5.0 Introduction

Numerous studies have analyzed the microflora of infected dental root canals [1,2]. Nevertheless, there is limited information with respect to which microorganisms persist and survive after completion of root canal treatment [3]. However, certain groups of microorganisms seem to be associated with persistent endodontic infections. Most endodontic infections are mixed and polymicrobial, with predominance of strict anaerobes, some facultative anaerobes and rarely aerobes [4-6]. In addition, recent studies have associated the presence of fungi with therapy-resistant endodontic infections [7,8]. Although fungi have occasionally been reported in untreated cases [9], they have been more associated with cases of failed endodontic treatment [5]. Besides *E. faecalis*, *C. albicans* is the other most predominantly identified species recovered from root canals undergoing endodontic treatment. *C. albicans* is also most often isolated from endodontic therapy failure cases with persistent infections [9,10]. *Candida* species may not respond to conventional cleaning and shaping procedures in the same way as other organisms respond.

Microbiological investigations have revealed that yeasts can be isolated from the root canal both in pure culture and together with bacteria [11]. It has been reported that yeasts exist in about 5% of cases of apical periodontitis. According to Grossman (1952), as much as 17% of infected root canals may contain *Candida* species. Hobson (1959) reported that *C. albicans* was often isolated from root canal infections, although the role of yeasts as potential pathogens in the root canal was not known. According to a case report, a pure culture of *C. albicans* caused acute pulpal-alveolar cellulitis. Nair *et al.* (1990) studied therapy-resistant root canal infections and found microorganisms in six of nine specimens. Bacteria were shown in four of the six cases, while yeast-like organisms were found in two cases as judged by electron
microscopy. The presence of intraradicular bacteria and fungi in root-filled human teeth was associated with persistent periapical lesions. Sen et al. (1995) observed bacteria and fungi by scanning electron microscopy in infected root canals and dentinal tubules. It was found that four out of ten root canals were heavily infected with yeasts. Hence, the root canal treatment aims at eliminating the predominate fungi (C.albicans) involved in root canal infection.

5.1 Methodology

5.1.1 Antibiofilm activity of CAMPs

The overnight cultures of C.albicans were centrifuged (about 3,000 rpm, 5-10 minutes). The cells were washed twice with sterile PBS and the final pellet was resuspended in about 20-25 mL of RPMI 1640 medium. For biofilm formation, 100 μl of the cell suspension (1x10^7cells/ml in PBS) was added to each well of 96 well micro plates. Plates were incubated at 37 °C for 90 minutes of adhesion phase. Wells were washed with sterile PBS to remove non-adhered cells and 200 μl of RPMI-1640 medium was added to the adhered cells. To determine the susceptibility of biofilm development, medium containing various concentrations between 1.565μM and 200μM of the CAMPs were added at the zero hour of biofilm formation i.e. immediately after adhesion phase and the plates were further incubated for 48 hours at 37°C [12]. Density of the cells that survived in biofilm formation was analyzed after staining with 0.1% crystal violet and then quantified by measuring O.D at 600 nm. The reduction in biofilm formation was also observed by using Scanning electron microscopy.
5.1.2 Quantification of microbial load on ex vivo dentinal tubule model

5.1.2.1 Preparation of tooth samples

Freshly extracted tooth samples were collected (IEC approval No: IEC-NI/09/DEC/13/37) and immersed in 5.25% sodium hypochlorite to remove the soft surface tissues and organic debris. The tooth samples were horizontally sectioned into coronal, middle and apical sections using carbide disc. The root canal of each specimen was enlarged with a No:10 round bur. The smear layer including organic and inorganic debris was removed by placing the samples in an ultrasonic bath containing 17% ethylene diamine tetra acetic acid, followed by rinsing with 5.25% NaOCl each for 5 min. The processed tooth samples were then sterilized by autoclaving at 121°C for 30 min.

5.1.2.2 Contamination of blocks

Sterilized tooth samples were immersed in C. albicans suspension and incubated for 7 days at 37°C. Samples which were not incubated with organisms served as negative control to confirm the sterility of samples. Medium was changed every day. After incubation, the samples were gently washed with saline to remove the excess culture from the surface of the sample. Nystatin served as positive control. Subsequently, 10 samples were used for each group and the inoculated samples were grouped as:

Group 1: Tooth samples treated with VSL2 & VS2 (10µM)

Group 2: Tooth samples treated with positive control (Nystatin 10µg)

Group 3: Tooth samples treated with positive control (2% CHX).

Group 4: Tooth samples without any treatment served as negative control.
After the incubation, the dentinal chips were harvested at 400 μm depth using Gates Glidden Drill and the microbial load was determined by measuring O.D at 600 nm.

5.1.3 Mechanism of action- SEM

*C. albinicans* was grown on Whatman No: 1 filter paper strips and incubated overnight at 37°C. After incubation, the strips were treated with peptides (VSL2&VS2) at 10µM for 24 h and followed by immersion in neutralizing broth to stop the action of peptides. The strips were dried and coated with gold. The observations were made on supra 55, Carl Zeiss SEM at the magnification of 100 KX [13].

5.1.4 Quantitative assessment of bacterial viability in dentin by confocal laser scanning microscopy (CLSM)

The dentin block with a length of 4 mm was horizontally sectioned from each tooth at 1mm below the cementoenamel junction. The root canals inside the blocks were enlarged to a size of Gates Glidden Drill #6. Each dentin block was fractured by making a thin groove in the middle of the specimen by using low speed hand piece with a small bur and then the specimens were fractured by a blade and hammer into semi cylindrical halves. The processed dentin was infected with *C. albinicans* for 7 days. The contaminated specimens were washed with sterile water and were divided into four groups: 2%CHX, VSL2 (10µM), VS2 (10µM) and untreated. The specimens were disinfected with each drug at their MIC concentrations and incubated for 24 hours at 37°C. After the disinfection, the dentin specimens were stained with fluorescein di acetate (FDA) which emits green fluorescence in live cells and propidium iodide (PI), which emits red fluorescence in dead cells, whose membranes are disrupted. The uninfected specimens were stained under the same protocol and used as negative controls. Fluorescence from the stained cells were viewed by using
confocal laser scanning microscopy (CLSM) and by staining with FDA and PI, the % of live and dead cells were computed. Post hoc multiple comparisons were used to compare the results at a significance level of $p > 0.05$.

5.1.5 Nuclear condensation in *Candida* cells treated with CAMPs treated *C. albicans*

Nuclear fragmentation was analyzed by DAPI staining [14]. *C. albicans* cells were suspended in RPMI-1640 medium containing peptides (VSL2 & VS2) at their MIC values (10µM), incubated in water bath set at 30 °C for 5–60 min. After incubation, cells were harvested by centrifugation, suspended in PBS and incubated in the dark with DAPI for 10 min. Untreated cells which were stained with DAPI served as controls. After incubation with DAPI, cells were then harvested and examined for nuclear condensation using fluorescence microscope.

5.1.6 Hyphal growth assay

Yeast-form cells of *C. albicans* were collected from cultures on Sabouraud glucose agar, washed with saline and suspended in RPMI medium at $10^5$cells/ml. 20 µL of *Candida* suspension was added to each well containing peptides (VSL2 & VS2) at their MICs (10µM) and were incubated at 37°C for 24 hours. The medium in the wells was discarded and the adhesive *Candida* mycelia were washed with 70% ethanol. The cells were stained with 0.02% crystal violet and washed with water. The microplates were dried and 150 µL of isopropanol was added to the wells and mixed adequately. Then, the reduction in hyphal growth was observed using the phase contrast microscope [15].
5.1.7 Statistical analysis

Comparison between treatment groups were conducted using the Kruskal-Wallis Test /analysis of variance on rank. Post hoc multiple comparison was done using Student-Newman-Keuls test. Comparison within each group was conducted by using Wilcoxon signed-rank test. Values of p<0.05 were considered to be statistically significant.

5.2 Results

5.2.1 Antifungal susceptibility testing of biofilms and membrane damage of *C.albicans*

Reduction in biofilm formation of *C.albicans* after treatment with peptides (VSL2&VS2) of varying concentrations was observed when quantified spectrophotometrically (figure: 5.1).The decline in the optical density at higher concentrations of CAMPs showed the dose dependent decrease in the reduction of biofilm formation of Candida cells. The same was also confirmed using scanning electron microscopy which showed that the spread of biofilm on the membrane was disturbed in the peptides treated samples. This in turn proved the antibiofilm activity of CAMPs (figure: 5.2). The effects of CAMPs on the morphology of Candida cells were investigated using SEM. A change in the cell morphology was observed in Candida cells treated with peptides (figure: 5.3).
Figure: 5.1 Quantification of biofilm development after treatment with antifungal agents
Figure: 5.2 Microscopic observation of biofilm formation by *Candida* cells
Figure: 5.3 Scanning electron micrographs of *C. albicans* showing cell wall damage in CAMPs treated cells.

CONTROL

NYSTATIN 10μg

VSL2 10μM

VS2 10μM
5.2.2 Quantification of microbial load on **ex vivo** dentinal tubule model

Antibacterial activity of VSL2 and VS2 were tested against *C. albicans* in dentinal tubule model by spectrophotometry. The activity of peptides was comparable to an antifungal agent nystatin and CHX. Peptides showed high inhibitory activity at 400 μm depth after 24 h of treatment. VSL2, VS2, Nystatin and CHX showed 74%, 78%, 84% and 85% of reduction of *C. albicans* after treatment for 24 hours, respectively (figure:5.4).

5.2.3 Assessment of bacterial viability in dentin by confocal laser scanning microscopy

The efficacy of AMPS on dentinal tubules penetrated with *Candida* cells were assessed by CLSM (figure: 5.5). The results shows 57% dead cells in VSL2 treated tooth samples and 57% dead cells after VS2 treatment which confirms the complete penetration of peptides inside the infected dentinal tubules.
Figure: 5.4 (a) Assessment of fungal viability in dentin by confocal laser scanning microscopy
Figure: 5.4 (b) Percentage of live and dead cells distributed in dentinal tubule after treatment with antifungal agents at their MICs

Figure: 5.5 Efficacy of CAMPs -Ex vivo dentinal tubule model
5.2.4 Nuclear condensation in peptides treated *C. albicans*

DAPI, a cell-permeable fluorescent dye, is commonly used to detect nuclear morphology changes, including nuclear condensation and fragmentation. It binds strongly and selectively to the minor groove of adenine and thymine-rich sequences of DNA. Therefore, any increase in the uptake of cells reflects the extent of DNA damage. DAPI staining of cells treated with VSL2 & VS2 showed increased uptake of dye in to the cells. The results indicated that peptides caused nuclear condensation in *Candida* cells (figure: 5.6).

**Figure: 5.6 Nuclear condensation by DAPI staining in CAMPs treated *Candida* cells**

![Figure 5.6](image_url)
5.2.5 Hyphal growth assay

The hyphal form has an important role in causing disease. Hyphae are defined as tubular projections from a mother cell that extend greater than one cell diameter from the mother cell. Hyphae germinate from a single cell and continue to extend, consisting of several septated hyphal cells. To determine the effects of CAMPs as an antifungal agent on hyphal formation, Candida cells were analyzed for hyphal formation in the absence and presence of CAMPs and the effect of the same was observed microscopically. A standard protocol for hyphal formation was used. In the presence of VSL2 &VS2, fewer yeast cells survived and almost no hyphae were observed. In the untreated sample, more hyphal and yeast cells were seen. Hence, it could be concluded that CAMPs completely inhibited the hyphal growth in Candida cells (figure: 5.7).

**Figure: 5.7 Phase-contrast micrographs of hyphal formation by C. albicans grown in the presence of antifungal agents**
5.3 Discussion

Microorganisms and their products are considered to be one of the main causes of pulpal and periapical diseases [16]. They can attach to the root canal walls and form biofilms [17]. Microbiological investigations of root canal infections have demonstrated that root canal infections with only one bacterial strain are rare [18]. The incidence of *C. albicans* in infected root canals has been shown to vary between 7% and 55% [19]. The ecology of the necrotic root canal may allow yeasts to grow in pure culture without the presence of bacteria. Because of the difficulty of ensuring adequate removal of the biofilm on the dentine wall using the mechanical procedures only, it is necessary to use the agents with antimicrobial properties [20]. In the present study, it was found that AMPs were found to be active against *C. albicans*. The antifungal activity of VSL2 &VS2 against clinical isolates of *C. albicans* was checked and was sensitive to AMPs at the MIC of 10µM.

Various studies reported that biofilms formed by *C. albicans* are resistant to most of the commonly used antifungal drugs [21]. But a reduction in biofilm formation of *C. albicans* after treatment with peptides was observed using SEM and quantified by spectrophotometric method. The aggregation of *Candida* cells was prevented by AMPs. This assay is significant because; the formation of biofilm is an important causative factor in dental infection. The effects of VSL2 &VS2 treatment on the morphology of *Candida* cells were investigated by SEM. A change in the cell morphology was observed in peptides treated *Candida* cells at their MICs (10µM). *Ex vivo* study on dentinal tubule model showed that designed AMPs showed a drastic reduction in microbial load at 400µm depth in root canal. It could serve as a useful intracanal medicament to eradicate *C. albicans*. The efficacy of AMPS against the organisms invading the root canal was also proved by CLSM. DAPI, a cell-permeable...
fluorescent dye, is commonly used to detect nuclear morphology changes, including nuclear condensation and fragmentation. It binds strongly and selectively to the minor groove of adenine and thymine-rich sequence of DNA. Therefore, any increase in uptake of dye in VSL2 & VS2 treated *C. albicans* reflects the extent of DNA damage. CAMPs also proved to inhibit hyphal formation by *Candida* wherein, the cells were grown to stationary phase (48 h) at 30°C followed by washing in PBS and resuspension to a final concentration of less than $10^5$ cells/ml at 37°C. Under these normal conditions, 90% of hyphal formation is observed. But CAMPs at 10µM concentration inhibited hyphal formation of *C. albicans* cells at 24 hours.

### 5.4 Conclusion

In conclusion, CAMPs are broad spectrum fungicidal agents and their mode of action in *C. albicans* is by disrupting the cell wall and by inhibiting biofilm formation. They also inhibit hyphal formation of yeast cells. Hence, these peptides hold good promise to be used as intracanal medicaments in the treatment of endodontic infections with *C. albicans*. 
5.5 Reference


