Original Article

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Dentistry Section

Efficacy of Natural and Allopathic Antimicrobial Agents Incorporated onto Guided Tissue Regeneration Membrane Against Periodontal Pathogens: An In vitro Study

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ABSTRACT

Introduction: Periodontal disease is one of the most prevalent afflictions worldwide. It is an infection of the periodontium as a result of subgingival colonization of the specific microbiota, leading to loss of attachment, which requires optimal care for regeneration to its pre-disease state. Guided Tissue Regeneration (GTR) is one of the successful treatment modalities in Periodontal Regenerative Therapy, but is vulnerable to bacterial colonization. The conflict between usage of classical antibiotics and plant origin antimicrobial agents has recently been in the limelight.

Aim: The aim of this study was to assess the in vitro antimicrobial activity of amoxicillin, metronidazole and green coffee extract loaded onto GTR membrane against periodonto-pathogens.

Materials and Methods: Pure form of amoxicillin, metronidazole and green coffee extract were obtained. One percent concentration of each antimicrobial agent was prepared by appropriate dilution with distilled water. GTR membrane was cut into a size of 1x0.5 cm under sterile conditions and was coated with the antimicrobial agents respectively and with distilled water as the negative control. Antimicrobial activity was checked against *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) and *Porphyromonas gingivalis* (*P. gingivalis*) using agar disc diffusion method. The statistical analysis was done using Kruskal Wallis ANOVA and Mann-Whitney U test.

Results: One percent amoxicillin showed level of significance ($p>0.05$) against both *A. actinomycetemcomitans* and *P. gingivalis*. Green coffee extract showed no zone of inhibition against both the bacterial species.

Conclusion: Loading of commercially available antimicrobial agents onto GTR membrane can prevent its bacterial colonization leading to better treatment outcomes for periodontal regeneration.

Keywords: Amoxicillin, *Aggregatibacter actinomycetemcomitans*, Green Coffee extract, Metronidazole, *Porphyromonas gingivalis*

INTRODUCTION

It has been well established that oral infections, particularly periodontitis, are associated with aerobic and anaerobic microbiota. The oral cavity harbors more than 700 different types of bacterial species that are associated with various oral diseases [1]. The major putative periodonto-pathogens are *A. actinomycetemcomitans* and *P. gingivalis*. *A. actinomycetemcomitans* is particularly associated with an inflammatory disease, localized aggressive periodontitis, which can result in early tooth loss [2]. *P. gingivalis* is one of the culprit organisms in chronic periodontitis and peri-implantitis [3,4]. An immune response is triggered by fibroblasts and macrophages as soon as the microbiota inhabits the periodontal tissues resulting in production of mediators of inflammation and immune response i.e., Interleukin (IL) 1, 6 and Tumour Necrosis Factor-α (TNF-α) [5].

GTR has been shown to be a successful treatment modality for periodontal regeneration in many clinical studies. The GTR technique prevents the apical migration of the gingival epithelium and allows the periodontal cells to repopulate in the area of the denuded root surface. Some of the human biopsy GTR studies have demonstrated new attachment level with bone fill [6,7]. The most common periodontal pathogens to inoculate on the GTR membrane are *A. actinomycetemcomitans* and *P. gingivalis* [8]. A clinical study [9], reported that within three minutes of the procedure there is bacterial contamination of the GTR membrane. Hence, incorporating antimicrobial agents in the GTR membrane may help in preventing the surface colonization.

Several studies [10-12] have been reported that there is drastic reduction in colonization of periodontal pathogens on the GTR membrane and improvement in clinical sites followed by allopathic antimicrobial coating. The main concept that has instigated the search of new antimicrobial alternatives of plant origin is the emanation of drug resistance in pathogenic bacteria and the undesirable side effects of synthetic antimicrobial agents [13].

GTR membrane incorporated with antimicrobial agents may be beneficial to control membrane-associated infections in GTR therapy. The aim of this in vitro study was to determine whether the growth of *A. actinomycetemcomitans* and *P. gingivalis* can be prevented by the surface treatment of GTR membrane with antimicrobial agents.

MATERIALS AND METHODS

An in vitro experimental design was formulated for the study. Institutional Ethics Committee of Army College of Dental Sciences, Secunderabad, Telangana, India, granted ethical clearance for conducting the study.

Pure form of amoxicillin and metronidazole was procured for the purpose of the study. Pure green coffee extract was procured from Top secret nutrition supplements, New York, USA containing 50% Chlorogenic Acid (CGA). The manufacturer certified it to be free from any form of bacteria, yeast or mold after microbial analysis. To obtain 10% concentration of antimicrobial agent, 1 gram of these antimicrobial agents was dissolved in 10 ml of distilled water. Further, appropriate amount of solvent was added to dilute

the concentrations to 1% amoxicillin, 1% metronidazole and 1% green coffee extract respectively. Microbial assay was done of the extracts. Distilled water, the solvent was used as negative control in this study.

GTR membrane that was used for the study was sterile reconstituted Type-I collagen membrane. The membrane was aseptically cut into the dimensions of 1x0.5 cm. Nearly, 10 ml of 1% amoxicillin, 1% metronidazole and 1% green coffee extract solution was poured into sterile petridishes respectively in which the GTR membrane was placed. The GTR membrane was allowed to soak in the concentrated solution for 15 minutes and was dried for five minutes.

Microbial Assay

Agar disc diffusion method was used to determine the antimicrobial activity of GTR membrane coated with 1% amoxicillin solution, 1% metronidazole solution and 1% green coffee extract solution respectively in vitro. Blood agar was used for culturing and examining different microorganisms. Colonies of microorganisms were transferred to the agar plate by means of an inoculation loop. For ensuring even distribution of microorganisms the plates were rotated at approximately 60º between every streak. For *A. actinomycetemcomitans* and *P. gingivalis*, similar overall procedure of inoculum preparation and inoculation of culture media was followed. Both the bacteria were inoculated on three different agar plates of 1% amoxicillin solution, 1% metronidazole solution and 1% green coffee extract solution respectively. Therefore, for both the bacteria a total of six plates were inoculated. The inoculated plates were allowed to stand for at least three minutes but no longer than 15 minutes, before placing the GTR membrane treated with different antimicrobial substances to be tested. Within 15 minutes of the placement of GTR membrane, the plates were incubated at 37ºC. For aerobic (*A. actinomycetemcomitans*) and anaerobic (*P. gingivalis*) bacteria, incubation was done for 48 hours. McIntosh and Filde's anaerobic jar was used for anaerobic microorganism culturing while incubator was used at 37ºC for aerobic microorganism for 48 hours. If the lawn of growth was confluent after the incubation period the plates were read. The diameter of zone of inhibition was measured to the nearest millimeter using Vernier's caliper. The microbiological procedure was repeated three times for each bacterium. The control for the study was, GTR membrane treated with distilled water.

STATISTICAL ANALYSIS

For statistical analysis of the study, software Statistical Package for Social Sciences (SPSS 20.0 version) was used. The statistical data was obtained and analyzed using Kruskal Wallis ANOVA and Mann-Whitney U test. Mann-Whitney U test was done to compare the antimicrobial property between various groups. Statistical significance level was established at $p<0.05$.

RESULTS

Zones of inhibition for 1% amoxicillin, 1% metronidazole, 1% green coffee extract and distilled water against *A. actinomycetemcomitans* and *P. gingivalis* are elaborated in [Table/Fig-1-3]. Distilled water and green coffee extract showed no zone of inhibition against both the bacterial species. While the widest zone of inhibition was displayed by 1% amoxicillin against both *A. actinomycetemcomitans* and *P. gingivalis*. For all the antimicrobial agents incorporated in the study the mean zone of inhibition for each bacterium was calculated for analysis [Table/Fig-4,5]. Amoxicillin, when compared to the other antimicrobial agents as well as negative control used in the study showed level of significance against both *A. actinomycetemcomitans* and *P. gingivalis* [Table/Fig-4]. Whereas, neither metronidazole nor green coffee extract showed any level of significance against *A. actinomycetemcomitans* using Mann- Whitney U test. Amoxicillin showed better results against *P. gingivalis* as compared to metronidazole (specific for anaerobic bacterial infections). Metronidazole showed level of significance against *P. gingivalis*.

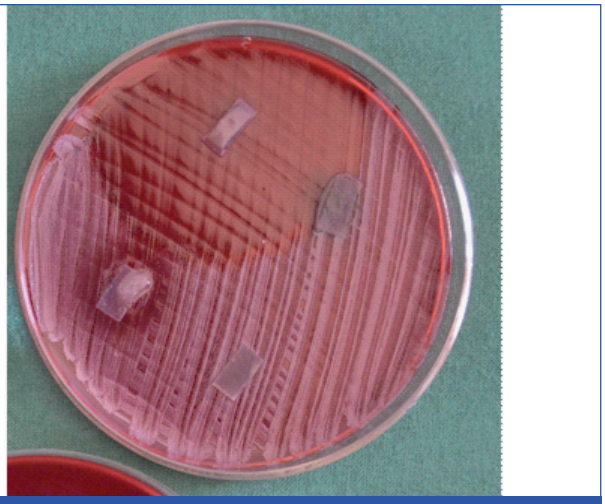
DISCUSSION

Microbial colonization of the GTR membrane adversely affects the treatment outcomes of the periodontal treatment [9]. Attachment of the periodontal pathogens onto the GTR membrane may expedite the recurrence of periodontitis and loss of alveolar bone. Direct incorporation of the antimicrobial agent in the GTR membrane may allow appropriate infection control. Previous studies of loading tetracycline and amoxicillin on GTR membrane have shown to drastically reduce the adherence of *A. actinomycetemcomitans* and *Streptococcus mutans* [14].

The present study consists of loading minimal concentration of antimicrobial agents onto reconstituted Type-I collagen GTR membranes and evaluating its antimicrobial efficacy on *A. actinomycetemcomitans* and *P. gingivalis*. The loading of GTR

Antimicrobial Extracts	Microorganisms Tested					
	A. actinomycetemcomitans			*P. gingivalis*		
Amoxicillin	42 x 46	43 x 48	41 x 45	22 X 18	30 X 24	24 X 18
Metronidazole	16 X 16	0 x 0	10 X 10	18 X 14	18 X 16	17 X 14
Green coffee extract	0 x 0	0 x 0	0 x 0	0 x 0	0 x 0	0 x 0
Distilled Water	0 x 0	0 x 0	0 x 0	0 x 0	0 x 0	0 x 0

[Table/Fig-1]: Zones of inhibition for various anti-microbial agents.



[Table/Fig-2]: GTR membrane loaded with antimicrobial agents placed on lawn of *A. actinomycetemcomitans* showing zone of inhibition.

[Table/Fig-3]: GTR membrane loaded with antimicrobial agents placed on lawn of *P. gingivalis* showing zone of inhibition.

Anti-Microbial Agents	Mean	SD	SE	Sum of Ranks	U-Value	Z-Value	p-value
Amoxicillin	1947.00	110.27	63.66	15.00	0.00	-1.9640	0.0495*
Metronidazole	118.67	129.02	74.49	6.00			
Amoxicillin	1947.00	110.27	63.66	15.00	0.00	-1.9640	0.0495*
Green Coffee	0.00	0.00	0.00	6.00			
Amoxicillin	1947.00	110.27	63.66	15.00	0.00	-1.9640	0.0495*
Distilled Water	0.00	0.00	0.00	6.00			
Metronidazole	118.67	129.02	74.49	13.50	1.50	-1.3093	0.1904
Green Coffee	0.00	0.00	0.00	7.50			
Metronidazole	118.67	129.02	74.49	13.50	1.50	-1.3093	0.1904
Distilled Water	0.00	0.00	0.00	7.50			
Green Coffee	0.00	0.00	0.00	10.50	4.50	0.0000	1.0000
Distilled Water	0.00	0.00	0.00	10.50			

[Table/Fig-4]: Pair wise comparisons of four anti-microbial agents with zone of anti-microbial activity of *A. actinomycetemcomitans* by Mann-Whitney U test.
*p<0.05

Anti-Microbial Agents	Mean	SD	SE	Sum of Ranks	U-Value	Z-Value	p-value
Amoxicillin	516.00	177.58	102.53	15.00	0.00	-1.9640	0.0495*
Metronidazole	259.33	25.79	14.89	6.00			
Amoxicillin	516.00	177.58	102.53	15.00	0.00	-1.9640	0.0495*
Green Coffee	0.00	0.00	0.00	6.00			
Amoxicillin	516.00	177.58	102.53	15.00	0.00	-1.9640	0.0495*
Distilled Water	0.00	0.00	0.00	6.00			
Metronidazole	259.33	25.79	14.89	15.00	0.00	-1.9640	0.0495*
Green Coffee	0.00	0.00	0.00	6.00			
Metronidazole	259.33	25.79	14.89	15.00	0.00	-1.9640	0.0495*
Distilled Water	0.00	0.00	0.00	6.00			
Green Coffee	0.00	0.00	0.00	10.50	4.50	0.0000	1.0000
Distilled Water	0.00	0.00	0.00	10.50			

[Table/Fig-5]: Pair wise comparisons of four anti-microbial agents with zone of anti-microbial activity of *P. gingivalis* by Mann-Whitney U test.
*p<0.05

membrane with commercially available antimicrobial agents can aid in elimination of locally residing pathogenic bacteria leading to better periodontal treatment outcomes. Usage of such a trivial amount of antimicrobial agents onto GTR membrane can lead to the possible improvement in its clinical implications. The ideology behind the present study was to check the antimicrobial property of various allopathic and herbal antimicrobial agents against periodontogenic pathogens at a minimal concentration of 1%. This is one of its kind studies in which green coffee extract was loaded onto the GTR membrane to evaluate its antimicrobial activity against *A. actinomycetemcomitans* and *P. gingivalis*.

Amoxicillin belongs to the family of β-Lactam antibiotics [15]. Amoxicillin exhibits its antimicrobial property by inhibiting the cross linkage between linear peptidoglycan polymer chains and hence, preventing the formation of bacterial cell wall synthesis [15]. According to Renvert S et al., there is selective elimination of periodontal pathogens after mechanical debridement in cases of advanced chronic periodontitis [16]. In his study, Renvert S et al., concluded that after mechanical debridement, *A. actinomycetemcomitans* could not be eliminated while there was significant reduction of *P. ginigivalis* from the positive sites [16]. Earlier, for eliminating subgingival population of *A. actinomycetemcomitan*, tetracyclines were used [17]. Markman C et al., determined the release of tetracycline hydrochloride from a cellulose GTR membrane and suggested that it may prevent the colonization of the bacterial species on it [10]. Zarkesh N et al., demonstrated additional gain in clinical attachment level by using tetracycline loaded e-Polytetrafluoroethylene (e PTFE) membrane [12]. However, various studies have been documented highlighting the failure of tetracycline therapy against *A. actinomycetemcomintans* [18-20]. Hung S-L et al., in a study demonstrated reduced adherence of *A. actinomycetemcomitans* and *S. mutans* onto GTR membrane loaded with tetracycline and amoxicillin which shows similar results to the present study [14].

Metronidazole is an antibiotic which belongs to the group, nitroimidazole. It was originally introduced to treat *Trichomonas vaginalis* infections but is presently used to treat anaerobic and protozoal infectons [21]. Metronidazole mediates its bactericidal action by producing toxic metabolites which result in the DNA breakage of the bacterial cell [21]. In the present study metronidazole when coated over GTR membrane showed better results against *P. gingivalis* as compared to *A. actinomycetemcomitans*. A similar study concluded that there was a marked improvement in clinical sites in patients when GTR membrane was surface coated with metronidazole [11]. Dowell P et al., concluded that it also helped in reducing post-operative discomfort to the patient [11]. Various authors reported similar findings following use of metronidazole as a subgingival irrigant [22,23] as well as topical application of sustained release antibiotics [24,25].

The market is presently laden with various popular therapeutic antimicrobial products. The quest for developing herbal remedies with extensive array of antimicrobial properties without the side effects of synthetic medications is still going on for treating periodontal disease [26]. Lately, green coffee extract has received much attention amid all the various herbal products because of its antimicrobial properties against both Gram-positive as well as Gram-negative organisms [27]. There are various components of coffee that have been documented to show antimicrobial properties, these include volatile and nonvolatile organic acids, caffeine, phenols and aromatic compounds. Growth of various Gram-positive microorganisms such as *Staphylococcus aureus, Bacillus cereus, Lactobacillus bulgaricus, Streptococus lactis* and *Streptococcus faecalis* and Gram-negative bacteria like *Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa* are inhibited by a non-volatile organic acid found in coffee i.e., CGA and caffeic acid [27]. An in vitro study recognized antimicrobial activity of green coffee extract against four periodonto-pathogens at a very low concentration. It was observed that Minimal Inhibitory Concentration (MIC) of green coffee extract was 0.2 µg/ml for *Prevotella intermedia*, *P. gingivalis*, and *A. actinomytemcomitans* [28]. Yi TL et al., contrasted the antimicrobial activity of 0.2% chlorhexidine mouthwash and different concentrations of green coffee extract against *P. gingivalis*, *P. intermedia, F. nucleatum* and *A. actinomycetemcomitans* [29]. The results of the study [29] concluded that chlorhexidine showed better results as compared to green coffee extract while, 20% and 15% concentration of green coffee extract showed antimicrobial activity against *P. intermedia*, *P. gingivalis* and *A. actinomycetemcomitans*. The present study showed negative results with green coffee extract, showing no antimicrobial property against both the bacterial species. To further evaluate the efficacy of green coffee extract, higher concentrations of extract should have been tested for the present study.

The present in vitro study was designed to evaluate the efficacy of 1% amoxicillin, 1% metronidazole and 1% green coffee extract loaded onto GTR membrane against *A. actinomycetemcomitans* and *P. gingivalis*. Zone of inhibition was measured in order to estimate the antimicrobial action while, incorporating attachment and penetration [30] of bacteria onto the antibacterial agent loaded GTR membrane would have given a better perspective to the present study. As green coffee extract showed negative results hence, higher concentrations of the extract should have been tested for their efficacy by loading onto the GTR membrane. Various other plant origin antibacterial agents should be tested and incorporated into non-surgical as well as surgical periodontal

therapy in order to avoid the side effects, due to commercially available synthetic antimicrobial agents.

CONCLUSION

Various studies have documented that GTR membrane is one of the successful methods for periodontal regeneration but it is prone for bacterial contamination thereby, impeding periodontal regeneration. Systemic administration of antibiotics may give rise to drug resistance as well as side effects. Therefore, loading of antimicrobial agents onto the GTR membrane may prevent its bacterial colonization. In the present study, 1% amoxicillin has shown significant antimicrobial effect against both aerobic and anaerobic bacteria. From the present study, it can be concluded that usage of commercially available antimicrobial agents in such a low concentration can effectively reduce the number of periodontal pathogens colonizing the GTR membrane.

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