**
Standard Deviation without Outliers:	**49508.5**	**0**	**7.56**	**22.36**

Table 4: Bacterial count with statistical values, prep. = preparation.

Discussion

Currently, active chlorine components are used in the oral cavity for local disinfection of patients with dental root treatment. Due to the high oxidation activity of these substances, severe inflammatory reactions with pain and reddening in the jaw area occur often. Therefore, new, locally applicable, but tolerable antimicrobial substances that can be used in dental interventions have been sought for a long time already.

One of the most frequent interventions in dentistry is dental root treatment with the goal of maintaining the tooth despite the damage. In that procedure, it is important to remove all bacteria and necrotic tissue from all the canals. For this, the tooth is flushed with a disinfecting solution after drilling and preparation of the canals. Commonly used rinsing solutions are, for example, natrium hypochloride or 3% hydrogen peroxide solution. The flushing serves above all for the disinfection of canal branching's that cannot be reached with instruments. As already mentioned, the disinfection agents currently used for this frequently cause undesired side-effects due to their high reactivity and cytotoxicity [15].

As an endogenous substance, NCT works less cytotoxically in comparison to the disinfection agents with antiseptic effects currently used. Through unspecific mechanisms of reaction of the active chlorine bonds, it loses little of its bactericidal activity in the presence of organic material. Therefore, NCT is essentially more stable in contact with biological substrates, and allergic side-effects and irritations are rather unlikely [3].

Both the bacterial colonization of the oral mucus membrane by *S. aureus* among others and also the irritations during dental interventions from the antiseptic rinsing solutions used are problems that have not been solved satisfactorily. A bactericidal NCT rinsing solution for dental root treatment and other interventions in the oral cavity could deliver clear advantages through its antiseptic properties with less additional stimulation. Precisely in root treatments, strong inflammatory irritations occur because of both the underlying disease and also the operative intervention itself.

On the basis of the pig tooth model used here, it was tested how well the bactericidal effect of NCT is suited for topical application in dental interventions. NCT, which is important for the human immune system, has already established itself well in previous studies, both in humans and also in the pig model. It has been shown that an inhalation of NCT was successfully applied for fighting strep in the lower airways and showed only minor side-effect effects [21]. Thereby, it attacks not only the pathogens themselves but also partially inactivates their toxins (for example the shiga toxin of *E. coli* [23]), supports the reduction of biofilms, and shows an anticoagulating activity [1].

NCT can be chemically synthesized relatively simply as a natrium salt ((Cl-HN-CH(2)-CH(2)-SO(3)Na) and is therefore very soluble in water. Without any further chemical modifications, NCT can be stored for 1 year at 4°C or for 3 weeks at 20°C. Thereby, a loss of activity against bacteria, fungi, viruses, and parasites of only 10% at most occurs. The surface chlorination brought about by incubation with sublethal NCT leads to a so-called post-antibiotic effect with a resulting loss of virulence from pathogens such as bacteria and yeast [24]. There is experience in humans with topical NCT as a 1% solution for illnesses of the eyes, skin, external auditory canal, nasal mucous membrane, paranasal sinuses, oral cavity, and bladder [24]. As a non-antibiotic disinfection agent, NCT attacks bacteria through the integration of active chlorine components in the outer protein matrix of the cell membrane. Even with sublethal doses, changes in the bacterial cell membrane and in the cytoplasm of *S. aureus*, for example, could be made visible with the aid of electron microscope scans (Figure 9).

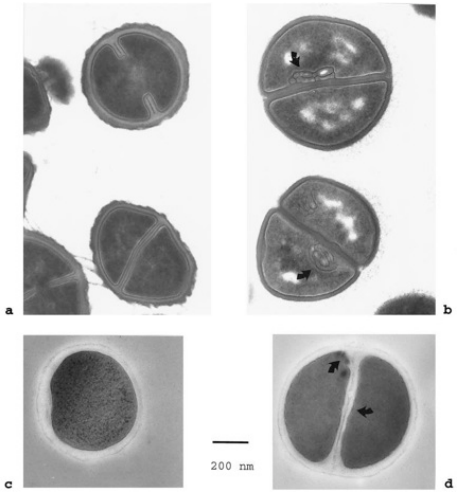

Figure 9: Transmission electron-microscopic record of *S. aureus* Smith diffuse. **a** and **c**: morphology of the control group, made visible after fixation, which was incubated in phosphate-buffered salt-solution for 120 min. (**b**) Arrow: membrane infolding and cytoplasm segregation in chemically fixated examples after 120 minutes of activity time with NCT (50 µM). **d**: Arrow: undulations and light infoldings of the bacterial cell membrane after 120 minutes of activity time with NCT [25].

In the present work, a clearly higher reduction of the bacterial count was recognizable in the dissected teeth rinsed with 1% NCT solution in comparison to those rinsed only with 0.9% cooking salt. After carrying out the outlier test according to Grubbs, the values from preparation 3 of approach II could be excluded from the statistical evaluation. The critical value according to Grubbs was (for a sample size of 8 preparations) below the ascertained value of the outlier ((gcrit.=) 2.1266 < 2.4749 (= prep. 3, II)). Similarly, the value of preparation 2 of approach III and preparation 1 of approach IV were excludable as outliers ((gcrit.=) 2.1266 < 2.4676 (= prep. 2, III); (gcrit.=) 2.1266 < 2.3241 (= prep. 1, IV)). On average, all 29027.50 bacteria were destroyed in approach II, which corresponds to a bacteria reduction of 100%. In approach III, a reduction of 99.99% was observed, and in approach IV, a reduction of 99.97% of the bacteria was determinable. Thus, a topical bactericidal effect of NCT on all dampened tooth surfaces must be assumed. *S. aureus* was selected because it is a problem bacterium occurring frequently in the human oral flora, which is co-responsible to a large degree for bacterially caused illnesses and complications in dental treatment.

In an experiment carried out in a not sterile environment, a high burden of foreign bacteria is to be expected. Despite this foreign bacterial burden, an overwhelming reduction of the bacterial count was documentable in this experimental approach in groups II, III, and IV, yet not in preparation 1 of group IV. In the 1st preparation of group IV, a reduction of *S. aureus* was indeed recorded, yet this plate was heavily burdened with foreign bacteria. Presumably, this burden was due to infiltrated condensed water, perhaps promoted through the preceding drilling under *in vitro* conditions. The capillary cracks thereby arising favored the infiltration of water and foreign bacteria under the covering with a damp cloth. Furthermore, in the 2nd preparation of group 3, there was also a burden of foreign bacteria but not in the amount seen in group IV. The count of *S. aureus* colonies was nonetheless clearly reduced here also after corresponding exposure to the rinse solution.

The unexpectedly frequently occurring foreign bacterial burden (see explanation of table 3) made it indeed difficult to evaluate the data, but nonetheless it was possible to precisely determine the bacterial count of *S. aureus*. The causes of the contaminations with foreign bacteria could be due to, among other things, the surfaces of the pig teeth with their cracks, clefts, and difficult to access canals, whose structure and form offer spaces that protect bacteria from disinfection agents. Furthermore, the occurrence of foreign bacteria could have been brought about by unsterile working conditions, for example through the lack of sterilization of the cover cloth, which nonetheless served to simulate the damp bacteria-colonized milieu in the mouth. Also, the necrotic tissue cannot always be entirely removed from the teeth, whereby a further hearth for foreign bacteria can arise. Moreover, extremely fine structured, inaccessible little canals, and above all capillary cracks from the drilling mentioned above, could not be reached with the needle, and so the effect of the disinfection agent was weakened.

Nonetheless, the contaminations did not hinder the experiment. In almost all the preparations, a clear reduction of the bacterial count was observed. Not only the decimation of only *S. aureus* but also for the most part complete extermination of other bacteria underlines the microbicidal effect of NCT. The results of the experiment make further experiments with other pathogenic bacteria of the mouth and nasopharyngeal zone conceivable with the same experimental model. As the next goal, it would thus be sensible to use this experimental model for studies with further microorganisms that are pathogenic for humans. Interesting organisms for the oral cavity for this would be, among others, the germs *Streptococcus* mutants and *Streptococcus sobrinus* that are mainly connected to tooth decay [14].

Summary

Topically applied 1% N-chlorotaurine with and without dexamethsone was able to mostly eliminate *S. aureus* from pig teeth that were inoculated under controlled conditions. Moreover, other foreign bacteria were also reduced in a substantial amount. Further, studies recording the microbicidal effect on further bacteria can and should be carried out with this experimental model, in order to find out for which other agents NCT could be applied, in which dosage, combination, and effect time. NCT 1% rinse-solution would thereby also be conceivable in the future as a mild

antiseptic with few side-effects for dental root treatments.

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