Journal of Lasers in Medical Sciences

J Lasers Med Sci 2016 Winter;7(1):21-25

Original Article

http://www.journals.sbmu.ac.ir/jlms

doi 10.15171/jlms.2016.05

An In Vitro Comparison of Antimicrobial Effects of Curcumin-Based Photodynamic Therapy and Chlorhexidine, on *Aggregatibacter actinomycetemcomitans*

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Published online 7 January 2016

Abstract

Introduction: Considering the importance of prevention in periodontal diseases and the important role of *Aggregatibacter actinomycetemcomitans* in induction and progression of these diseases, the aim of the present in vitro study was to compare the antimicrobial effects of chlorhexidine digluconate (CHX), curcumin and light-emitting diode (LED) laser, on this bacterium.

Methods: Antimicrobial activity of curcumin (5 mg/ml), CHX (2%), LED (120 J/cm^2) and LED + curcumin (120 J/cm^2 + 2.5 mg/ml) against *A. actinomycetemcomitans* were tested in vitro, using micro-broth dilution test. One-way analysis of variance (ANOVA) and Tukey's HSD tests served for statistical analysis.

Results: Regarding the minimum inhibitory concentration (MIC), CHX had a significantly lower MIC than curcumin ($P<0.05$). Sorted out by bacterial growth from lowest to highest, were CHX, LED + curcumin, curcumin, and LED groups. All the differences were found to be statistically significant ($P<0.05$) except for the LED group.

Conclusion: We conclude that curcumin is an effective substance in preventing the growth of *A. actinomycetemcomitans*, whose impact is reinforced when used simultaneously with photodynamic therapy (PDT).

Keywords: Aggregatibacter actinomycetemcomitans; Chlorhexidine; Curcumin; Photodynamic therapy.

Introduction

Periodontal diseases are referred to as inflammatory processes in periodontal tissues in response to the aggregation of bacteria on the teeth. The aggregation rarely leads to evident infection, but the inflammatory response caused in the gingival tissue is responsible for the gradual loss of dental collagen's attachment to the alveolar bone. Left overlooked, this phenomenon might end in increased dental mobility and eventually edentulism.[1] Production of destructive metabolites by gram negative and positive bacteria of the microbial plaque in the oral cavity causes gingivitis, which can play a key role in the progression of inflammation to periodontal diseases.[2]

Aggregatibacter actinomycetemcomitans is an immobile microaerophilic, facultative anaerobic, gram negative coccoid rod,[3] strongly associated with pathogenesis of periodontal diseases, particularly aggressive generalized periodontitis.[4] Some of this bacterium's membranous proteins play an important role in attachment to and penetration in tissue cells.[5] Other virulence factors secreted by this bacterium include an agent that inhibits the reproduction of fibroblasts and a leukotoxin that kills the leukocytes.[6]

Despite the many complications caused by periodontitis, no treatment protocol is known to be able to fully control this disease. Currently, a combination of mechanical treatments and systemic antibiotics are used as treatment[7]; however, periodontal pathogens are not completely removed by the mechanical removal of the biofilm; and there are problems associated with the use of antibiotics,

Please cite this article as follows: Najafi S, Khayamzadeh M, Paknejad M, Poursepanj G, Kharazi Fard MJ, Bahador A. . An in vitro comparison of antimicrobial effects of curcumin-based photodynamic therapy and chlorhexidine, on Aggregatibacter actinomycetemcomitans. *J Lasers Med Sci*. 2016;7(1):21-25. doi:10.15171/jlms.2016.05.

including weak compliance of patients,[8] difficulty maintaining therapeutic antimicrobial concentrations in the periodontal plaque,[9] and eventual complications related to antibiotics such as allergic reactions, gastrointestinal problems and bacterial resistance.[10] Therefore, researchers are trying to find alternative methods for treatment of periodontal diseases.

Photodynamic therapy (PDT) has been used mostly in the treatment of tumoral lesions, and is now considered as one of the alternative methods. PDT involves three main components: visible light, a photosensitizer (PS) and oxygen molecules.[11] In this method the PS, which is a photoactivatable substance selective for the prokaryotic target cells, binds to the cells and is activated by light at a specific wavelength. Free radicals are formed, such as oxygen species that have a toxic effect on cells.[11-14] PDT is a repeatable procedure which costs less than other methods, has a broad spectrum microbicidal effects, is safe for the host tissue, and can reach inaccessible sites.[15]

Another alternative method to treat periodontal diseases is to find substances with anti-plaque effects that can inhibit bacterial reproduction. Curcumin is one of these potential substances, derived from the rhizome of Curcuma longa, which has been reported to show anti-inflammatory,[16] anti-oxidant,[17] anti-microbial[18] and wound healing[19] properties. The first three mentioned properties make curcumin a favorable substance for the treatment of periodontal diseases. Other than its direct effects, curcumin has also been evaluated as a photosensitizer for PDT. It has a rather broad absorption peak in the 300 to 500 nm range and shows no toxic effects in a number of cell cultures and animal studies, which makes it a suitable candidate as a PS.[20]

Considering the importance of prevention in periodontal diseases and the important role of *A. actinomycetemcomitans* in their induction and progression, the aim of the present in vitro study was to compare the antimicrobial effects of curcumin and light-emitting diode (LED) laser on this bacterium, with chlorhexidine digluconate (CHX), as the gold standard mouthwash.

Methods

Bacterial Strain and Culture Conditions

Lyophilized *A. actinomycetemcomitans* (ATCC cultures 33384) were obtained from Rayen Biotechnology Co. Ltd., Tehran, Iran. The bacteria were rehydrated in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany); and incubated in a microaerophilic atmosphere at 37°C for 48 hours. For assays requiring cultures on plates, cultures grown in supplemented BHI broth were transferred onto BHI agar (Merck, Darmstadt, Germany) plates. Fresh BHI bacterial culture, in the logarithmic growth phase, was adjusted to a concentration of 10^6 CFU (colony-forming units)/ml as verified by colony counting.

Materials

Curcumin (Sigma-Aldrich, South Korea) (5 mg/ml) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, South Korea), just before each treatment.[20] The final concentration of DMSO in the medium was 0.5%. Solutions with a concentration of 2% CHX were prepared by diluting 20% CHX (Sigma-Aldrich, South Korea) into the appropriate concentration with purified water.

Light Source

A blue light LED system (DB-685 Penguin, COXO, China) with a real intensity of 400 mW/cm^2 and a wavelength of 420-480 nm was applied for 5 minutes (energy density of 120 J/cm^2), in PDT producer.

Minimum Inhibitory Concentration

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of curcumin and CHX against *A. actinomycetemcomitans,* as described previously.[17] Briefly, testing was done using 96-well flat-bottomed microplate (Nunc, Denmark), with an assay volume of 200 µl/well. First, Mueller–Hinton broth (MHB) (Merck, Darmstadt, Germany) was added (90 µl) to each well. The curcumin (5 mg/ml) and CHX 2% were added (90 µl/well) to wells and serially diluted two-fold across the plate. The plates were then inoculated with a 20 µl/well of fresh MHB bacterial cultures, with a concentration of 10^6 CFU/ml for *A. actinomycetemcomitans*. The final bacterial cell concentration in the wells was 10^5 CFU/ml. Then the microplates were incubated for 48 hours at 37°C, under microaerophilic conditions. The MIC was defined as that concentration of the substance that will inhibit the visible growth of microorganism after 48 hours of incubation.

To determine whether LED alone caused any reduction of cell viability, bacterial suspensions were exposed to LED at 1, 3, and 5 min (energy density of 24, 72 and 120 J/cm^2), as described previously.[17] The lowest of LED exposure time that prevented bacterial growth was considered to be the minimum inhibitory LED irradiation dose. All tests were repeated at least 3 times.

Antimicrobial Effect of Treatments Against A. actinomycetemcomitans

To determine the antimicrobial effect of treatments on *A. actinomycetemcomitans*, samples were distributed to 5 groups as follow: (1) Groups C^+L^+ (treated with MIC of curcumin and LED), (2) Group C^+L^- (treated only with MIC of curcumin), (3) Group CH^+L^- (treated only with MIC of CHX), (4) Group $C^-CH^-L^+$ (treated only with LED), and (5) Group $C^-CH^-L^-$ (negative control; no exposure to either curcumin, CHX or LED light). The C, CH and L indicate "curcumin," "CHX" and "light," respectively. Tests were performed using previously described procedure.[17] For each group the experiment was repeated 12 times.

Statistical analysis

SPSS version 20 software served for statistical analysis. One-way analysis of variance (ANOVA) was used to analyze the differences between means, and the post hoc test

of Tukey's HSD served to evaluate the significance of all paired groups comparisons, with a *P* value of less than 0.05 considered as statistically significant.

Results

Based on the results obtained for the MIC of the experimental substances, CHX had the lowest MIC, which was 0.12%. The MIC measured for curcumin was 2.5 µg/ml. Our data revealed no effect of LED intensity up to 120 J/cm^2 on the survival of *A. actinomycetemcomitans*.

No growth was observed in our negative control groups. Table 1 shows the logarithm of the CFU/ml of bacteria in all 12 experiments of each group. Descriptive statistics of these results are shown in the Table 2.

As it can be seen in Table 2, the mean of the 12 experiments is the lowest in CHX group with 0.93. After that in the second place with the highest potency and least calculated mean is the LED + curcumin group, with 5.56. Groups curcumin, LED and the control group follow the first two mentioned groups with 6.89, 8.1 and 8.5, respectively. The differences between these figures were evaluated by one-way ANOVA and the post hoc test of Tukey's HSD, the results of which are presented in Table 3.

According to these results, CHX had the greatest potency with significant differences compared to other groups. Simultaneous use of LED with curcumin was found to have the highest potency after CHX, which was also statistically different from the other groups. Following them were curcumin, LED and the control group. The analysis showed that the differences between the curcumin group and the LED group were significant, but the LED group was insignificantly different from the control group with a *P* value of 0.095.

Table 1. Logarithm of the CFU/ml of the Bacteria for All the 12 Experiments of Each Study Group

No.	Control	Curcumin	Curcumin+LED	LED	CHX
1	8.90	6.56	5.39	8.26	0.84
2	8.78	7.19	6.48	8.16	1.14
3	8.12	6.93	4.55	7.78	1.04
4	8.19	7.28	6.12	8.01	1.36
5	8.16	6.33	5.46	7.68	1.14
6	8.93	6.83	5.09	8.31	1.04
7	8.87	7.65	5.82	8.42	1
8	8.02	6.45	5.61	7.71	1.04
9	8.92	6.61	6.26	7.94	0.69
10	8.65	6.72	5.62	8.37	0.90
11	8.23	7.62	5.01	8.11	0.50
12	8.98	6.56	5.33	8.52	1.04
Mean	8.56	6.89	5.56	8.10	0.93

Discussion

Many studies have been conducted aiming to find alternative treatments to mechanical removal and antibiotic administration for periodontal diseases. Among all the bacteria involved in the etiology of these oral health problems, *A. actinomycetemcomitans* has an important role in aggressive generalized periodontitis, and therefore has been paid much attention by many researchers. In this survey we aimed to evaluate the effects of curcumin and LED on this specific bacterium by comparing them to CHX, currently used worldwide. The overall results yielded from our survey were promising; but as could be expected, the MIC results showed greater preventive potency of CHX compared to curcumin on bacterial growth.

The antimicrobial effects of curcumin and LED laser have been evaluated on different strains of bacteria. In 2013, Paschoal et al[21] evaluated this preventive method on *Streptococcus mutans* in a planktonic culture. They found that simultaneous use of blue light LED and curcumin had better antibacterial effect on *S. mutans*. Their results were congruent with ours. We worked on *A. actinomycetemcomitans* since it seems to be a more important bacterium considering its complications. We also aimed to compare the efficacy of this method with the use of each component alone, and we found that simultaneous application of curcumin and LED laser inhibited the growth of *A. actinomycetemcomitans* more efficiently than using curcumin or LED laser individually. Another survey by Mattiello et al[22] assessed the effectiveness of 0.01% toluidine blue accompanied by diode laser of Aluminum Gallium Indium Phosphorus (AlGaInP), on A. *actinomycetemcomitans*. They also found a positive effect of PDT on this bacterium, which was compatible with our results. They evaluated a synthetic substance whose application might cause various complications. Thus, we chose curcumin, a natural derivative of medicinal plants, with fewer complications expected.

Abdul Azeez et al[23] also mentioned in 2014, the synergistic effect of visible blue light emitted from a light-cure laser and 0.2% CHX mouthwash against *A. actinomycetemcomitans*. Since oral complications are observed in chronic use of CHX, it is more favorable to find natural substances with fewer side-effects. Thus, we aimed to evaluate the effects of curcumin. In the same year Moslemi et al[24] conducted a study on the antimicrobial effects of Radachlorin with PDT on *A. actinomycetemcomitans*. They also reported the synergistic effects, due to a simultaneous use of these methods. Moreover, they found that Radachlorin had a greater inhibitory effect on the growth of these bacteria, compared to toluidine blue. They also

Table 2. Descriptive Statistics of the Mean of the Logarithm of Bacteria's CFU/ml in the Study Groups

Study group	N	Mean	SE	SD	Range	95% CI for Mean
Curcumin	12	6.8942	0.12880	0.44619	6.33-7.65	6.6107-7.1777
LED+curcumin	12	5.5617	0.15952	0.55259	4.55-6.48	5.2106-5.9128
LED	12	8.1058	0.08208	0.28433	7.68-8.52	7.9252-8.2865
CHX	12	0.9358	0.09760	0.33811	0.00-1.36	0.7210-1.1507
Control	12	8.5625	0.11017	0.38163	8.02-8.98	8.3200-8.8050

Table 3. The Results of Tukey's HSD Test

Group	N	Subset for Alpha = 0.05			
		1	2	3	4
Ch	12	0.9358			
Cu/LED	12		5.5617		
Cu	12			6.8942	
LED	12				8.1058
Control	12				8.5625
P value		1.000	1.000	1.000	.095

focused on synthetic materials, whose effects in vivo should be carefully evaluated for eventual complications or side-effects.

The antibacterial effect of curcumin and LED laser on other bacteria including *S. mutans* and *Lactobacillus acidophilus* was assessed in a study conducted by Araújo et al in 2014.[25] They also reported the same results indicative of a better effect in a simultaneous use of these methods. As mentioned before, the more aggressive complications of *A. actinomycetemcomitans* make this bacteria a more important target in research, which was focused on in our survey.

Other than the in vitro experiments mentioned, researchers have also assessed these methods in vivo. Waghmare et al[26] evaluated the effects of CHX and curcumin mouthwashes in prevention of plaque formation and inflammation in 2011. Their results suggested these mouthwashes as effective complementary measures alongside mechanical removal for periodontal diseases, showing a comparable effect between curcumin and CHX. Behal et al[27] assessed the effect of a 2% gel form of curcumin on periodontal problems and they found a significant decrease in plaque formation and inflammation, and proposed this method as a complementary treatment for scaling and root planning.

In a study conducted by Suhag et al[28] in 2007, the effect of curcumin as a subgingival irrigator was evaluated, which showed promising results compared to CHX and normal saline.[28] A significant decrease was observed in bleeding on probing and gingival redness in the patients treated with curcumin irrigator in the first days of treatment, while the differences with chlorhexidine and normal saline were not significant in the later recall visit. These three studies failed to compare the effects of curcumin and CHX with simultaneous application of these materials with LED laser. Also in vivo experiments such as these cannot evaluate the effects of a substance on a specific bacterium.

Our results pointed out the effectiveness of curcumin on preventing the growth of *A. actinomycetemcomitans,* which was reinforced when used simultaneously with PDT.

In this survey, we only evaluated the effects of mentioned methods on one specific strain of bacteria, which can be referred to as one of the limitations in our study. Also we did not assess the effects of various concentrations of substances or doses of radiation or even radiations with different wavelengths and durations. The most important part of the researches aiming to find substitute treatments should be conducted on in vivo environments, so that the complications are discovered as well as the positive effects. So putting it all together, further investigations are required to cover all the limitations of our survey.

Conclusion

It can be concluded that curcumin is an effective substance in preventing the growth of *A. actinomycetemcomitans*, whose impact is reinforced when used simultaneously in PDT procedure.

Conflict of Interest

The author has no conflict of interest to declare.

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