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The Antifungal Effect of Endocyn Against *Candida albicans* Biofilm

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**Abstract**

**Introduction:** Endocyn, a super-oxidized water-based irrigation solution, has been reported to be non-toxic against mammalian cells, yet highly efficacious against bacteria; however, its effect against fungi and fungal biofilms remain unknown. This study evaluated the antifungal efficacy of Endocyn compared to sodium hypochlorite (NaOCl) and chlorhexidine (CHX).

**Methods:** An *in vitro* biofilm system was used to assess the antifungal efficacy of Endocyn, NaOCl, and CHX based irrigants against *Candida albicans* biofilm at 1, 5, 10, 15, 30, and 45 min using the XTT metabolic assay. PBS served as the control. Additionally, the irrigants were tested in extracted teeth inoculated with *C. albicans* with antifungal efficacy monitored quantitatively by microbiological plate count and qualitatively by confocal microscopy using Live/Dead staining. XTT data was analyzed by two-way analysis of variance (ANOVA) and Bonferroni post-test and CFU data analyzed using a one-way ANOVA and Kruskall-Wallis posttest.

**Results:** XTT results revealed that Endocyn demonstrated similar antifungal activity as NaOCl or CHX at the 10 min exposure interval (*p* < 0.05) but was less effective at 1 min. For the *ex vivo* tooth model, while no irrigants completely eliminated fungal burden, NaOCl and CHX showed better antifungal activity than Endocyn (*p* < 0.05). Staining patterns assessed by confocal microscopy confirmed these results.
**Conclusion**: Endocyn demonstrated similar inhibition of *C. albicans* biofilms as NaOCl and CHX *in vitro*. However, Endocyn was not as effective as NaOCl and CHX at reducing fungal burden in an *ex vivo* endodontic infection model.

**Introduction**

Fungal microorganisms, including *Candida albicans*, can dwell within root canal systems with failed endodontic treatments, often as biofilms (1, 2). Biofilm formation by *C. albicans* is clinically relevant in endodontics since a fungal biofilm can readily form in tooth dentin (3), evading host immune defenses (4) and antifungal drugs (5). Hence, elimination of *C. albicans* biofilms can be a difficult clinical task (6).

The chemo-mechanical process of elimination of fungi includes conventional endodontic irrigation (7). Ideal irrigating agents used for non-surgical endodontic procedures should ideally be antibacterial, antiviral, and antifungal (8, 9). They should also be non-toxic to periapical tissues, inexpensive, and easy to use. Thus sodium hypochlorite (NaOCl) is the most frequently used agent for irrigation due to its cost-effectiveness and broad-spectrum antimicrobial activity (10). However, despite the effectiveness of NaOCl for the elimination of fungi, accidental extrusion into the periapical tissue commonly damages host tissue, progressing to necrosis and edema (11, 12).

Chlorhexidine (CHX) in liquid form can also be used as an endodontic irrigant (13). Substantivity is one benefit of using CHX as its antimicrobial effects last for at least 72 hours following endodontic treatment (14).

Endocyn, a new super-oxidized water (Microcyn) with hypochlorous acid (HOCl)
as the key ingredient, could play an important role as an endodontic irrigant because of its purported antimicrobial effects and extremely low toxicity to mammalian cells (15). The aim of this study was to compare the antifungal efficacy of Endocyn with NaOCl and CHX for the elimination of *C. albicans* biofilm.

**MATERIALS AND METHODS**

**CULTURE**

A frozen stock of *C. albicans* (strain SC5314) was maintained at -80°C, thawed then streaked onto Yeast Peptone Dextrose (YPD) agar prior to use, and grown overnight in liquid YPD medium at 30°C. The culture was then washed twice by centrifugation with sterile PBS.

**XTT ASSAY**

Cells were grown as described, counted on a hemocytometer and diluted to 6.67×10^6 CFU/mL in RPMI (Roswell Park Memorial Institute) medium. Biofilms were grown using a robust *in vitro* system on 96-well polystyrene plates. Each well was inoculated with 150 μL of standardized cell suspension resulting in 1×10^6 CFU per well. The 96-well plate was placed in a humidified chamber on an orbital shaker (75 rpm) and incubated at 37°C for 24 h.

Following the incubation period, biofilms were gently washed twice with 200 μL sterile PBS (phosphate buffered saline) to remove non-adherent cells. Each row was then replenished with 200 μL sterile PBS. At designated time points (1, 5, 10, 15, 30,
45 min) the PBS was removed and replaced with 200 μL of drug (Endocyn, CHX, NaOCl). Wells containing only PBS served as the control. After completion of the time course, drugs and PBS were aspirated and then the wells washed twice with 200 μL PBS to remove traces of drug. In order to assess viability, the XTT assay was used as previously described (16). A 200 μL of freshly prepared XTT working solution was added to each well and the plates incubated statically at 37°C for 2 h (or until color developed). Following XTT incubation, 100 uL from each well was transferred to a fresh microtiter plate and the absorbance measured at 490 nm using a VersaMax microplate reader (Molecular Devices).

**EX VIVO TOOTH MODEL**

A randomized collection of 34 de-identified human incisors was obtained from LSUHSC Tooth Bank. Tooth inclusion criteria included: mature roots, caries-free, non-previous endodontic treatment, and intact dentinal walls without resorption defects. All teeth were decoronated at the most apical portion of the CEJ followed by canal preparation to the size of 40/04 or greater. Canals were irrigated with 5 ml 6% NaOCl, 3 ml 17 % EDTA, and 5 ml 6% NaOCl. A final irrigation of 5 ml 5% sodium thiosulfate then 5 ml of phosphate buffer solution (PBS) was accomplished. The apical foramen was then coated with two-layers of commercial fingernail polish.

Additionally, six teeth were prepared for confocal microscopy analysis using a split-tooth model system. These teeth were prepared as described above, fixed in Ortho-Jet clear cold cured acrylic resin (Lang) and sectioned in a sagittal plane using a Struers Accutom-50 fine saw. The blocks were tightly secured using 20mm length
bolts. All prepared teeth, including the split-tooth models, were sterilized using a high-pressured steam sterilization cycle at 121°C for 20 min.

As described, planktonic *Candida albicans* was grown then washed and re-suspended to 5x10^8 CFU/mL 1X RPMI medium without phenol red. All teeth samples were secured in an upright position. Sterile paper points were used to dry the canals. Teeth were then inoculated with 10μL of the adjusted cell suspension (an inoculum size of 5x10^6 CFU), then carefully moved to a humidified chamber and incubated at 37°C without shaking for 30 min. Following the incubation period, teeth were washed with 0.2ml of PBS in order to remove the non-adherent cells. Inoculated teeth were submerged in separate wells of a 24-well plate containing 2 ml of sterile RPMI media. In order to achieve mature fungal biofilm formation, the plate was placed in a humidified chamber and incubated at 37°C with gentle shaking (75 rpm) for 48 h. Following the incubation period, teeth were washed with PBS to remove non-adherent cells. Additionally, the root canal space was flushed with 0.2ml of sterile PBS. Four groups (n = 7 each) were included in this study: PBS for Group I (control); 6% NaOCl for Group II; 2% CHX for Group III; and Endocyn for Group IV. The irrigation solution (0.5 mL) was allowed to remain within the canal space for 10 min. A final flush was performed with 0.2 mL of sterile PBS to remove residual irrigant.

Following the irrigation protocol, all teeth were dried using a single-use sterile paper point (Sybronendo). A single paper point was vigorously probed for 1 min into the canal to optimally absorb intraradicular fungal growth. Paper points were
immediately placed into 15 mL conical tubes containing 1 ml of sterile PBS. And vigorously vortexed for 1 min at maximum speed. Aliquots were removed and plated onto YPD plates containing 50 μg/mL chloramphenicol (YPD-CAM). All plates were incubated at 37°C for 24 hours and CFUs enumerated.

CONFOCAL SCANNING LASER MICROSCOPY

Each split-tooth model (Endocyn, n =2; 2% CHX, n= 2; 6% NaOCl, n =1; PBS. n =1) was inoculated with 10μL of the adjusted cell suspension (an inoculum size of 5x10⁶ CFU). For biofilm growth, the inoculated blocks were submerged in 50ml RPMI and incubated in a humidified chamber at 37°C with gentle shaking (75 rpm) for 48 hours. Following the incubation phase, the split-tooth blocks were submerged into a sterile bath of PBS to remove any non-adherent cells from its surface. An endodontic side-vented needle was used to flush the canals with 0.2ml of PBS to remove non-adherent cells. The BacLight Live/Dead stain composed of fluorescent dyes Syto9/Propidium iodide (Invitrogen) was prepared according to manufacturer’s instructions. Tooth canals were stained twice with 0.2 mL of BacLight for 10 min. Following staining, tooth models were rinsed with PBS to remove unbound stains. Bisected teeth were imaged using the Flowview FV 1000 confocal microscope with FITC/Texas Red filter sets.

STATISTICAL ANALYSIS

All experiments were conducted in triplicate unless indicated otherwise. All data were plotted and analyzed for statistical significance by using GraphPad Prism
software. Data for the in vitro biofilm model system (XTT assay) was compared by using a two-way analysis of variance (ANOVA) and Bonferroni posttest. CFU data was analyzed using a one-way ANOVA and Kruskal-Wallis posttest. Graphs were annotated to denote significance levels: * p < 0.05; ** p < 0.01; *** p < 0.001.

**Results**

The objective of this study was to assess the antifungal efficacy of Endocyn, a novel endodontic irrigant solution, against *C. albicans* biofilms. Using an in vitro microtiter plate biofilm model system and the XTT metabolic assay, it was determined that Endocyn had similar efficacy as NaOCl and CHX against *C. albicans* biofilms at exposure times of greater than 5 min. Endocyn demonstrated slightly less efficacy against *C. albicans* at a 1 min exposure as compared to NaOCl and CHX. However, near total metabolic inhibition required 10 min exposure to Endocyn (Fig. 1).

In order to determine more clinically relevant efficacy of Endocyn, an ex vivo human extracted tooth model was utilized. Teeth were inoculated with *C. albicans* then challenged with Endocyn and control irrigant solutions. Fungal burden was significantly reduced with all irrigant treatments (Fig. 2A,B). While no irrigant completely eliminated fungal burden, NaOCl and CHX showed better antifungal activity than Endocyn (p < 0.05). Supportive qualitative data comparing antimicrobial efficacies was obtained by confocal microscopy using a split-tooth model. Live/Dead staining of Endocyn-, CHX-, and NaOCl-treated biofilms demonstrated increased red staining of the fungal biomass, indicating cell death. The drug-free control showed little propidium iodine uptake (Fig. 3A,B). Moderate PI staining was observed during
Endocyn treatment, while CHX treatment induced robust PI uptake. Staining of NaOCl-treated biofilms revealed weak Syto9 and PI staining, indicating a lack of cells. This is likely due to corrosion and dissolution of microbes upon NaOCl treatment. The staining patterns confirmed similar results as CFU analysis.

**Discussion**

Candida *albicans* biofilm presents a significant challenge when dealing with the pathogenesis of periapical lesions (17). *C. albicans* is a polymorphic fungus, observed mainly as round blastospore or elongated hypha, the latter form is important for adhesion, tissue invasion, and biofilm formation (18). Indeed, this fungal organism has the ability to thrive and form biofilm within dentinal tubules and therefore conventional chemo-mechanical procedures may not eliminate them completely (19). Further underscoring their role in endodontic infection, some immunocompromised patients may have an increased risk of fungal infection in the root canal system (20).

Irrigation during canal preparation is an essential step in eliminating the surviving microorganisms after mechanical preparation (21). Sodium hypochlorite (NaOCl) is the most commonly used agent because of its ability to dissolve necrotic tissues (22). Despite its effective antifungal and tissue dissolution properties, accidental extrusion beyond the confinements of the canal space can damage the periapical tissues and exposed oral mucosa (23). Aside from NaOCl, chlorhexidine (CHX) is another popular and commonly used irrigant solution. A concentration of 2% Chlorhexidine gluconate
has shown to be bactericidal and bacteriostatic with a wide spectrum of activity against bacteria and fungi (24).

While NaOCl is highly effective at reducing organism burden, “NaOCl accidents” remain a serious concern regarding its use (25). Accidental extrusion of NaOCl can cause severe pain or permanent nerve and tissue damage to patients undergoing endodontic therapy. Therefore, less toxic, yet highly effective, irrigant solutions are desperately needed. Thus, Endocyn could play a significant role as an endodontic irrigant. Super oxidation of purified water is achieved by passing it through anode and cathode chambers that are separated from the idle salt (NaCl) chamber by an ionic membrane (26). It is made up of 99.99% water and contains other electrically charged atoms (27). Purified water and salt are the sole input materials for its production (28). Contrary to misconception, Endocyn is not watery bleach (29). The ingredients include water, sodium chlorite, sodium sulfate, and monobasic sodium phosphate. The use of super oxide water has been known in the medical community to treat skin infections and ulcers (30), and cases of peritonitis and intraperitoneal abscesses (31). Our results demonstrated that Endocyn has in vitro efficacy against C. albicans biofilm comparable to both CHX and NaOCl. However, its efficacy was greatly reduced compared to these other irrigants using an ex vivo tooth model. It is unlikely that Endocyn failed to adequately reach the canal, as other irrigants were in most cases highly effective at eliminating fungal burden. That said, variations in canal architecture (32) may influence irrigant accessibility. It is also possible that Endocyn is sensitive to breakdown in the presence of complex biologic material (such as dentin) and may rapidly lose antimicrobial efficacy. Therefore, further studies on
Endocyn stability and antimicrobial spectrum are required.

**Conclusion**

Endocyn may be useful as a new endodontic irrigant because of its non-toxic properties and its potential antifungal effect in dental applications. In this study, Endocyn demonstrated similar inhibition of *C. albicans* biofilms as NaOCl and CHX *in vitro*. However, Endocyn was not as effective as NaOCl and CHX at reducing fungal burden in an *ex vivo* split-tooth model.

**References**

Figure 1. Endocyn exhibits rapid antifungal efficacy in vitro. *C. albicans* biofilms were cultivated on polystyrene, washed, and treated with Endocyn (white bar) over a time course. Biofilms were also treated with chlorhexidine (CHX, light gray bar) and sodium hypochlorite (NaOCl, dark gray bar) as controls. Cell viability was assessed using the XTT assay and all values compared to untreated controls. All experiments were repeated in biological triplicate using four wells per group. All data was combined, averaged, and reported as the mean ± SEM. Values were assessed using a two-way ANOVA and Bonferroni post-test. * denotes p < 0.05.
Figure 2. Endocyn demonstrates limited efficacy in an ex vivo split tooth model system. (A) Deidentified human teeth (n=8 per group) were prepared as described and inoculated with *C. albicans*. After 48 h of growth, canals were washed extensively and irrigated with Endocyn, chlorhexidine (CHX), sodium hypochlorite (NaOCl), or PBS control. Quantitative CFU counts were obtained by plating on selective media. Horizontal line denotes the median and statistics generated using a one-way ANOVA and Kruskal-Wallis post-test. ** p < 0.01, *** p < 0.001. (B) Qualitative images of plates utilized for CFU enumeration.
Figure 3. Endocyn demonstrates limited capacity to kill *C. albicans*. Deidentified human teeth were prepared, inoculated with *C. albicans*, and treated with various irrigants, including: Endocyn, chlorhexidine (CHX), sodium hypochlorite (NaOCl), and PBS control. Teeth were stained with BacLight Live/Dead (Syto9, propidium iodide) stain to assess killing. Teeth were analyzed by confocal microscopy using FITC/Texas Red filter sets. Images were captured with (A) 20X and (B) 60X objectives.