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FULL ARTICLE

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Effects of cold atmospheric pressure plasma and disinfecting agents on *Candida albicans* in root canals of extracted human teeth

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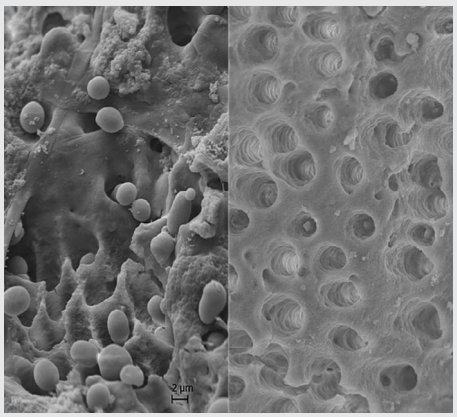
Abstract



Reinfection in endodontically treated teeth is linked to the complexity of the root canal system, which is problematic to reach with conventional disinfection methods. As plasma is expected to have the ability to sanitize narrow areas, the aim of this study was to analyze the effect of cold atmospheric pressure plasma (CAP) on *Candida albicans* in root canals of extracted human teeth. CAP was applied as mono treatment and in combination with standard endodontic disinfectants (sodium hypochlorite, chlorhexidine and octenidine). Disinfection efficiency was evaluated as reduction of the logarithm of colony forming units per milliliter ($\log_{10}$ CFU/mL) supported by scanning electron microscopy as imaging technique. Plasma alone showed the highest reduction of $\log_{10}$ CFU, suggesting the best disinfection properties of all tested agents.

KEYWORDS

Candida albicans, cold atmospheric pressure plasma, human teeth, medical plasma, root canal

Abbreviations: *C. albicans*, *Candida albicans*; CAP, cold atmospheric plasma; CFU/ml, colony forming units per milliliter; CHX, chlorhexidine; *E. faecalis*, *Enterococcus faecalis*; EDTA, ethylenediaminetetraacetic acid; OCT, octenidine; YPD, yeast extract peptone dextrose.

1 | INTRODUCTION

A successful endodontic treatment highly depends on a sufficient reduction of the number of microorganisms [1]. Although after chemo-mechanic and medical preparation of the tooth a significant reduction of the microbial load could usually be achieved in the root canal [2], not all microorganisms are susceptible to conventional therapy [3] or can be reached by contemporary endodontic instruments. [4] In the majority of all treated roots, microbes remain in the root canal system [5] and may cause reinfection. Reinfection of the root canal system is linked to specific microorganisms. The most common bacteria in reinfected root canals are *Enterococcus faecalis* [6] and the most commonly found fungus is *Candida albicans* [7]. *C. albicans* belongs to the commensal flora in some humans' gastro-intestinal tracts [8] but may become infectious in patients whose immune system is suppressed or whose microbiome is unbalanced [9]. *C. albicans* is not susceptible to standard drugs in endodontic therapy, like calcium hydroxide [3]. Dentine tubules act as reservoirs for reinfection of *C. albicans* [10].

Because *C. albicans* is linked to reinfection of the root canal, it is desirable to find additional therapeutic options to reduce the load of *C. albicans*.

Cold atmospheric plasma (CAP) shows potential to become a promising tool in a number of applications in dentistry including inactivation of oral pathogens, the treatment of periodontitis, gingival wound healing, esthetic dentistry and cavity treatment [11]. Plasma is a gaseous matter in which molecules are divided into free electrons and ions. The charged carriers are able to move freely. Therefore, plasma can be formed by magnetic and electric fields [12]. CAP within physiological temperatures can be used on vital human tissue [13]. Gases frequently used to produce CAP are helium, argon, nitrogen, ambient air or mixtures of them [13]. Studies indicate a higher effectiveness when using an argon/oxygen [14] or argon/air mixture [15]. The effect of plasma is based on reactive oxygen and nitrogen species supported by electric field, temperature and radiation [16] and depends on of the liquid surroundings of the cells [13]. The composition of the plasma components can be controlled and therefore optimized according to the intended treatment optimized.

Plasma is able to reach narrow niches and hence able to reduce vital microorganisms in such reservoirs [17]; its endodontic effectiveness was frequently tested on *E. faecalis*. Experiments with CAP on *E. feacalis* showed satisfying but not overwhelming results both in disinfecting the root canal system as well as in disinfecting dentine tubules [17–20].

A former study from our lab reported a beneficial effect of an argon-plasma in combination with a disinfectant on monospecies (*Streptococcus mutans)* and multispecies (saliva, subgingival) dental biofilms on titanium discs [21]. It is unknown whether this beneficial effect also occurs in endodontic treatment.

This study analyses the effect of CAP with argon admixed 1% oxygen as working gas on *C. albicans* in human root canals in comparison to conventional disinfectants. In addition, the combination of conventional disinfectants with CAP is tested to investigate the potential additive effect of CAP treatment, and the influence of the test cycle was analyzed. The hypothesis of this study was that CAP treatment is more or comparable effective than the commonly used antiseptic solutions.

2 | MATERIALS AND METHODS

2.1 | Specimens

Extracted human single rooted teeth (maxillary incisors and mandibular premolars) were used. They had to have neither caries within the root canal nor longitudinal cracks. The teeth were provided by the tooth bank of the Greifswald Dental School. The study was approved by the local ethics committee (BB 30/11).

The teeth were mechanically prepared up to ISO 30/06 (Flex Master, VDW, Munich, Germany) according to manufacturers' instructions and decoronated to a standard length of 15 mm. All teeth were sealed with nail varnish (Rival de Loop, Berlin, Germany) and embedded into plastic blocks (Technovit 4071, Heraeus Kulzer, Hanau, Germany). During the preparation period the teeth were stored in physiological saline solution (NaCl). Before starting the test series, detritus was removed through 2 minute ultrasonic baths (Elmasonic S 30 H, EMAG AG, Mörfelden-Walldorf, Germany) in 17% EDTA (University Pharmacy Greifswald, Germany) and subsequently in 3% sodium hypochlorite (NaOCl, Hedinger, Stuttgart, Germany). All specimens were autoclaved for initial sterility (2540 EK, Tuttnauer, Breda, Netherlands).

The root canals were filled to the edge with yeast extract peptone dextrose broth (YPD, Y1375-250 g, Sigma, Hamburg, Germany) inoculated with the strain *C. albicans* ATCC 10231 (Rockville, Maryland). The suspension's initial fungi concentration was 4×10^7 colony forming units per milliliter (CFU/mL). All specimens were incubated aerobically for 7 days at 37°C (incubator BE400, Memmert, Schwabach, Germany). All procedures were performed under sterile conditions (biological safety cabinet Herasafe KS 12, Thermo-Scientific, Waltham, Massachusetts).

2.2 | Plasma device and experimental groups of antiseptic treatments

The cold atmospheric-pressure plasma jet kINPen 08 (INP, Greifswald, Germany) was used as plasma source [22, 23]. The argon gas flow was set to 5 slm (standard liters per minute) with an admixture of 1% oxygen (Plasma/O_2). The flow rate was controlled by a flow controller (MKS Instruments, Munich, Germany). The tip of the plasma device was placed 1 to 2 mm apart from the canal entrance (Figure S1).

The specimens were sorted randomly into 15 groups containing 10 teeth each (Table 1). For mono treatments, teeth of six groups were treated for 6 min and teeth of additional six groups were treated for 12 min with 0.9% NaCl (negative control), gas, 2% chlorhexidine (CHX), 5.25% NaOCl, 0.1% octenidine (OCT), and plasma/O_2, respectively. The combined teeth treatments of the three remaining groups were carried out with (a) CHX and plasma/O_2, (b) NaOCl and plasma/O_2 and (c) OCT and plasma/O_2. At first, the chemical agent was applied for 6 min, followed by the plasma/O_2 treatment for another period of 6 min. Altogether 150 teeth were tested. For practical reasons, the experiment was divided into four identical test cycles, spread over 4 weeks. The 16 additional teeth for scanning electron microscopy were chosen randomly and treated in a separate cycle. Chemical agents were applied directly into the root canal with a sterile gauge needle (B. Braun, Melsungen, Germany). After the treatment OCT and CHX were neutralized for 20 min with 20% Lipofundin MCT (B. Braun, Melsungen, Germany) and NaOCl with an inactivator solution containing 96.1% YPD Broth, 3% Tween 80, 0.1% histidine, 0.3% Lecithin and 0.5% sodium thiosulfate. The inactivator was certified by the quantitative suspension test of DIN EN 1040 (German Institute for Standardization). Roots treated with a combination of chemical agent and subsequent plasma/O2 treatment were neutralized after the treatment with plasma/O_2 to investigate potential enhancing effects of plasma/O_2 on chemical disinfection.

After each treatment, the decade logarithms of the colony forming units per ml (CFU/mL) were assessed. To obtain the content of each root canal, the canal was brushed with five sterile paper points (ISO 30, Loser & Co, Leverkusen, Germany). The material on the paper points was dissolved in 1 mL sterile 0.9% NaCl solution. From this suspension, a serial dilution in 0.9% NaCl solution was prepared and 100 µL of the dilutions from 10^{-5} to 10^{-7} were plated on YPD-agar (Y1500-250 g, Sigma, Hamburg, Germany). After incubation at 37°C for 48 hours the CFU were counted to recalculate the $\log_{10}$ CFU/mL for statistical analyses.

2.3 | Statistics

CFU values were $\log_{10}$ transformed (referred to as $\log_{10}$ CFU). Means and SD were reported. First, for treatment periods of 6 min, we tested whether $\log_{10}$ CFU values after mono treatment procedures were significantly lower compared to $\log_{10}$ CFU values of the negative control using one-sided unpaired *t* tests. Then, the effects of mono treatment procedures (ref. NaCl) on $\log_{10}$ CFU values were further quantified using linear regression analyses. Linear regression coefficients (B) and 95% confidence intervals (CI) were reported. To detect significant effect differences between mono

TABLE 1 Description of treatment groups and exposure times

Mono treatment	Exposure time	Treatment
Negative control	6 min, 12 min	NaCl (0.9%)
Gas	6 min, 12 min	Non-ignited mixture of argon and 1% oxygen
NaOCl	6 min, 12 min	NaOCl (5.25%)
CHX	6 min, 12 min	Chlorhexidine (2%)
OCT	6 min, 12 min	Octenidine (0.1%)
Plasma/O_2	6 min, 12 min	Argon +1% oxygen plasma
Combined treatment		
NaOCl + plasma/O_2	12 min	6 min NaOCl (5.25%) followed by 6 min argon +1% oxygen plasma
CHX + plasma/O_2	12 min	6 min CHX (2%) followed by 6 min argon +1% oxygen plasma
OCT + plasma/O_2	12 min	6 min OCT (0.1%) followed by 6 min argon +1% oxygen plasma

Note: Each group comprises 10 teeth.

treatment procedures, posthoc linear combinations were calculated. Second, for mono treatment procedures it was tested whether $\log_{10}$ CFUs values after treatment periods of 12 minutes were lower compared to $\log_{10}$ CFU values after treatment periods of 6 minutes using one-sided unpaired *t* tests. Third, for treatment periods of 12 minutes, it was tested whether $\log_{10}$ CFU values of mono and combined procedures were lower compared to $\log_{10}$ CFU values of the negative control using one-sided unpaired *t* tests. Afterward, the effects of mono treatment procedures (ref. NaCl) on $\log_{10}$ CFU values were further quantified using multilevel linear regression analyses including a random factor for examination day ($P = .0015$ from Likelihood Ratio test, favoring multilevel over ordinary linear model). To detect significant effect differences between plasma/O_2 and combined treatment procedures, posthoc linear combinations were calculated.

To adjust for multiple testing within each step, *P* values were corrected according to Bonferroni. Statistical differences were considered significant if $P < .05$. All statistical analyses were performed with Stata/SE Version 14.1 (StataCorp LP, Texas, USA).

2.4 | Scanning electron microscopy

One specimen per treatment group was notched with a microtome (Leica SP 1600, Leica Biosystems, Nußloch, Germany) without opening the root canal and then split into half. Each specimen was fixed (1% glutaraldehyde, 4% paraformaldehyde, 0.2% picric acid, 50 mM NaN_3 in 5 mM HEPES) for 1 hour at room temperature and then stored at 4°C until further processing. After that, samples were treated with 2% tannic acid in buffer (100 mM cacodylate buffer [pH 7.0], 1 mM $CaCl_2$, 50 mM NaN_3) for 1 hour, with 1% osmium tetroxide in buffer for 1 hour, and 1% thiocarbohydrazide for 30 min at room temperature—with washing steps in between. After treatment with 1% osmium tetroxide in buffer over night at 4°C, the samples were dehydrated in a graded series of aqueous ethanol solutions (10%, 30%, 50%, 70%, 90% 100%) on ice for 15 minute each step. Before the final change of 100% ethanol, samples were allowed to reach room temperature and then critical point-dried with liquid CO_2. Finally, samples were mounted on aluminum stubs, sputtered with gold/palladium and examined with a scanning electron microscope EVO LS10 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Scanning electron micrographs were taken from the transition zone between upper and middle third of the teeth and illustrate the effects after 6 minute treatment time. All micrographs were edited by using Adobe Photoshop CS6.

3 | RESULTS AND DISCUSSION

Plasma/O_2 treatment achieved the highest $\log_{10}$ CFU reduction rates, both after 6 and 12 minute treatment time (Tables 2-4).

TABLE 3 $\log_{10}$ CFU values for different treatments comparing 6 with 12 min treatment time

Treatment	6 min	12 min	*P*[a] value
Negative control	6.79 ± 0.35	6.55 ± 0.56	0.81
Gas	6.81 ± 0.33	6.73 ± 0.25	0.99
NaOCl	5.33 ± 0.76	5.14 ± 0.83	0.99
CHX	5.57 ± 0.38	5.60 ± 0.27	0.99
OCT	5.51 ± 0.50	5.22 ± 0.67	0.84
Plasma/O_2	4.65 ± 0.84	3.84 ± 1.38	0.40

Note: Means ± SD are given.
Abbreviations: CFU, colony forming unit; CHX, chlorhexidine; OCT, octenidine.
[a]One-sided unpaired *t* test, *P*-values corrected according to Bonferroni.

TABLE 2 $\log_{10}$ CFU values of *Candida albicans* after 6 min treatment according to different procedure compared to the negative control

Treatment	$\log_{10}$ CFU/mL	*P*[a] value versus NaCl	B (95% CI)
Negative control	6.79 ± 0.35	—	0.00 (ref.)
Gas	6.81 ± 0.33	0.99	0.02 (−0.48; 0.53)
NaOCl	5.33 ± 0.76	<0.001	−1.46 (−1.96; −0.95)
CHX	5.57 ± 0.38	<0.001	−1.22 (−1.73; −0.72)
OCT	5.51 ± 0.50	<0.001	−1.27 (−1.78; −0.76)
Plasma/O_2	4.65 ± 0.84	<0.001	−2.13 (−2.64; −1.63)

Note: Means ± SD and linear regression coefficients (B) with 95% confidence intervals (CI) are given, indicating treatment effects compared to NaCl.
Abbreviations: CFU, colony forming unit; CHX, chlorhexidine; OCT, octenidine.
[a]Two-sided unpaired *t* test, *P*-values corrected according to Bonferroni.

3.1 | Comparison of CFUs after 6 minute treatment time with the negative control

After treatment time of 6 minute, $\log_{10}$ CFUs/mL were significantly lower for plasma/O_2 (4.65 ± 0.84, $P < .001$), NaOCl (5.33 ± 0.76, $P < .001$), OCT (5.51 ± 0.5, $P < .001$) and CHX (5.57 ± 0.38, $P < .001$) compared to NaCl (6.79 ± 0.35 $\log_{10}$ CFU). Gas (6.81 ± 0.33, $P = .999$) achieved no significant reduction of CFUs. Plasma/O_2 obtained the highest reduction of CFU (−2.13 $\log_{10}$ CFU) within this group (Table 2). Post hoc analyses indicated a significantly larger reduction of $\log_{10}$ CFUs by plasma/O_2 than by NaOCl, CHX and OCT ($P = .01$).

3.2 | Comparison of CFUs after 6 and 12 minute treatment times

Neither a chemical agent nor plasma/O_2 achieved significantly lower CFUs after 12 minute of treatment compared to 6 minute of treatment. Still, plasma/O_2 showed the highest reduction of CFUs (−0.81, $P = .4$) after 12 minute treatment (3.84 ± 1.38) when compared to 6 minute treatment (4.65 ± 0.84) (Table 3).

3.3 | Comparison of CFUs after 12 minute treatment time after combined and mono plasma/O_2 treatment with the negative control

After application of 12 minute treatment, $\log_{10}$ CFUs were significantly lower for plasma/O_2 (3.84 ± 1.38, $P < .001$), NaOCl (5.14 ± 0.83, $P < .001$), OCT (5.22 ± 0.67, $P < .001$) and CHX (5.6 ± 0.27, $P < .001$) compared to NaCl (6.55 ± 0.56 $\log_{10}$ CFU). Gas did not achieve any significant reduction of CFU (6.73 ± 0.25, $P = .999$). Plasma/O_2 obtained the highest reduction of CFU (−2.71 $\log_{10}$ CFU) after 12 minute of treatment.

TABLE 4 Comparison of $\log_{10}$ CFU values of *Candida albicans* after 12 min treatment with mono and combined treatments

Treatment	$\log_{10}$ CFU	P^a value versus NaCl	B (95% CI)
Negative control	6.55 ± 0.56	—	0 (ref.)
Gas	6.73 ± 0.25	0.99	0.17 (−0.58; 0.93)
NaOCl	5.14 ± 0.83	<0.001	−1.41 (−2.17; −0.66)
CHX	5.60 ± 0.27	<0.001	−0.95 (−1.70; −0.20)
OCT	5.22 ± 0.67	<0.001	−1.33 (−2.08; −0.58)
Plasma/O_2	3.84 ± 1.38	<0.001	−2.71 (−3.49; −1.94)
NaOCl + Plasma/O_2	4.09 ± 1.08	<0.001	−2.46 (−3.22; −1.71)
OCT + Plasma/O_2	4.65 ± 1.01	<0.001	−1.90 (−2.65; −1.14)
CHX + Plasma/O_2	4.65 ± 0.97	<0.001	−1.90 (−2.67; −1.12)

Note: Means ± SD and linear regression coefficients (B) with 95% confidence intervals (CI) are given, indicating treatment effects compared to NaCl.
Abbreviations: CFU, colony forming unit; CHX, chlorhexidine; OCT, octenidine.
[a]One-sided unpaired *t* test, *P*-values corrected according to Bonferroni.

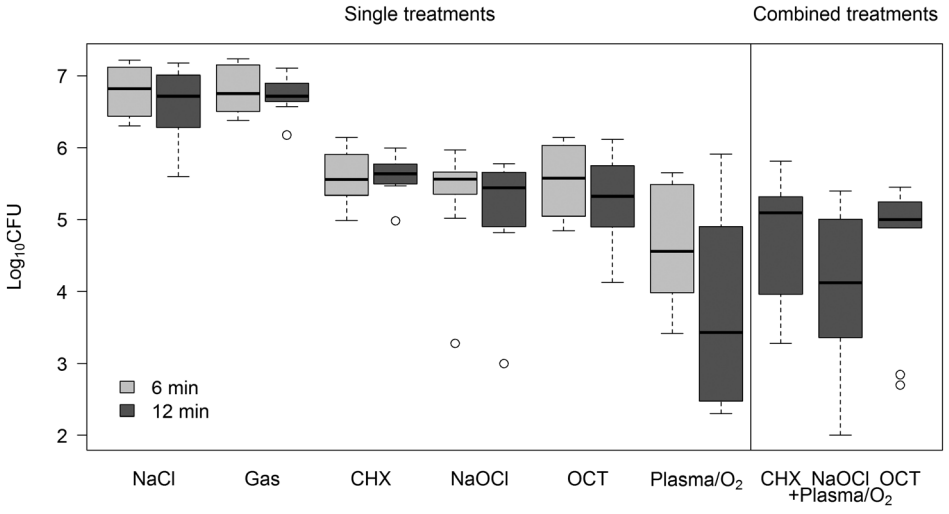


FIGURE 1 Box plot of mean $\log_{10}$ CFU count sorted by treatment and treatment time (6 or 12 min). CFU, colony forming unit; CHX, chlorhexidine; OCT, octenidine

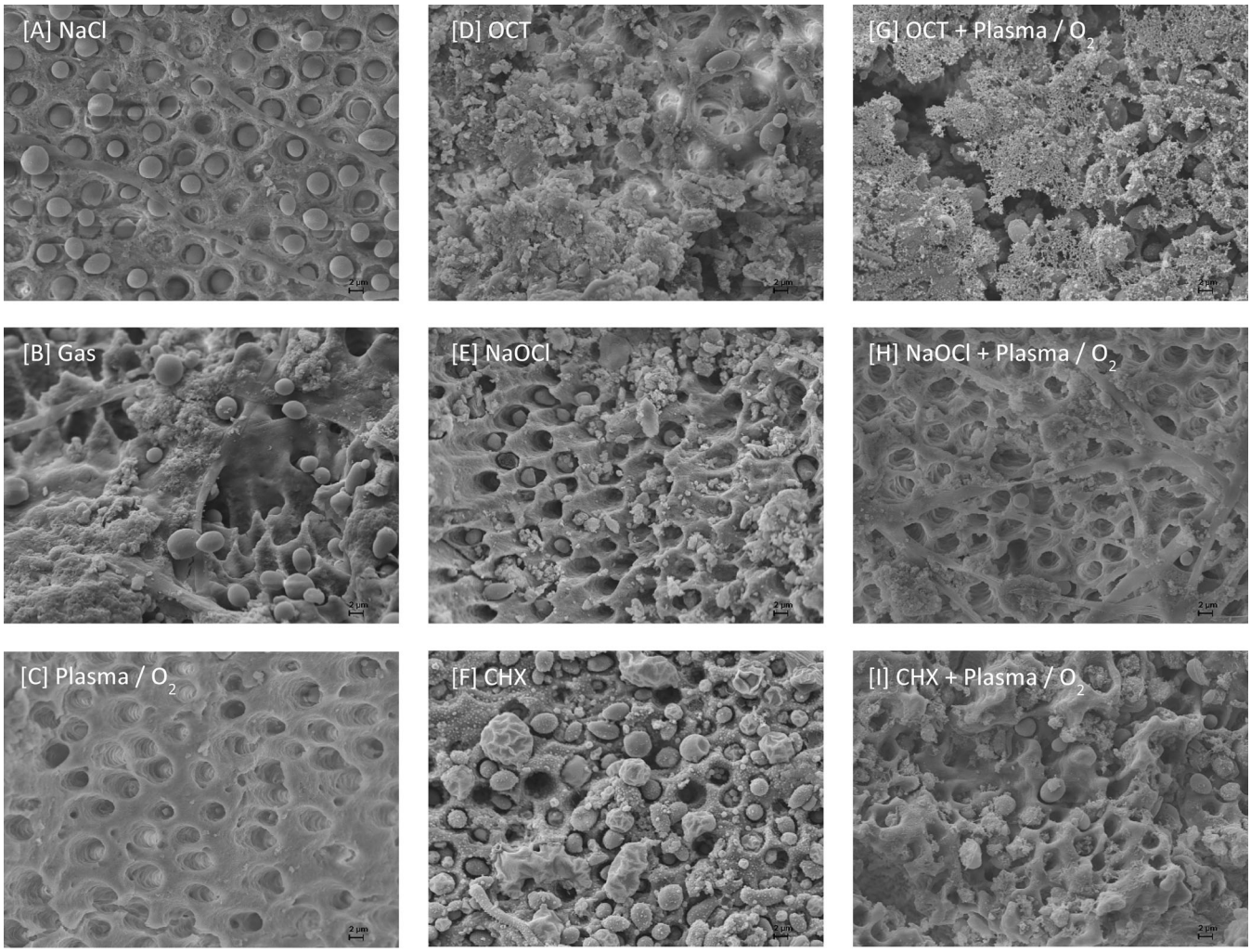


FIGURE 2 Scanning electron micrographs of the transition zone between the upper and the middle third of the root canal of extracted and *Candida albicans* infected teeth of the negative controls after 6 min treatment with A, 0.9% NaCl; B, gas (argon +1% oxygen) and C, plasma/O_2, of the positive controls after 6 min treatment with D, octenidine; E, NaOCl and F, chlorhexidine and of samples after combined treatment with G, octenidine and Plasma/O_2; H, NaOCl and plasma/O_2 and I, chlorhexidine and Plasma/O_2; CHX: chlorhexidine, OCT: octenidine, Scale bar = 2 μm

All combined treatments achieved significant reductions of $\log_{10}$ CFU when compared to NaCl (NaOCl + plasma/O_2: 4.09 ± 1.08, $P < .001$; CHX + plasma/O_2: 4.65 ± 0.97, $P < .001$; OCT + plasma/O_2: 4.65 ± 1.01, $P < .001$) (Table 4). NaOCl + plasma/O_2 achieved the highest $\log_{10}$ CFU reduction within combined treatments (Table 4). Post hoc analyses showed significantly less effects on $\log_{10}$ CFUs for CHX + plasma/O_2 (B = 0.82 [0.14; 1.50]; $P = .019$) and OCT + plasma/O_2 (B = 0.81 [0.14; 1.47]; $P = .017$) as compared to plasma/O_2 alone.

3.4 | Influence of the test cycles

For combined treatments, test cycles 2 and 3 showed significantly higher reduction rates of $\log_{10}$ CFU than test cycles 1 and 4. In any test cycle, the SD was higher when using plasma/O_2 was used (±1.06 $\log_{10}$ CFU) compared to chemical agents alone (±0.6 $\log_{10}$ CFU) (Figure 1).

3.5 | Scanning electron microscopy

Scanning electron micrographs of treatment with NaCl and gas showed colonization by fungi within the dentinal tubules (Figure 2A and B). Some micrographs display a dense layer of extracellular matrix covering almost the complete surface of the root canal (Figure 2B, D, G).

The scanning electron micrographs of teeth treated with plasma/O_2 revealed a considerable reduction of cell number (Figure 2C).

Chemical agents seemed to affect the morphology of the cells. Treatment with CHX and NaOCl led to misshaping of the cells (Figure 2F,E). Larger amounts of extracellular matrix were preserved after treatment with OCT (Figure 2D). The cell number was reduced in Figure 2E,D.

Combined treatments revealed changes in the cell morphology similar to mono treatment with chemical agents. The cell number seemed to be reduced compared to negative controls and to NaOCl + plasma/O_2 and CHX + plasma/O_2-treated samples (Figure 2H,I). The greatest reduction in visible cell number among combined treatments was achieved after treatment with NaOCl + plasma/O_2 (Figure 2H). In concordance to mono treatments, OCT + plasma/O_2 displayed remaining extracellular matrix (Figure 2G).

3.6 | Discussion

The aim of the present study was to investigate the effectiveness of plasma/O_2 in reducing the cell count of *C. albicans* in root canals of extracted human teeth. In our experiments, 12 minute teeth treatment with plasma/O_2 was the most effective disinfecting treatment compared to the treatment time of 6 minute, conventional chemical agents and the combination of chemical agents with plasma/O_2, that confirm our hypothesis (Tables 2-4).

Our and other labs have reported a reduction of CFUs after the application of plasma/O_2 on *C. albicans* before [24–26]. Corresponding to our reduction rates (−2.13 $\log_{10}$ CFU/mL after 6 minute, −2.71 $\log_{10}$ CFU/mL after 12 minute), Handorf et al. achieved a reduction rate of −2 $\log_{10}$ CFU/ml after 5 minute treatment time. [26] In contrast to this study, Handorf et al. used the kINPen09 with 99.99% argon gas on *C. albicans* biofilms grown on Sabouraud agar within 96-well plates. [26] Agar plates are flat surfaces that do not provide the cavity structure of natural teeth, which putatively acts as shelter for single cells diminishing CFU reduction. Duske et al. compared the disinfection capacity of different plasma sources on *Staphylococcus epidermidis* biofilms grown on cover slips, demonstrating a higher reduction rate using the kINPen08 compared to the kINPen09 or kINPenMED, which is one reason why our group favored the kINPen08 in this study [27]. Another reason is that we wanted to ensure comparability with our previous studies with the kINPen08 in the area of endodontics [17, 19]. The main difference between kINPen09, kINPenMED and kINPen08 is the higher power of the kINPen08 [27]. Since our results do not differ from the reduction rates achieved by Handorf et al. using a comparable plasma device on flat surfaces, we assume that a future version of the plasma source kINPen08 could be used as an adjuvant treatment in endodontics and suitable instrument for root canal disinfection [26]. At the moment it is a pure laboratory device, which is not intended for clinical use.

In former experiments on dental biofilms grown on titanium discs Koban et al. observed a significant additive effect when applying combined treatments [21]. In contrast, Hüfner et al. as well as this study did not show any beneficial effect of combined treatments [19]. Both latter studies used natural teeth with the disinfectant forming a liquid column in the tooth whereas on titanium discs the fluid formed a thin film. Both experimental setups illustrate different mechanisms. Koban et al. suspected an additive effect in the sense of destruction of the biofilm by the disinfecting agents and subsequent destruction of the cells by plasma [21]. In natural teeth we assume that the plasma effect is limited to the fluid. Plasma effects on disinfecting liquids are currently unknown. Experimental settings with water, saline solution and cell culture media indicated that plasma is leading to an acidification in liquids and an inlet of reactive oxygen and nitrogen species, with special emphasis on hydrogen peroxide (H_2O_2) [28]. It was also demonstrated repeatedly that such plasma-treated liquids are antimicrobially effective [20, 28–33]. In 2015, Simoncelli et al. demonstrated a relevant cell reducing effect of plasma activated water on *E. faecalis* in model root canals of artificial teeth. A relevant antimicrobial effect of plasma activated water could be demonstrated on planctonic *C. albicans*, as well. Nevertheless, they emphasized that the direct antimicrobial effect of the plasma source within the root canal is superior to the antimicrobial effect of plasma activated water, particularly in a dry environment. In addition, plasma-activated water is a medication, whereas treatment with a medical device, such as a plasma source, also has advantages when it comes to further approval. For this reason, our study refrained from an investigation of the effects of plasma activated water on *C. albicans*.

Liquid disinfectants are susceptible to not reaching the apex due to their surface tension. Hence, reactive species in the disinfectant would not reach the apex either. This hypothesis is corroborated by the data. Each disinfectant in combination with plasma/O_2 showed reduction rates slightly higher than the chemical disinfectant alone, but significantly less reduction of CFU/mL than plasma/O_2 treatment. In a promising experimental setup on dentinal discs, Du et al. demonstrated a significant additive effect when adding 2% CHX into the gas inlet so that the gas and the chemical agent were excited at the same time [34]. Further experiments could give more insight into whether this set up is capable of exceeding the effect of plasma/O_2 in extracted teeth.

SEM showed no colonization in the dentinal tubules after using plasma/O_2. Similar effects were shown by

others and our working group [18, 19]. Unlike our CFU analysis, scanning electron micrographs indicate an additive effect of the combined therapies. We consider the data from of the CFU analysis more reliable, because pictures were taken only from the upper third of the root canals. Therefore, a surface-near effect that does not perpetuate deeper into the root canal could be depicted.

Antifungal activity of plasma/O_2 was time-dependent. Earlier studies could demonstrate similar observations [19, 26]. Previous studies indicated oxidation processes caused by reactive oxygen species to be responsible for the disinfecting effect of plasma [14]. With longer treatment time more reactive oxygen species reacted with the cell surfaces making a destruction of membrane molecules more probable, hence the time-dependent disinfection effect.

Our results indicate that plasma is a promising method in endodontic therapy, but some limitations were identified. Although plasma/O_2 was unambiguously identified as the technique with the highest disinfecting effect, the standard deviations among the results for all groups using plasma/O_2 were higher than for groups using chemical disinfectants alone. We assume that in our set-up the plasma jet could not reproducibly access all surfaces inside the root canal evenly. It has to be borne in mind that the plasma jet is positioned manually, thus our experimental method is prone to inaccuracies. The correct application angle decides on whether the plasma plume is drawn into the canal or swirls at its entrance, which is to be seen when using transparent plastic blocks. It appears to be necessary to design a plasma source or a guiding pin for the plasma plume that can be deeply inserted into the root canal. In an experiment of our lab a different plasma device (hairline plasma devices, plume length 15 mm) showed promising results to reach the apex of natural teeth [35]. Liang et al. tested a polytetrafluoroethylene filament with multiple radially emitted plasma plumes inserted into an agar tube (6 mm in length, 44 mm in diameter) [36]. The results indicated a significantly higher antimicrobial efficacy on *Bacillus aureus* than conventional axial plasma sources.

Anatomical niches, such as the apical region of the root canal, narrow side canals or deeper regions of dentinal tubules, are often responsible for reinfection. Unfortunately, these areas could not be scrutinized with our scanning electron micrographs or investigated with our CFU sample acquisition method. As expectations are for CAP to sanitize narrow areas more efficiently than conventional chemical agents, further studies have to concentrate on a more precise examination of those niches. Within our experimental set up the CFU reduction rates of the liquid disinfectants appeared to be unusually low. We have to assume, that the teeth dried out during the 7d incubation period despite the water saturation within the incubation chamber. It is known that the wetting properties of dehydrated dentine deteriorate significantly [37]. This circumstance is not transferable to the clinical situation where the teeth are hydrated by saliva. Further research with molars and/or multispecies biofilms is needed. If CAP produces more reliable results in more complex root anatomies, an in vivo study is necessary to examine its clinical suitability.

4 | CONCLUSION

This study showed that plasma/O_2 treatment significantly reduced the number of living cells of *C. albicans* in extracted human teeth. Therefore, we assume it to be an effective extension to treatment options in endodontic therapy. Concerning the precise application of the plasma plume into the root canal, we emphasize the need of a plasma source that reliably extends into the entire root canal applicable even in the spatially limited oral cavity.

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AUTHOR CONTRIBUTIONS

Anne Kerlikowski was involved in conceptualization, investigation and writing of the original draft. Rutger Matthes and Lukasz Jablonowski were involved in conceptualization, investigation and reviewing/ editing of the article. Christiane Pink and Birte Holtfreter were involved in statistical analysis and reviewing of the article. Rabea Schlüter was involved in SEM analysis and reviewing of the article. Klaus-Dieter Weltmann, Thomas von Woedtke, Thomas Kocher and Heike Steffen were involved in reviewing and editing of the article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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