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Effect of dental films containing amoxycillin and metronidazole on periodontal pathogens: microbiological response

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Biodegradable dental films containing a combination of amoxycillin and metronidazole were prepared by a dispersion method. The aim of the present study was to determine the antimicrobial activity of this formulation against the pathogens which are commonly implicated in periodontal infections. Films showed sustained release *in vitro* for a period of 16 days. *In situ* release studies carried out using fresh bovine buccal mucosa in a flow through cell, showed that drug concentrations were maintained above MIC values for the entire period of the release studies. Samples from these studies were capable of inhibiting the growth of most of the test strains. The combination of amoxycillin and metronidazole using carrier polymer poly-L (lactide co-glycolide) not only showed an extended spectrum of antimicrobial activity but also showed a synergistic effect against *Eubacterium limosum*, which had earlier been reported resistant to metronidazole.

1. Introduction

Periodontitis is a general term used to describe several pathological conditions that affect the supporting structures/tissues of the teeth [1]. The principal etiological factor in periodontitis is the pathogenic bacterial flora that causes the formation of a periodontal pocket (lesion between teeth and junctional epithelium). Traditionally, treatment of disease was based on mechanical or surgical methods and administration of systemic or local antimicrobials. The role of local drug delivery in the management of periodontal disease has been investigated by several scientists [2–4]. This is because use of systemically administered antibiotics is associated with several drawbacks including low patient compliance, requirement for frequent dosing, side effects including gastrointestinal and central nervous system disturbances and superinfection [5, 6].

Combination therapy based on amoxycillin and metronidazole has been widely investigated in clinical dental practice due to its activity against a wide range of microbial species associated with periodontitis. The rationale for the use of this combination is that metronidazole is very active against anaerobic microorganisms and is known to act synergistically with penicillin [7]. The combination of both the drugs covers a wide range of microflora, with metronidazole inhibiting the anaerobes and amoxycillin inhibiting the facultative and aerobic bacteria [8]. Furthermore both drugs are bactericidal which may be essential for the elimination of subgingivally occurring periodontal pathogens [9].

Van Winkelhoff et al. investigated the use of a metronidazole and amoxycillin combination in periodontitis. They claimed that an *in vivo* synergistic effect between metronidazole and amoxycillin was responsible for the effectiveness of the combination therapy as eradication of *Actinoba-*

cillus actinomycetemcomitans was not achieved with either of the antibiotics when used alone [9]. Pavicic et al. evaluated the microbiological and clinical effects of mechanical debridement with the combination of metronidazole and amoxycillin therapy in patients with *A. actinomycetemcomitans* associated periodontitis and showed that it was successful in suppressing the microbes below cultivable levels for a long period of time [10]. Lopez et al. conducted clinical studies to assess the effectiveness of metronidazole plus amoxycillin in patients with moderate to advanced progressive adult periodontitis and concluded that it produced a significant improvement in clinical conditions [11, 12].

In the present study the antimicrobial activity of biodegradable dental films containing a combination of amoxycillin and metronidazole along with poly-L (lactide co-glycolide) was investigated against periodontal pathogens commonly found in periodontal infections. These included: *Bacteroides melaninogenicus*, *Bacteroides oralis*, *Bacteroides fragilis*, *Peptostreptococcus assachrolyticus*, *Peptostreptococcus* species, *Eubacterium limosum*, *Propionibacterium acnes*, *Staphylococcus aureus* and *Escherichia coli*. For this purpose microbiological evaluation procedures were developed depending on the type of microorganisms and the media on which they grow. These were based on modification of standard techniques namely agar dilution method, broth dilution method and cylinder plate method [13–15].

2. Investigations and results

In the present study, the microbiological response of stock solutions containing amoxycillin and metronidazole in different concentrations, *in situ* release study samples and

drug loaded films was determined. The agar dilution technique demonstrated that the stock solutions were able to inhibit the growth of BML, *B. fragilis*, *B. oralis*, *Peptostreptococcus* species, *P. asachrolyticus*, *A. viscosus* and *P. acne* when the concentration of amoxycillin and metronidazole was 20 µg/ml and 15 µg/ml respectively. However, *E. limosum* was resistant. When concentrations of metronidazole and amoxycillin were only 12.5 µg/ml and 30 µg/ml, respectively, in solution *B. fragilis* and *E. limosum* were not inhibited. Similar results were observed when the solution contained less than these concentrations of amoxycillin and metronidazole. Solutions containing metronidazole and amoxycillin in a concentration of 6 µg/ml and 25 µg/ml, respectively were not able to inhibit the growth of BML, *B. fragilis*, *Peptostreptococcus*, *P. asachrolyticus* and *E. limosum*. However, *B. oralis*, *A. viscosus* and *P. acnes* were inhibited at such low concentrations. When drug loaded films were tested it was seen that *Peptostreptococcus* species, *E. limosum* and *P. asachrolyticus* were not inhibited after 48 h of incubation when amoxycillin and metronidazole were present in a concentration of 5 mg each per film. However other anaerobes were inhibited. After 72 h of incubation all the anaerobes including *Peptostreptococcus* species and *E. limosum* were inhibited. In *in situ* release study samples, it was observed that when the concentration of metronidazole fell to 14.77 µg/ml, *B. fragilis* was not inhibited. However its growth was inhibited when the studies were done with solutions containing metronidazole in a concentration of 19 µg/ml and more. However, the amoxycillin concentration was almost the same in all the samples indicating that the concentration of amoxycillin did not influence the results. However, *E. limosum* was not inhibited in any of the samples. Even the concentration of metronidazole as high as 21 µg/ml was not sufficient to inhibit its growth. Similar results were seen when we retested our samples using the broth dilution technique. It was seen that *E. limosum* was inhibited even after 48 h of incubation. The other microbes also showed inhibition after 48 h as seen with the agar dilution technique. In situ release study samples also showed the same results in the broth dilution technique as seen before with the agar dilution technique. Studies on aerobic strains using the cylinder plate method showed that *S. aureus* and *E. coli* were inhibited even when metronidazole was present in a concentration as low as 6 µg/ml along with amoxycillin in a concentration of 25 µg/ml. However, a maximum zone of inhibition was seen when metronidazole was present in a concentration of 12.5 µg/ml along with amoxycillin in a concentration of 30 µg/ml. For aerobes it was seen that when the concentration of amoxycillin increased from 35.19 µg/ml to 35.94 µg/ml, there was marginal increase in zone of inhibition, i.e. from 30 mm to 31 mm. One interesting finding is that as concentration of metronidazole increased from 19 to 20.05 µg/ml, the zone of inhibition increased as well. Good zones of inhibition were seen with drug loaded films also.

3. Discussion

In the present study, the antimicrobial activity of dental films containing a combination of amoxycillin and metronidazole, stock solutions and *in situ* release study samples was studied on aerobic and anaerobic strains commonly implicated in periodontal infections. Microbiological evaluation carried out by the agar dilution technique, showed that films inhibited the growth of all the anaerobic strains after 72 h. The concentration of drugs released was well above the Mini-

mum Inhibitory Concentration (MIC). The *in situ* release study samples were successful in inhibiting all the strains except *E. limosum*. Various mixed standards containing different concentrations of two drugs were capable of inhibiting all the strains except *E. limosum* and *B. fragilis*. The microbiological evaluation was repeated using the broth dilution technique and similar results were obtained. *E. limosum* and *B. fragilis* were found to be resistant even to 5 µg metronidazole disk used in AIIMS laboratory. However, the drug-loaded films were successful in inhibiting *E. limosum*, which had been reported resistant to metronidazole earlier [16]. Thus it can be concluded that a synergistic effect between amoxycillin and metronidazole at a particular concentration was responsible for the activity against *E. limosum*. It is also possible that the concentration of drug released from the films when placed directly on the plates was higher than the concentration of drug released from *in situ* release samples. It is possible that high concentration of metronidazole along with amoxycillin is leading to synergistic action and is capable of inhibiting the growth of *E. limosum*.

Drug-loaded films, stock solutions and *in situ* release study samples were tested against the aerobic organisms *S. aureus* and *E. coli* by the cylinder plate method. The results showed inhibition of growth. The results of microbiological evaluation carried out on *in situ* release study samples showed that dental films were capable of achieving and maintaining concentrations well above the MIC value against most of the periodontal pathogens for a period of about 16 days.

It can be concluded that with its biodegradable and antimicrobial properties, amoxycillin and metronidazole containing films can be a successful low-dose, targeted drug delivery system in periodontal therapy.

4. Experimental

4.1. Materials

Amoxycillin and metronidazole were obtained as gift samples from Lark Laboratories (India) Ltd, New Delhi. Biodegradable polymer poly-L (lactide-co-glycolide) was obtained as gift sample from Purac Biochem bv, Holland. Microbiological media used for aerobic and anaerobic studies was purchased from Hi Media, Mumbai.

4.2. Preparation of media

4.2.1. Preparation of Brain Heart Infusion agar medium (BHI agar)

BHI agar (dehydrated media 52 g, yeast extract 5 g, proteose peptone 5 g, hemin 10 ml, L-lysine 0.5 to 1 g and agar 5 g) was dissolved in 1 l of distilled water. The solution was boiled and the pH of the clear solution was adjusted to 7.4. It was autoclaved at 15 lbs for 15 min after filtration and was cooled to 50 to 60 °C.

5–10% human blood was added. 20 ml of this media was poured in petriplates and the plates were stored in refrigerator at 4 °C.

The media is prereduced Brain Heart Infusion Broth, supplemented with 0.0005% hemin, 0.05% cysteine, 0.002% menadione and 0.5% yeast extract (BHI-S).

4.2.2. Preparation of Mueller Hinton agar medium

88 g of Mueller Hinton agar was suspended in 1000 ml of distilled water and heated to dissolve it completely. It was then sterilized by autoclaving at 121 °C and 15 lbs pressure for 15 min.

4.3. Preparation of dental films

Films were prepared by a dispersion method [17]. An accurately weighed quantity of polymer poly-L (lactide/co-glycolide) was dissolved in methylene chloride and diethyl phthalate was added as a plasticizer. Amoxycillin and metronidazole previously sieved through a 80–120 mesh sieve were homogeneously dispersed in the polymer solution by vortexing for 2 to 10 min. The resulting dispersion was then poured into glass rings placed over aluminum foil and films were dried initially at –5 to –10 °C for 8 to 10 h and further at room temperature for another 24 h.

4.4. Preparation of stock solutions

Standard solutions of metronidazole (200 µg/ml) and amoxycillin (400 µg/ml) were separately prepared in alkaline borate buffer of pH 8.1. Different dilutions were prepared from the stock solution. All these dilutions were passed through bacteria proof Millipore membrane filter (0.2 µm), which was autoclaved at 15 lbs for 20 min before use.

4.5. Standardization of inoculum size

Standardization of inoculum size was done to achieve a 0.5 Mac Farland standard. In BHI broth a loopful of the inoculum was added and it was vortexed to allow the uniform distribution of the culture. The turbidity in the tubes was matched with standard tubes containing a microbial concentration of 1500 million/ml (0.5 Mac Farland standard).

4.6. Microbiological evaluation against anaerobes

The stock solutions, the *in situ* release study samples and the drug-loaded films were subjected to microbiological evaluation using various techniques i.e. the agar dilution technique and the broth dilution technique. Different techniques were used to standardize the method of microbiological evaluation against the strains.

4.6.1. Agar dilution technique

Stock solutions containing 200 µg/ml metronidazole and 400 µg/ml amoxycillin were prepared in alkaline borate buffer of pH 8.1. Mixed standards containing 12 µg/ml, 20 µg/ml, 25 µg/ml and 40 µg/ml of metronidazole and 50 µg/ml, 40 µg/ml, 60 µg/ml and 30 µg/ml of amoxycillin respectively, were prepared from the stock solution. 10 ml of each mixed standard was added to 10 ml of molten Brain Heart Infusion (BHI) agar medium (containing 4% agar) and this was immediately poured into different petri dishes and allowed to set. The plates were then inoculated with the previously mentioned anaerobic strains. The resulting concentrations of the solutions after dilution with BHI agar were 6 µg/ml, 10 µg/ml, 12.5 µg/ml and 20 µg/ml of metronidazole and 25 µg/ml, 20 µg/ml, 30 µg/ml and 15 µg/ml of amoxycillin respectively. The plates were kept in Mac-Intosh Fildes anaerobic jars, which had been vacuumised and filled with gases (nitrogen 80%, hydrogen 10% and carbon dioxide 10%). The jars were kept in the incubator for 48 h. Control evaluation was also carried out simultaneously. Observations are recorded in Table 1.

BHI agar plates were prepared according to section 4.2. Plates were inoculated with the anaerobic strains mentioned previously, by the streaking

method. Dental films (each containing 5 mg amoxycillin and 5 mg metronidazole) were aseptically placed over the inoculated areas with the help of a sterilized spatula. Plates were incubated anaerobically for a period of 48 and 72 h. Observations are recorded in Table 2. Control tests were similarly done using placebo films in place of drug-loaded films.

The release of drugs from the dental films was determined using a flow through cell. Fresh bovine buccal mucosa was placed in the cavity of the lower chamber of the cell and a drug-loaded film was placed over it. Alkaline borate buffer (pH 8.1) simulating the pH of Gingival Crevicular Fluid (GCF), was continuously pumped through the cell at a flow rate of 20 ml per day using a peristaltic pump. The flow rate corresponded to the flow rate of GCF during periodontitis, which is above 3.5 ml per day [6]. Samples were collected on the fourth, eighth, twelfth and sixteenth day and concentrations were determined spectrophotometrically in multicomponent mode using a Shimadzu 1601 UV-visible spectrophotometer. The Samples were then filtered through a Millipore membrane filter (0.2 µm) and subjected to microbiological evaluation. The samples were added to BHI agar plates which were inoculated with the anaerobic strains mentioned above and incubated for 48 h. Results of the study are recorded in Table 3.

4.6.2. Broth dilution technique

Pre-reduced BHI broth 5 ml was taken in a series of test tubes and autoclaved at 15 lbs for 15 min. The tubes were cooled and inoculated anaerobically with the culture to achieve a 0.5 Mac Farland standard. Different dilutions of the stock solution of amoxycillin and metronidazole were prepared as given in Table 4. 3 ml of each dilution was added to 5 ml of BHI broth contained in the test tubes.

The resulting drug concentrations after dilution with BHI broth were 6 µg/ml, 10 µg/ml, 12.5 µg/ml and 20 µg/ml for metronidazole and 25 µg/ml, 20 µg/ml, 30 µg/ml and 15 µg/ml for amoxycillin respectively. For each strain tested, control tests were run simultaneously. Tubes were incubated at 37 °C in an anaerobic jar under 80% nitrogen, 10% carbon dioxide and 10% hydrogen for a duration of 48 h. Observations are recorded in Table 4.

Pre-reduced BHI broth (5 ml) was taken in a series of test tubes and autoclaved at 15 lbs for 15 min. Tubes were cooled and inoculated anaerobically with culture to achieve a 0.5 Mac Farland standard. One drug-loaded film was added aseptically to each tube and these were then incubated anaerobically for a period of 48 h. For each strain tested, control tests were run simultaneously and observations are recorded in Table 5.

The samples collected at different time intervals (fourth, eighth, twelfth and sixteenth day) were tested on the previously mentioned strains. 5 ml of pre-reduced BHI broth was taken in a series of test tubes and autoclaved at

Table 1: Effect of metronidazole and amoxycillin stock solutions on the anaerobic strains (agar dilution technique)

S. No	Concentration of the solution (µg/ml)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
1	6 (M) + 25 (A)	G	G	NG	G	G	NG	G	NG
2	10 (M) + 20 (A)	NG	G	NG	NG	NG	NG	G	NG
3	12.5 (M) + 30 (A)	NG	G	NG	NG	NG	NG	G	NG
4	20 (M) + 15 (A)	NG	NG	NG	NG	NG	NG	G	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus*; A: amoxycillin; G: growth

Table 2: Effect of drug -loaded film on the anaerobic strains

Dose of the drugs (mg)	Duration of incubation (h)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
A (5) + M (5)	48	NG	NG	NG	G	G	NG	G	NG
A (5) + M (5)	72	NG	NG	NG	NG	NG	NG	NG	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus*; A: amoxycillin; G: growth

Table 3: Effect of *in situ* release study samples on the anaerobic strains (agar dilution technique)

S. No	Concentration of the solution (µg/ml)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
1	19 (M) + 35.19 (A)	NG	NG	NG	NG	NG	NG	G	NG
2	21 (M) + 33.66 (A)	NG	NG	NG	NG	NG	NG	G	NG
3	20.05 (M) + 35.94 (A)	NG	NG	NG	NG	NG	NG	G	NG
4	14.77 (M) + 38.07 (A)	NG	G	NG	NG	NG	NG	G	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus*; A: amoxycillin; G: growth

Table 4: Effect of metronidazole and amoxycillin mixed standards on the anaerobic strains (Broth Dilution Technique)

S. No	Concentration of the solution (µg/ml)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
1	6 (M) + 25 (A)	G	G	NG	G	G	NG	G	NG
2	10 (M) + 20 (A)	NG	G	NG	NG	NG	NG	G	NG
3	12.5 (M) + 30 (A)	NG	G	NG	NG	NG	NG	G	NG
4	20 (M) + 15 (A)	NG	NG	NG	NG	NG	NG	G	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus* A: amoxycillin; G: growth

Table 5: Effect of drug-loaded films on the anaerobic strains

Dose of the drugs (mg)	Duration of incubation (hours)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
A (5) + M (5)	48	NG	NG	NG	NG	NG	NG	NG	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus*; A: amoxycillin; G: growth

Table 6: Effect of *in situ* release study samples on the anaerobic strains (broth dilution technique)

S. No	Concentration of the solution (µg/ml)	Effect on the anaerobic strains							
		BML	<i>B. fragilis</i>	<i>B. oralis</i>	<i>Peptostreptococcus</i> species	<i>P. asacchrolyticus</i>	<i>A. viscosus</i>	<i>E. limosum</i>	<i>P. acnes</i>
1	19 (M) + 35.19 (A)	NG	NG	NG	NG	NG	NG	G	NG
2	21 (M) + 33.66 (A)	NG	NG	NG	NG	NG	NG	G	NG
3	20.05 (M) + 35.94 (A)	NG	NG	NG	NG	NG	NG	G	NG
4	14.77 (M) + 38.07 (A)	NG	G	NG	NG	NG	NG	G	NG

M: metronidazole; NG: No growth; BML: *B. melaninogenicus*; A: amoxycillin; G: growth

15 lbs for 15 min. They were cooled and inoculated anaerobically with the culture to achieve a 0.5 Mac Farland standard. Each sample was added to BHI broth contained in test tubes, which were incubated anaerobically for a period of 48 h. For each strain tested, control tests were run simultaneously and observations are recorded in Table 6.

Table 7: Effect of metronidazole and amoxycillin stock solutions on the aerobic strains (cylinder plate method)

S. No	Concentration of the drug solution ((µg/ml)	Diameter of zone of inhibition (mm) ± S.D	
		<i>S. aureus</i>	<i>E. coli</i>
1	6 (M) + 25 (A)	16 ± 1.8	22 ± 2.3
2	10 (M) + 20 (A)	18 ± 1.5	22 ± 1.2
3	12.5 (M) + 30 (A)	21 ± 2.1	24 ± 1.5
4	20 (M) + 15 (A)	14 ± 3.4	18 ± 0.8

M: metronidazole; A: amoxycillin

Table 8: Effect of drug-loaded film on the aerobic strains

Dose of the drugs (mg)	Diameter of zone of inhibition (mm) ± S.D	
	<i>S. aureus</i>	<i>E. coli</i>
A (5) + M (5)	30 ± 1.0	22 ± 0.8

M: metronidazole; A: amoxycillin

Table 9: Effect of *in situ* release study samples on the aerobic strains (cylinder plate method)

Time of release of the drug (in days)	Concentration of the drug solution ((µg/ml)	Diameter of zone of inhibition (mm) ± S.D	
		<i>S. aureus</i>	<i>E. coli</i>
4	19 (M) + 35.19 (A)	30 ± 0.5	18 ± 1.4
8	21(M) + 33.7 (A)	29 ± 1.2	14 ± 1.1
12	20.05 (M) + 35.94 (A)	31 ± 3.1	20 ± 1.4
16	14.8 (M) + 38.07 (A)	30 ± 0.6	15 ± 1.2

M: metronidazole; A: amoxycillin

4.7. Microbiological evaluation against aerobes

The test was done against *S. aureus* and *E. coli* as these aerobes are implicated in oro-dental infections. The stock solution, the *in situ* release study samples and the drug-loaded films were subjected to microbiological evaluation. The cylinder plate method was used to carry out the evaluation. Mueller Hinton agar plates were prepared according to the method described in section 4.2.

4.7.1. Microbiological evaluation of stock solution

The plates were inoculated with the microorganisms by the lawn culture technique. Culture was applied over agar surface with the help of sterilized swabs. Uniformly spaced 10 mm diameter wells were made in the agar plates using a sterilized cork borer. Filtered mixed standards of different concentrations (as given in Table 7) were pipetted into the wells. Distilled water was used as control. The petriplates were incubated aerobically at 37 °C for 18 h and diameter of zone of inhibition was measured.

4.7.2. Evaluation of drug loaded dental films

Mueller Hinton agar plates were inoculated with the microorganisms by the lawn culture technique. Drug loaded films were aseptically placed over the inoculated surface with the help of a sterilized spatula and incubated aerobically for 18 h and diameters of zone of inhibition were measured. An inoculated plate having a placebo film served as control. Observations are recorded in Table 8.

4.7.3. Evaluation of *in situ* release study samples

Mueller Hinton agar plates were inoculated with the microorganisms mentioned above by the lawn culture technique and uniformly spaced wells of

10 mm diameter, were made. Filtered *in situ* release study samples were pipetted into the wells. The samples collected at different time intervals (fourth, eighth, twelfth and sixteenth day) were tested on the previously mentioned strains. The petri plates were incubated aerobically at 37 °C for 18 hours and diameters of zone of inhibition were measured. Distilled water was used as the control and observations are recorded in Table 9.

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