Characterization of Novel Antimicrobial Agent for Endodontic Applications

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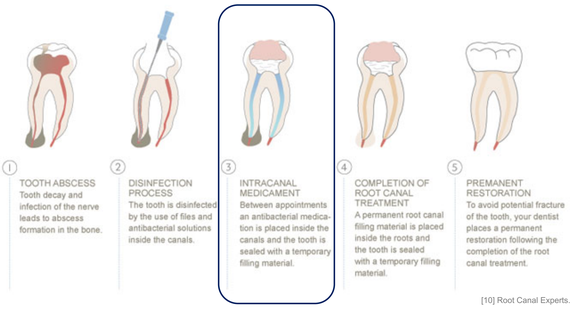
Endodontic Infections

- Endodontic infections arise from the entrance of microbes into the root canal system through periodontal disease, dental trauma, and tooth erosion, all of which **affect 99% of the global population** at some point in their lives. [1-4]
- In the US alone, there are more than 20 million cases of endodontic treatment annually, over **6 million** of these treatments for endodontic infections. [3]
- Untreated or recurrent endodontic infections can be **painful** and have a **direct impact on daily life** and cause **abscesses**, **cellulitis**, and **periradicular inflammatory response**. [5] More severely, these microbes have the potential enter the bloodstream through the root canal to cause **chronic diseases**. [6]
- The infection-causing microbes include bacteria and fungi of various genera including *Lactobacillus*, *Actinomyces*, *Streptococcus*, *Staphylococcus*, *Escherichia*, *Enterococcus*, and *Candida*. [7]

Current Treatment



[10] Root Canal Experts.

Figure 1: Illustration of root canal procedure. The developed antimicrobial agent would most likely function as an intracanal medicament.

- Patients must under undergo a root canal procedure to remove infected pulp tissue and disinfect the affected area.
- **Calcium hydroxide** ($Ca(OH)_2$) is used as an intracanal medicament in root canal procedures to **eliminate microbes** from the root canal system and prevent recurrent infections.

The Problem with $Ca(OH)_2$:

- Calcium hydroxide is **ineffective antimicrobial** against persistent microbes *Enterococcus faecalis* and *Candida albicans*. [8]
- Calcium hydroxide is **cytotoxic** to the dental pulp, which causing dental pulp necrosis, inflammation, and **delays tissue healing.** [9]

Product Development

CASA

Novel compound based on calcium hydroxide*

*Details of product undisclosed due to legal reasons

- Based on calcium hydroxide, we developed an antimicrobial compound which has enhanced antimicrobial efficacy compared to calcium hydroxide while having limited cytotoxicity.
- The pH value of the compound is near the neutral range.
- The compound was synthesized with the goal of being a **Class II medical device** by FDA standards.

Objectives

1. Develop a new antimicrobial agent to replace calcium hydroxide that more effectively eliminates root canal infections and prevents related diseases.

2. Characterize various properties of the antimicrobial agent that are relevant to its application as an intracanal medicament clinical settings.

Antimicrobial Properties

- Calcium hydroxide, and CASA were each deposited into the wells of separate agar plates containing tested microbes: *Candida albicans* SC5314, *Lactobacillus salivarius* 11741, *Actinomyces viscosus* 15987, *Streptococcus gordonii* 49818, *Staphylococcus aureus* 25923, *Escherichia coli* 25922, and *Enterococcus faecalis* 19433
- Larger zones of inhibition indicate greater antimicrobial effect.

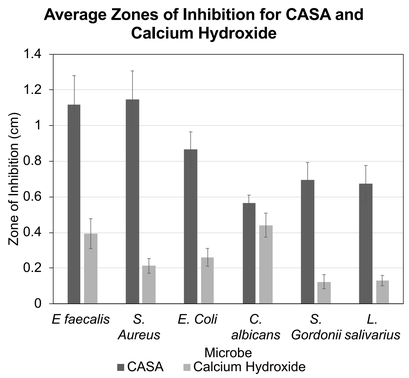


Figure 2: Graph displaying zones of inhibition of CASA and calcium hydroxide for common pathogens found in the root canal.

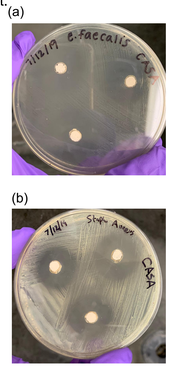


Figure 3. Zones of Inhibition for (a) *E. faecalis* and (b) *S. aureus* in TBSY agar after 24 hour incubation with CASA.

CASA and calcium hydroxide successfully inhibited growth of all tested endodontic microbes, with CASA yielding significantly larger ($p>0.05$) zones of inhibition than calcium hydroxide. On average, CASA produced zones of inhibition that were approximately four times larger than those of calcium hydroxide. Zones were too large to be quantified for *Actinomyces viscosus 15987* for CASA; for calcium hydroxide, inhibition zones averaged 0.714 cm.

DPSC Morphology

- On days 0, 3 and 4 of DPSC incubation, 0.20mL of 0.25 mg/mL of CASA and CaOH were ejected into cell media. Fluorescence staining was added to observe cell morphology.

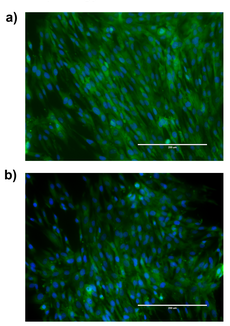


Figure 6: Fluorescent micrographs of DPSC nuclei (blue) and microtubules (green) of the **a)** control and **b)** CASA-exposed cells. Scale bar 200 µm.

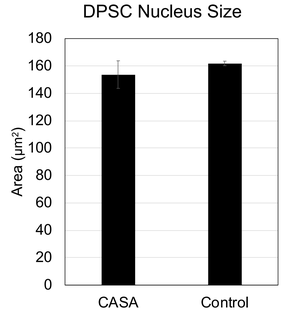


Figure 7: Graph showing average nucleus sizes of DPSC exposed to CASA.

Observation of DPSC under fluorescent microscope with DAPI and alexa fluor staining found similar cellular morphologies. The difference between DPSC nucleus size between CASA and the control is not statistically significant.

X-Ray Diffraction Pattern

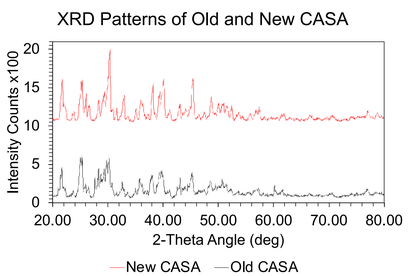


Figure 10: X-ray diffraction patterns of newly synthesized CASA and three-day old CASA. Both samples have similar peaks, suggesting both samples have similar crystalline structures.

- Powdered CASA synthesized day of testing and 3 days prior were used to determine X-ray diffraction (XRD) patterns using the copper Kα line as a radiation source with λ = 1.54184 Å.
- CASA that had been made three days prior generated a similar X-Ray diffraction pattern to that of CASA made the day of XRD testing.

Cytotoxicity Assay

- On the initial day DPSC plating, 0.2mL of 0.25 mg/mL of CASA, and calcium hydroxide were ejected into cell solutions.
- Following 48 hours of incubation, DPSC were counted, and additional 0.2mL of 0.25 mg/mL of CASA, and calcium hydroxide were ejected into cell solutions.
- Following another 24 hours of incubation, DPSC were counted.

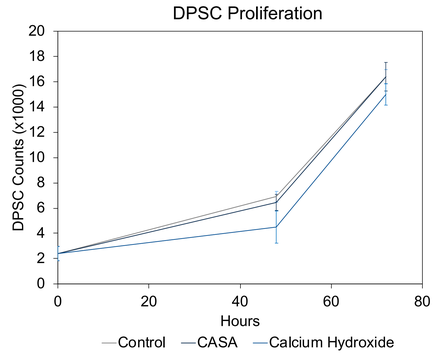


Figure 4: Graph showing cell counts of DPSC incubated with CASA or calcium hydroxide.

Average DPSC Doubling Times

	Hours
Control	24.67
CASA	24.37
$Ca(OH)_2$	24.7

Figure 5: Table of doubling times of DPSC incubated with CASA or calcium hydroxide.

DPSC growth over the four-day period show statistically significant cell count differences between calcium hydroxide and the control, but not between those of the control and CASA. Doubling times were statistically similar for the control and CASA.

Viability Staining: Precipitate and Supernatant

- Because CASA separates into a precipitate and supernatant, the antimicrobial properties of each component were investigated. Increasing amounts of CASA precipitate or supernatant were added to Brain Heart Infusion Broth containing either *Enterococcus faecalis* or *Candida albicans.* Then, the ratio of dead to live microbes was determined using fluorescence microscopy.

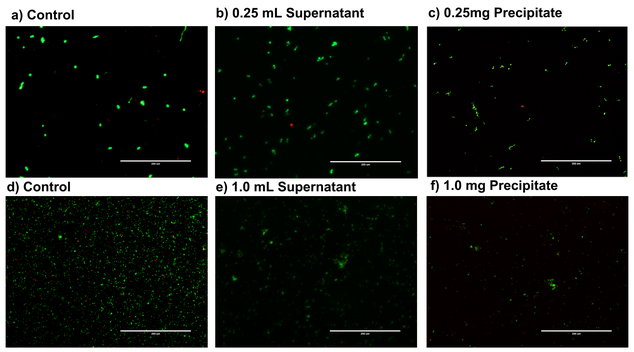


Figure 8: Fluorescence imaging of live (green) and dead (red) *C. albicans* **(a-c)** and *E. faecalis* **(d-f)** exposed to CASA precipitate or supernatant. Scale bar 200 µm.

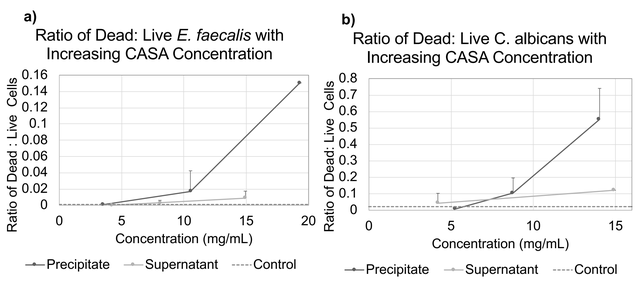


Figure 9: Graph comparing ratio of dead: live **g)** *E. faecalis* and **h)** *C. albicans* incubated with CASA precipitate or supernatant.

Both precipitate and supernatant had larger ratios of dead to live microbes with increasing concentration. In comparison to the supernatant, the precipitate yielded higher ratios of dead to live microbes for both *C. albicans* and *E. faecalis* (Fig. 5).

Dosage Dependence

- To determine whether the antimicrobial effects of CASA are dose-dependent, increasing amounts of CASA were added to Brain Heart Infusion Broth containing both *S. aureus* and *E. faecalis.*

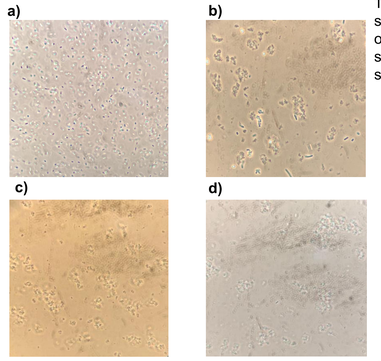


Figure 11: Optical microscopy images of *S. aureus* and *E. faecalis* with **a)** 0 mg, **b)** 0.20 mg, **c)** 0.50 mg, and **d)** 1.0 mg of CASA.

These prepared liquid suspension solutions of bacteria were injected onto agar plates and incubated. The same process was repeated with sodium salicylate (control).

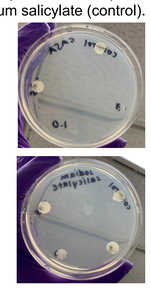


Figure 12. *S. aureus* and *E. faecalis* growth on agar after exposed to varying concentrations of **a)** CASA or **b)** sodium salicylate.

As more CASA was added, fewer bacteria were observed suggesting the antimicrobial properties of CASA are dose-dependent. Field growth of bacteria on all sodium salicylate samples. Bacterial samples with 0.5mg of CASA what is postulated to be a few isolated colonies of mutated bacteria. There was no bacterial growth with the highest concentration of CASA with 1.0 mg.

Discussion

- CASA produced significantly larger zones of inhibition compared to those of CaOH suggesting that CASA is the more efficacious antimicrobial.
- Results of the cytotoxicity assay suggest CaOH is also toxic to DPSC whereas CASA is non-cytotoxic to DPSC, a promising result for potential clinical applications.
- Morphological analysis of DPSC with fluorescent microscope staining indicated both CASA does not affect DPSC morphology, as nucleus sizes of the measured DPSC exposed to each compound were not statistically different from the control.
- Viability staining of CASA precipitate and supernatant affirm that the antimicrobial properties of CASA are derived from the synthesized compound and not excess acid in the supernatant.
- Dose-dependency testing with *S. aureus* and *E. coli* indicate that CASA is dose-dependent with larger concentrations of CASA resulting in greater bacterial lethality. *E. coli* on agar plates containing the highest concentration of sodium salicylate suggest that the sodium salicylate is bacterial-static. In contrast, the lack of bacterial growth exposed to the highest concentration of CASA suggests that CASA is bacteriolytic. For future endodontic applications, appropriate clinical concentrations must be carefully determined such that CASA kills all bacteria within the root canal, thus preventing endodontic reinfection.
- XRD patterns of three-day old and newly synthesized CASA are similar, but old CASA had smaller peak intensities, the variation likely due to powder orientation or variation in salicylic acid and calcium hydroxide measurements in CASA synthesis. Thus, CASA largely retains its crystalline structure over a three day period and does not need to be synthesized just before application.

Conclusion

- The biocompatibility and antimicrobial properties of CASA suggest it is a more efficacious antimicrobial agent than calcium hydroxide, which is currently used in endodontics. Furthermore, CASA, which is dosage dependent, can kill all bacteria found within the root canal owing to its bacterial-lytic properties.

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