CHAPTER 10

Microbiological Screening

10.1 INTRODUCTION

Antibiotics have historically been used as an adjunct to periodontal treatment in the discipline of infectious periodontal diseases caused due to various pathogenic bacteria, either by systemic or local administration. Currently, antibiotic treatment for periodontal infections and exacerbations is limited to systemic administration. Adult periodontitis has been shown to be associated with the presence of specific bacteria like; Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum, Eikenella corrodens, Campylobacter rectus, etc. Commonly prescribed antibiotics against periodontal infectious diseases include tetracyclines (e.g., minocycline), penicillins (e.g., penicillin V, amoxicillin), erythromycins (e.g., erythromycin stearate), lincosamides (e.g., clindamycin) and cephalosporins (e.g., cephalexin), clindamycin, tetracycline, neomycin, metronidazole or canamycin; antimicrobial agents such as iodine, sulfonamides, mercurials, bisbiguanidines, or phenolics; anti-inflammatory agents such as indomethacin or hydrocortisone; immune reagents such as immunoglobulins, or antigen binding fragments of immunoglobulins or immunomodulatory agents such as methotrexate.

Systemic administration of the active medicament relies upon circulatory elements to bring active drug to the infected site. However, the absence of normal vasculature near infected and/or inflamed periradicular tissue and necrotic pulpless teeth renders systemic administration inefficient, particularly when it is combined with the knowledge that to be effective, an antibiotic must be in contact with the targeted microorganisms (Grossman, 1951). These facts clearly compromise the potential utility of systemically administered prophylactic antibiotics.

In contrast, the local delivery of antibiotics confers the therapeutic benefit of delivering the medicament directly to the targeted tissue space. Furthermore, the ability to establish substantial local concentrations of an antibiotic also minimizes the risk of contributing to the development of drug resistant pathogens. One of the major contraindications to the use of systemic antibiotics is the theoretical possibility that bacteria remains ineffective at relatively low concentrations of the antibiotic achieved by oral administration will give rise to strains having multiple drug resistance along with a high risk of toxicity.

Mainly antibiotic resistance among many periodontal pathogenic microbes has been increasing during the last decade, which is mostly associated with;

- Overuse of antibiotics in outpatient settings
- Unwarranted use of very broad spectrum antibiotics
- Poor standards for streamlining bacterial identification and patient monitoring
- In-effective hospital infection control over nosocomial transmission of resistant strains.

A critical reevaluation of the merits of delivery devices, vehicles, techniques, and medicaments which have been historically utilized for periodontal drug delivery methods reveals that the use of medicaments intraperiodontaly and in particular the use of antibiotics, has been criticized for inadequate spectrum of activity and short duration of effectiveness (Yoshida et al., 1987). The former issue has been addressed by improved microbiological sampling techniques, particularly anaerobic culturing techniques, which now provide practitioners with an accurate profile of the bacterial species associated with periodontal infections. As a result, the short duration of effectiveness emerged as the major flaw of periodontal drug delivery protocols.

An attempt has been made to study the resistance of these infectious microbes to minocycline hydrochloride/ clindamycin phosphate by using Staphylococcus aureus ATCC 29213 (an aerobic gram positive cocci) and Escherichia coli ATCC 25922 (aerobic gram negative cocco bacilli).

Staphylococci are facultative spherical, gram positive bacteria, which are immobile and form grape like clusters. Staphylococcus aureus ATCC 29213, a normal inhabitant of the skin and mucous membranes in the nasal and oral mucosa of a healthy human, mainly grow by aerobic respiration or fermentation that produces lactic acid.

Escherichia coli ATCC 25922 are normal inhabitant of the intestines of all animals, including humans. When aerobic culture methods are used, E. coli is the dominant species found in feces. Normally E. coli serves a useful function in the body by suppressing the growth of harmful bacterial species and by synthesizing appreciable amounts of vitamins. A minority of E. coli strains are capable of causing human illness

by several different mechanisms. E. coli serotype O157:H7 is a rare variety of E. coli that produces large quantities of one or more related, potent toxins that cause severe damage to the lining of the intestine. Escherichia coli ATCC 25922 are also reported to be the infective causative organism in most of the infectious periodontal diseases.

In the present investigation the antibiotics minocycline hydrochloride/ clindamycin phosphate used are reported to be highly effective against many of the bacterial taxa commonly associated with periodontal infectious diseases like; Actinomyces, Eubacterium, Fusobacterium, Propionibacterium, microareophilic Streptococci, Peptococcus, Peptostreptococcus, Veillonella, Prevotella, and Porphyromona. Depending on the effective concentration of the antibiotics achieved at the site of infection they act either as bacteriostatic or bacteriocidal.

Minocycline hydrochloride, a semi-synthetic tetracycline class of derivative, is bacteriostatic in nature with wide spectrum of activity (Stratton and Lorian 1996). Along with antibiotic nature minocycline hydrochloride also acts as a chelating agent by chelating Ca^{2+}, Mg^{2+}, Al^{3+} ions in gut. Minocycline hydrochloride mainly exerts antimicrobial activity by inhibiting the protein synthesis. Due to lipophilic nature, minocycline hydrochloride passes directly through the lipid bilayer of the bacterial cell wall. Once inside the bacterial cell minocycline inhibits protein synthesis and cause phosphorylation in microorganisms by binding specifically to the 30S ribosomes and reversibly to 50S ribosomal subunits. The drug appears to prevent access of aminoacyl tRNA to the acceptor site on m-RNA ribosome complex (Green et.al, 1976). This prevents the addition of amino acids to the growing peptide chain. In high concentrations minocycline impairs protein synthesis in mammalian cells (Pato ML 1977). Minocycline is partially degraded to microbiologically inactive metabolites by chemical conversion in the body (MacDonald H et.al, 1973). Minocycline hydrochloride, have similar antimicrobial spectra of activity like that of tetracycline against a wide range of gram-positive and gram-negative organisms. In vitro susceptibility testing has shown that most of the organisms associated with periodontal disease, are susceptible to minocycline hydrochloride at a concentration of 8 μg/ml (Slots and Rams 1990).

Clindamycin phosphate, a water soluble ester of semi-synthetic antibiotic, possess wider spectrum of activity than other lincomycin derivatives, which includes Staphylococcus species, Streptococcus species (except Streptococcus faecalis), and Mycoplasma species, as well as anaerobic organisms, such as Bacteroides species, Fusobacterium species, Clostridium perfringens, Actinomyces species, Peptostreptococcus species, and many Propionibacterium species. As anti-infective, clindamycin phosphate also inhibits bacterial protein synthesis at the level of bacterial ribosome by binding preferentially to the 50S subunit of bacterial ribosome and interfering with peptidyl transfer, which prevents elongation of peptide chains and ultimately suppresses bacterial protein synthesis. (AHFS Drug Information Reference, 388-393 (1997)). The short half life (2.4-3.2 hr) of clindamycin phosphate causes its rapid conversion into active clindamycin. About 10% of bioactivity is reported to be excreted in the urine and a very less amount of about 3.6% is excreted in the feces.

The minimum inhibitory concentration (MIC) of clindamycin for most susceptible aerobic and micro aerophilic bacteria is 1.0-4.0 μg/ml, and is often observed to be much lower than the corresponding penicillin or erythromycin MIC.

10.2 EXPERIMENTAL

10.2.1 Source of culture medium

Staphylococcus aureus ATCC 29213 was received as a gift sample from Food & Drug Laboratory, Vadodara.

Escherichia coli ATCC 25922 was also obtained from Food & Drug Laboratory, Vadodara.

10.2.2 Preparation of Culture medium

Required amount (13gm) of nutrient broth powder (HiMedia Labs, Mumbai) was accurately weighed and dissolved in 1000 ml of distilled water. After complete dissolution, the nutrient broth medium was subjected to sterilization by autoclaving in autoclave (Modern Industries Corp., Mumbai) at 121°C for 15 minutes.

10.2.3 Preparation of nutrient agar plates

Nutrient broth was prepared in the similar manner as described earlier. pH was adjusted to 6.75. To this 3 % Agar powder (Hi-Media Labs, Mumbai) was added with continuous heating. Then nutrient agar was subjected to sterilization by autoclaving at 121°C for 15 minutes. The nutrient agar plates each containing 25ml of sterile nutrient agar were prepared by directly pouring the media into sterile petridishes aseptically in the laminar flow and subjected to solidification. The petridishes were again incubated at 37±1°C for 24 hours to check for their sterility. The medium was seeded with the organism by pour plate method using sterile top agar (5ml) containing 0.1 ml culture of microbes (0.5 MacFarland standards) implicated in periodontal infections. Borewells of suitable size were made with the help of a sterile borer sterilized by dipping in ethanol and passing through the flame.

10.2.4 Preparation of minocycline hydrochloride/ clindamycin phosphate solutions

Accurately 0.1ml of the drug samples of minocycline hydrochloride/ clindamycin phosphate of known concentration (1µg/ml and 2µg/ml respectively) were filtered through sterilized millipore membrane filters (0.2 mm) and added in wells bored in inoculated solidified media in petridishes. About 0.1 ml of minocycline hydrochloride/ clindamycin phosphate at a concentration of 1µg/ml and 2µg/ml respectively was taken as standard. These plates were allowed to incubate at 37±1°C for 24h in an incubator. Then the diameter of the zone of inhibition was noted. The same procedure was also adopted for placebo formulations. Results given in table no.10.02 and 10.03 are the mean of three observations.

10.2.5 Preparation of solutions of minocycline hydrochloride/ clindamycin phosphate loaded periodontal formulations

Accurately 0.2ml of the samples of minocycline hydrochloride/ clindamycin phosphate loaded periodontal formulations of known concentration (1µg/ml and 2µg/ml respectively) obtained from ex vivo release studies were filtered through sterilized

millipore membrane filters (0.2 mm) and added in wells bored in inoculated solidified media in petridishes in the manner similar to that of plain drug dilutions. About 0.1 ml of minocycline hydrochloride/ clindamycin phosphate at a concentration of 1μg/ml and 2μg/ml respectively was taken as standard. These plates were allowed to incubate at 37±1°C for 24h in an incubator. Then the diameter of the zone of inhibition was noted. Results given in table no.10.05 to 10.12 are the mean of three observations.

10.2.6 Study of Minimum Inhibitory Concentration (MIC) by agar diffusion method

Minimum Inhibitory Concentration (MIC) of the antibiotics can be defined as the minimum concentration of substance in the external medium at which the antibiotic inhibits the growth of the microorganisms completely. Simple dilution method, agar dilution method and broth dilution method are useful to determine the MIC. In the present study agar diffusion method was used to determine MIC.

Nutrient agar plates were prepared as given earlier. Bore were made on the medium using sterile borer and 0.1 ml of different concentrations of minocycline hydrochloride/ clindamycin phosphate and their periodontal formulations were added to the respective bores. The petridishes were kept in the refrigerator at 4°C for 30 min. and then incubated at 37±1°C for 24 hrs. Finally the zones of inhibition in each of the samples were observed and measured using a scale. Results tabulated in table no.10.01, 10.02 and 10.03 are mean of three observations.

The concentration at which there was no visually detectable bacterial growth was observed was taken as the MIC. Results are tabulated in table no. 10.01. Distilled sterile water was taken as the control. The control activity was deducted from the test and the result obtained was reported.

Table 10.01: MIC of Minocycline hydrochloride/ Clindamycin phosphate and their periodontal formulations against S. aureus ATCC 29213 and E. coli ATCC 25922

Name of the dilution used	Minimum inhibitory concentration	
	S. aureus ATCC 29213	E. coli ATCC 25922
MnHCl	0.25-1.0µg/ml	0.5-2.0 µg/ml
MGF02	0.5µg/ml	1.0µg/ml
MSF27	1.0µg/ml	1.0µg/ml
MMF01	0.5µg/ml	1.5µg/ml
ML14	1.0µg/ml	1.0µg/ml
$ClPO_4$	3.0-5.0µg/ml	1.0-3.0µg/ml
CGF54	2.5µg/ml	2.5µg/ml
CSF27	2.0µg/ml	2.0µg/ml
CMF01	2.0µg/ml	2.0µg/ml
CL14	2.0µg/ml	2.0µg/ml

n=3

As obtained from the results given in table no.8.01, it can be concluded that minocycline hydrochloride is effectively active against S. aureus and E. coli at a concentration within 0.25-1µg/ml and 0.5-2µg/ml respectively. Similarly this can also be concluded that clindamycin phosphate is active against S. aureus and E. coli at a concentration within 2-5µg/ml and 1-3µg/ml respectively, which is similar as those reported.

All the minocycline hydrochloride/ clindamycin phosphate loaded periodontal formulations were tested to determine zone of inhibition to check their antimicrobial activity.

Table 10.02: Study of antimicrobial activity of Minocycline hydrochloride and their periodontal formulations against S. aureus ATCC 29213 and E. coli ATCC 25922

* Dilutions used	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
MnHCl	27.0-29.0	22.0-24.0
MGF02	26.0-28.0	21.0-23.0
MSF27	24.0-26.0	19.0-21.0
MMF01	25.0-27.0	20.0-22.0
ML14	23.0-25.0	19.0-22.0

* Concentration of the dilution used: 1µg/ml, n=3.

Table 10.03: Study of antimicrobial activity of Clindamycin phosphate and their periodontal formulations against S. aureus ATCC 29213 and E. coli ATCC 25922

* Dilutions used	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
$ClPO_4$	25.0	19.0
CGF54	28.0	19.0
CSF27	29.0	18.0
CMF01	30.0	22.0
CL14	27.0	21.0

* Concentration of the dilution used: 2µg/ml,n=3.

Table 10.04: Study of antimicrobial activity of formulation additives and the solvent against S. aureus ATCC 29213 and E. coli ATCC 25922

Dilutions used	* Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
Distilled water	-	-
Pluronic F127	-	-
Poly Vinyl Alcohol	-	-
Ethyl cellulose	-	-
Soya PC	-	-
Cholesterol	-	-

* "-" No zone if inhibition, n=3

Table 10.05: Study of antimicrobial activity of minocycline hydrochloride loaded periodontal thermoreversible gel formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
0.50	26.0	20.0
1.00	26.0	19.0
1.50	26.0	19.0
2.00	27.0	2.0
2.50	25.0	21.0
3.00	26.0	23.0
3.50	25.0	22.0
4.00	28.0	25.0
5.00	29.0	22.0
6.00	28.0	19.0
7.00	29.0	24.0
8.00	32.0	25.0

* Concentration of the dilution used: 1µg/ml, n=3

Table 10.06: Study of antimicrobial activity of clindamycin phosphate loaded periodontal thermoreversible gel formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
0.50	24.0	18.0
1.00	25.0	19.0
1.50	24.5	21.5
2.00	26.0	18.0
2.50	25.0	19.0
3.00	28.0	21.0
3.50	29.0	17.5
4.00	26.0	19.5
5.00	27.0	20.0
6.00	25.0	21.0
7.00	28.5	19.0
8.00	29.0	23.0

* Concentration of the dilution used: 2µg/ml, n=3

Table 10.07: Study of antimicrobial activity of minocycline hydrochloride loaded periodontal mucoadhesive strip formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	27.0	19.0
2.0	25.0	20.0
4.0	26.0	21.0
6.0	27.0	21.5
8.0	28.0	22.0
12.0	29.0	23.0
24.0	30.0	24.0

* Concentration of the dilution used: 1µg/ml, n=3

Table 10.08: Study of antimicrobial activity of clindamycin phosphate loaded periodontal mucoadhesive strip formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	24.0	19.0
2.0	24.5	20.0
4.0	25.0	18.0
6.0	26.0	18.5
8.0	27.0	21.0
12.0	28.0	22.0
24.0	29.0	23.0

* Concentration of the dilution used: 2µg/ml, n=3

Table 10.09: Study of antimicrobial activity of minocycline hydrochloride loaded periodontal mucoadhesive sphere formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	25.0	20.0
2.0	25.5	20.0
4.0	26.0	21.0
6.0	26.0	21.5
8.0	26.5	22.0
12.0	27.0	23.0
24.0	29.0	24.0

* Concentration of the dilution used: 1µg/ml, n=3

Table 10.10: Study of antimicrobial activity of clindamycin phosphate loaded periodontal mucoadhesive sphere formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	24.0	18.0
2.0	24.5	20.0
4.0	25.0	21.0
6.0	26.0	21.5
8.0	27.0	22.0
12.0	27.5	23.0
24.0	28.0	22.0

* Concentration of the dilution used: 2µg/ml, n=3

Table 10.11: Study of antimicrobial activity of minocycline hydrochloride loaded periodontal mucoadhesive liposomal gel formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	24.0	21.0
2.0	24.5	21.0
4.0	25.0	20.0
6.0	23.0	20.5
8.0	23.5	21.5
12.0	26.0	22.0
24.0	26.0	23.0

* Concentration of the dilution used: 1µg/ml, n=3

Table 10.12: Study of antimicrobial activity of clindamycin phosphate loaded periodontal mucoadhesive liposomal gel formulation against S. aureus ATCC 29213 and E. coli ATCC 25922 at different time interval

* Time in hour	Zone of Inhibition in mm	
	S. aureus ATCC 29213	E. coli ATCC 25922
1.0	23.0	19.5
2.0	23.5	20.0
4.0	24.0	18.0
6.0	25.0	20.5
8.0	25.5	21.0
12.0	26.5	22.0
24.0	27.0	22.5

* Concentration of the dilution used: 2µg/ml, n=3

10.3 RESULTS AND DISCUSSION

The microbes S. aureus and E. coli are the major pathogens with very high rates of prevalence of resistance against various periodontal pathogenic bacteria. Hence the study of the development of resistance of both the antibiotics minocycline hydrochloride/ clindamycin phosphate against both types of the aerobic microorganisms; gram positive Staphylococcus aureus ATCC 29213 and gram negative Escherichia coli ATCC 25922 were made to confirm the activity of the minocycline hydrochloride/ clindamycin phosphate and their periodontal formulations against periodontal pathogenic bacteria.

The data shown in table no.10.01 evidenced that the minimum inhibitory concentration of minocycline hydrochloride and its periodontal formulations for the antimicrobial activity against Staphylococcus aureus ATCC 29213 was obtained to be within 0.25-1μg/ml and within 0.5-2μg/ml for E. coli. However, the concentration of clindamycin phosphate poses MIC within 1-3μg/ml against Escherichia coli ATCC 25922 and within 2-5 μg/ml for S. aureus.

As shown from the data obtained from table no.10.02 it is evident that minocycline hydrochloride showed the zone of inhibition within the range 24.0-29.0mm for S. aureus and 19.0- 24.0mm for E. coli, which is almost similar as that of the standard reported one (25.0-30.0 mm for S. aureus and 19.0-25.0 mm for E. coli) (Minocin, The Monograph). From the results obtained in table no. 10.03 it is evident that clindamycin phosphate posses the zone of inhibition within 32.0-35.0 mm for S. aureus and 8.0-11.0 mm for E. coli, which is also similar to that of the reported one (24.0-30.0 mm for S. aureus and 17 mm for E. coli) (Cleocin, The monograph).

However, all the formulation additives and the solvent used when checked for presence of any of the antimicrobial activity resulted to posses no zone of inhibition thereby indicating absence of any antimicrobial activity or interference in the activity of the drugs tested.

All the mucoadhesive periodontal formulations when checked for their antimicrobial activity at the different sampling points of drug release were found to posses the zone of inhibition. Therefore this can be concluded that minocycline hydrochloride/ clindamycin phosphate and their periodontal formulations posses the required antimicrobial activity against both gram positive Staphylococcus aureus ATCC 29213 and gram negative Escherichia coli ATCC 25922 organisms. Hence they can be further considered for further investigations.

10.4 CONCLUSION

From the above study it can be concluded that the different mucoadhesive periodontal formulations such as thermoreversible gel, strips, spheres and liposomal gel showed in vitro antimicrobial activity against Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 organisms which are some of the responsible organisms for the periodontal diseases. Minocycline hydrochloride loaded periodontal formulations were evaluated for the antimicrobial activity at a concentration of 1µg/ml and clindamycin phosphate loaded periodontal formulations were evaluated for the antimicrobial formulations at a concentration of 2µg/ml. Formulations were also evaluated for the antimicrobial activity at each of the points of drug release, which were also resulted to posses the diameter of zone of inhibition, which indicates the release of minocycline hydrochloride and clindamycin phosphate from the periodontal formulations were above the MIC.

Hence it can be concluded that all selected drug loaded mucoadhesive periodontal formulations meant to be placed locally into the periodontal pocket posses the promising antimicrobial activity against staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 organisms. The selected drug loaded periodontal formulations showed antimicrobial activity at all the drug release point of in vitro release study, which made them good candidate for drug delivery system through periodontal route for the treatment of infectious periodontal diseases. Therefore, from the antimicrobial activity point of view, the conclusion can be made that all the selected minocycline hydrochloride/ clindamycin phosphate loaded periodontal formulations were found to be a promising delivery system for infectious periodontal diseases.

10.5 REFERENCES

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