ABSTRACT

Infections have been the major cause of disease throughout the history of human population. The emergence of pathogenic bacterial resistance to available conventional antibiotics is a major threat in treatment of infectious diseases. It has been recognized that oral infections, may affect the course and pathogenesis of a number of systemic diseases, such as cardiovascular disease, bacterial pneumonia, diabetes mellitus, and low birth weight. Endodontic infection is the infection of the dental root canal system and the major etiologic agent of apical periodontitis. Almost 700 bacterial species can be found in the oral cavity, with any particular individual harboring 100–200 of these species. *E. faecalis* is a gram positive cocci and a facultative anaerobe is the predominant microbe in 4-40% of endodontic infection responsible for dental plaque and biofilm formation. Besides *E. faecalis* the other predominant microbe *C. albicans*, has been repeatedly identified as the species most commonly recovered from root canals undergoing treatment, in cases of failed endodontic therapy and canals with persistent infections.

Recent studies have shown that more than 70% of the clinical isolates of dental infections are resistant to existing antibiotics. The resistance developed by bacteria has been attributed to target site alteration, drug inactivation, or changes in the metabolic pathway. The current treatment of eradicating these microbes in endodontics involves irrigating the root canal with powerful irrigants like *chlorhexidine* (CHX) and sodium hypochlorite (NaOCl). Treatment failure occurs despite the usage of these irrigants due to the resistant microbes harboring the root canal. These agents can damage the surrounding tissues over long residence in the root canal. The reason for the endodontic treatment failure has been attributed to restricted or inadequate residence time of the powerful antiseptics in the root canal,
due to its toxicity, resulting in incomplete cleaning of the root canal & thereby leaving resident microbes, which can colonize & cause reinfection. Hence complete removal of microbe from root canal is expected to provide good results in combating majority of endodontic infection by a new class of agents which can overcome these limitations.

Peptides comprising about or less than 50 amino acids and possessing a net positive charge are grouped as Cationic antimicrobial peptides (CAMPs). They exhibit a broad spectrum of antimicrobial activity similar to that of naturally occurring peptides and hence resistance to such agents is relatively rare. Synthetic AMPs also offer the flexibility for inclusion of unnatural amino acids such as α,β-didehydrophenylalanine (ΔPhe), D-amino acids, aminoisobutyric acid (Aib), N-methylated amino acids during the synthesis and such modifications leads to better stability of these peptides toward proteolysis. AMPs with modified amino acids are thus reported to have enhanced antimicrobial efficacy for potential clinical application. The main advantage of AMPs resides in the mechanism of action which is different from that of conventional antibiotics currently used in endodontic infection. Hence the hypothesis of the study is that AMPs may probably be a better agent in eradicating resident microbe thereby preventing the possibility of reinfection.

**Aim:**

To determine the antimicrobial efficacy of the cationic synthetic peptides

**Objectives:**

- Screening a panel of CAMPs against various pathogens
- To determine the anti-bacterial efficacy of the best CAMPson chosen microbes
- To determine the *in vivo* efficacy of CAMPs in animal models
✓ To study the biocompatibility of CAMPs by *in vitro* methods

✓ To determine genotoxicity of CAMPs using *Drosophila melanogaster* and cytogenetic techniques

The thesis comprises of eight chapters. The **first chapter** summarizes about different peptides used for various applications, their mode of synthesis and characterization. It also discusses the organisms involved in endodontic infections, about current treatment in particular the reason behind recurrent infection and treatment failures. Furthermore the mechanisms of action of peptides against microbes are also discussed. The chapter also explains about different models chosen to study the efficacy and toxicity of the peptide to formulate the hypothesis.

The **second chapter** describes in detail the materials and methods for the study including real time PCR analysis for reduction in microbial load; *ex vivo* dentinal tubule model, cytotoxicity on mammalian cell lines and genotoxicity on *Drosophila melanogaster* among others. The chapter also deals with statistical analysis used in the various assays.

Solid phase peptide synthesis (SPPS) was adopted for the synthesis AMPs. The general principle of SPPS is one of repeated cycles of coupling-deprotection wherein the free N-terminal amine of a solid-phase attached peptide is coupled to a single N-protected amino acid unit. Using Fmoc (9-fluorenylmethoxycarbonyl) chemistry on rink amide MBHA(4-methylbenzhydrylamine hydrochloride salt) resin with DIPC(Diisopropylcarbodiimide) and HoBT (Hydroxybenzotriazole) as coupling agents. This unit is then deprotected, revealing a new N-terminal amine to which a further amino acid may be attached followed by cleavage of the peptide from the resin support with the concurrent cleavage of all side chain protecting groups to give the crude free peptide. Pure peptides were obtained by reverse-phase high-performance
liquid chromatography. The mass of the peptide was confirmed by electro spray ionization mass spectrometry and the results are given in **chapter three**.

The **fourth chapter** presents the screening efficacies of peptides using agar diffusion method and micro dilution assay by spectrophotometric technique. Eleven peptides were screened against various organisms of which two peptides **VSL2 & VS2** were found to be more potent and were taken up for further studies. The bactericidal activity of CAMPs was determined by minimal bactericidal concentration (MBC). The killing kinetics was studied using time kill assay. To investigate further more on efficacies of these peptides an *ex vivo* tooth model was developed in which the *E. faecalis* infected tooth samples were treated with AMPs and the efficacy was determined by quantifying the bacterial load reduction using 16s rRNA at two different depths(200µm &400µm) in the dentinal tubule by Real time PCR. The mechanism of action of CAMPs was observed using scanning electron microscopy. The chapter also presents efficacy studies documented by Confocal laser scanning microscopy (CLSM) and Fluorescence activated cell sorter (FACS). This chapter deals with the efficacy of synthesized peptides against resistant strains of *E. faecalis* to common antibiotics. The endodontic treatment failed tooth samples were collected from the dental college at SRU and the resistant *E. faecalis* was isolated and characterized. The efficacies of the peptides were determined using Agar diffusion and micro dilution technique. The results showed that CAMPs were potent against those isolated resistant strains which were resistant to other antibiotics.

Once the *in vitro* efficacies and mechanism of actions of peptides were performed, *in vivo* efficacy of AMPs was carried out using female BALB/c mice (IAEC approval No: IAEC/XXII/SRU/300/2013). To mimic *E. faecalis* infection, the bacterial bolus was injected into the left thigh of mice and the infected mice were treated with
Peptide (VSL2) and vancomycin and the mice were euthanized after 24 hours. The infected tissues were removed, homogenized, and plated for determination of viable bacterial count (CFU). The Samples of dissected organs from the infected animals were fixed in formalin and stained using hematoxylin and eosin and were analyzed qualitatively under light microscope for microscopical changes. The results are discussed in chapter five. 

*Candida albicans* is the most frequently isolated species from infected tooth samples besides other isolates: *C.glabrata, C.guilliermondii, C.inconspicua, C.krusei, C.parapsilosis, C.tropicalis, C.crusei* and treating them is important. Hence screening of same peptides (VSL2&VS2) against *C. albicans* were performed by agar diffusion and the reductions in biofilm formation of *C.albicans* were also determined by qualitative and quantitative method. The mechanism of action of peptides on the fungal cell membrane was proved using scanning electron microscopy.

The efficacy of peptides in *C.albicans* infected tooth model was also determined by spectrophotometric method. Furthermore the penetration of peptides in the infected dentinal tubules was analyzed using CLSM. Nuclear fragmentation of the fungi treated with peptide was analyzed by DAPI staining technique and examined by microscopy. The results of the same are discussed in chapter six.

The biocompatibilities of chosen peptides to mammalian cells were assessed using different techniques. The cytotoxicity of the AMPs (VSL2 & VS2) was determined using MTT assay on L6, rat myoblast cells ;in this assay equal number of cells were treated with at 1X and 5 X MIC of peptides and the percentage of viability was measured at the end of 24 hours by spectrophotometric method. To determine the hemocompatibility of the peptides, RBCs from healthy volunteers were treated with peptides and the percentage of hemolysis was calculated in comparison to known
controls. The effect of peptides to platelets were determined by incubating the peptides with isolated human platelets from healthy volunteers and the aggregating properties of peptides were analyzed and compared with controls. The results of these biocompatibility tests are shown in chapter seven.

To determine the genotoxicity of peptides, cytokinesis blocked micronucleus assay (CBMN) was performed on human peripheral blood mononuclear cells (PBMCs). In this assay, the PBMCs from healthy volunteers were collected and exposed to different concentration of peptides. After the exposure, the cells were harvested at specified time point and stained with Giemsa and a total of thousand binucleated cells were scored and the MN frequency was computed. The genotoxicity of peptides on *Drosophila melanogaster* was determined by WING SPOT assay. The results of genotoxicity of AMPs are discussed in chapter eight and nine.

The overall results suggest that the CAMPs VSL2 and VS2 showed potent activity against *E. faecalis* and *C. albicans* majorly involved in root canal infections. These CAMPS were also found to be biocompatible and not genotoxic and hence all these properties prove that these CAMPs can be developed as worthy candidates to treat various infections.