ORIGINAL ARTICLE

Potentiating Antibacterial Effect of Locally Deliver Caffeine Nanoparticles on Systemically Used Antibiotics in Periodontal Treatments

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Abstract

Aims: Subinhibitory concentration of antibiotics at periodontal sites may increase the microbial resistance development; hence, this study was carried out to support the hypothesis that antimicrobial as well as anti-inflammatory action of caffeine and its locally deliver nanoparticles, which can deeply penetrate into the periodontal sites might potentiate and synergize the antibacterial effect of systematically used antibiotics for the treatment of periodontitis. **Materials and Methods:** In this study, the caffeine-loaded low-molecular-weight chitosan nanoparticles were prepared by ionic gelation methodology. *Ex vivo* antimicrobial activity of prepared nanoparticles was carried out by periodontitis patient's stimulated saliva sample. **Result:** Our finding showed that caffeine nanoparticles in combination with amoxicillin affect the growth of periodontitis microorganisms. Periodontitis microorganism grew on a nutrient agar medium in Petri plates. Agar cups were filled in combination of different concentrations of amoxicillin with or without fixed caffeine concentration containing nanoparticles. High amoxicillin concentration (0.5 µg/ml) with 1 mg of caffeine-containing nanoparticles showed maximum zone of inhibition (1.81 ± 0.24 cm). On the other hand, low amoxicillin concentration (0.3 µg/ml) with 1 mg of caffeine-containing nanoparticles demonstrated significant potentiating inhibitory zone (1.54 ± 0.15 cm) as compare to high amoxicillin concentration (0.5 µg/ml) alone (1.50 ± 0.21 cm). **Conclusions:** It was shown that caffeine nanoparticles potentiate antibacterial effect of amoxicillin.

Key words: Amoxicillin, caffeine nanoparticles, *Ex vivo* antibacterial activity, periodontitis microorganisms

INTRODUCTION

Microbial colonization on surface of tooth, margin of gingival, and environment of subgingival may cause to periodontal diseases.[1] It may cause inflammation and destruction of the dentogingival complex.[2] As per the previous study, utilization of antioxidant therapy may help in maintenance of the periodontal health and minimization of inflammatory levels.[3-5] At submillimolar concentrations, caffeine shows noticeable effects on variety of microorganisms.[6,7] These include increasing of intracellular amount of cAMP by phosphodiesterases inhibition; direct or indirect effects on calcium concentrations at intracellular level; and adenosine receptors antagonism. Caffeine also inhibits the ataxia-telangiectasia mutated kinase activity and therefore elimination of G2/M DNA damage checkpoint.[8,9] The ionic radiation is being widely utilized in the treatment of cancer treatment. Caffeine can effectively potentiate the lethal effects of ionic radiation.[10,11]

In early days for the treatment of periodontal diseases, systemic antibiotics such as metronidazole, doxycycline, amoxicillin (in combination with or without clavulanic acid), tetracycline, clindamycin, spiramycin, and azithromycin were used.[12-14] In case of aggressive periodontal conditions,

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severe and progressive forms of periodontal diseases, utilization of systemic antibacterial agents should be under optimal conditions or restricted.[15] In addition, subinhibitory concentrations of antimicrobials at periodontal pockets may facilitate bacterial resistance development.[16-20]

Recent study suggested that utilization of antioxidant-loaded nanoparticles may inhibit inflammation and bone resorption.[21,22] Moreover, nanoparticles contain multiple simultaneous actions toward various microbes and so, normally microbial cells cannot develop resistance against them.[23-26] In view of the abovementioned information's, the current research is carried out to investigate the effect of locally deliver caffeine nanoparticles along with systemic antimicrobial for the treatment of periodontal diseases. For this purpose, we have selected amoxicillin as a model antibacterial agent and to carry out antimicrobial experiments, we have used periodontitis patient's saliva sample.

The salivary microbes have been proposed as a diagnostic marker for dental caries[27,28] and periodontal disease.[29] As previously studied,[30] stimulated saliva contains 3 times higher microbial species as compared to unstimulated saliva. Hence, we had collected stimulated saliva samples using sterile paraffin.

The aim of this research was to examine the enhancing effect of antibacterial agent with caffeine nanoparticles on periodontitis salivary microbiota.

MATERIALS AND METHODS

Materials

Chitosan (low molecular weight, deacetylated chitin, and poly[D-glucosamine]), sodium tripolyphosphate (technical grade, 85%), and pure caffeine (solubility: 15 mg/ml) were purchased from Sigma-Aldrich (Bengaluru, India). Acetic acid (extra pure) was purchased from FINAR® (Ahmedabad, India). Nutrient agar was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Double-distilled water was utilized during all experiments. All other chemicals utilized as received were of at least reagent grade.

Drug polymer compatibility

To prepare the KBr pellet, the test sample was kindly mixed with a suitable quantity of micronized powder of KBr and disc was prepared. Using FT-IR spectrometer (Bruker, Germany), infrared spectra were performed.

Preparation of caffeine nanoparticles

As early prescribed,[31-33] ionic gelation method was used for the preparation of caffeine-loaded nanoparticles. In brief, low-molecular-weight chitosan (LMC) was dissolved in 0.75% (V/V) acetic acid (0.1 M) solution and then further diluted to prepare various concentrations (3.3, 1.6, 0.95, 1.4, and 2.1 mg/ml) of LMC. A cross-linking agent, sodium tripolyphosphate (STPP) was dissolved in double-distilled water to prepare a concentration of 1 mg/ml. The pH 4.4 was maintained by utilizing sodium hydroxide (0.5 M), which was done in dropwise addition. After this, 100 ml of STPP solution was dropwise added to the 100 ml of LMC solution at room temperature under vigorous magnetic stirring. The mixture was then centrifuged at 20,000 rpm for 15 min to collect nanoparticles. A 1 mg/ml concentration containing caffeine-loaded LMC nanoparticles was prepared by first dissolving 100 mg of caffeine into 100 mL of STPP solution. Then, this mixture was dropwise added to different concentrations containing 100 mL of LMC solution [Table 1]. Prepared nanoparticles were freeze-dried (Allied Frost, New Delhi), kept at cool and dry place for further studies.

Mean particle size and zeta potential analysis

As prescribed earlier,[34] using a dynamic light scattering (Malvern Zetasizer, UK), particle size and zeta potential were carried out. All samples were diluted using distilled water to different intensity concentration and examination was carried out at a temperature of 25°C at a scattering angle of 90°.

Drug incorporation efficiency and loading capacity

As prescribed earlier,[35] for the determination of drug incorporation efficiency, precisely weighed 10 mg of nanoparticles were added to 10 ml of acetic acid (0.1 M) and dissolved them completely. Clear supernatant was obtained using centrifugation (10,000 rpm, 10 min). Then, the clear supernatant was filtered (Whatman paper No. 41) and 1 ml of filtrate was mixed with 4 ml of acetic acid (0.1 M). The free concentration of caffeine in the supernatant and the resulting caffeine-loaded nanoparticles concentration were carried out by ultraviolet spectroscopy (UV-1800; Shimadzu, Germany) at 282 nm. The drug incorporation (DI) was calculated as DI = $(\text{Caffeine}_{\text{total}} - \text{Caffeine}_{\text{supernatant}})/\text{Caffeine}_{\text{total}} \times 100\%$ and the drug loading (DL) was calculated as DL = $(\text{Caffeine}_{\text{total}} - \text{Caffeine}_{\text{supernatant}})/\text{excipients} \times 100\%$.

Table 1: Particle size and polydispersity index of different batches of caffeine nanoparticles

Batch	LMC (mg/mL)	STPP (mg/mL)	Particle size (nm±SD)	Polydispersity index (±SD)
A	3.3	1	677.0±17.98	0.140±0.05
B	2.1	1	450.0±12.40	0.019±0.05
C	1.6	1	325.6±8.98	0.141±0.07
D	1.4	1	281.3±3.82	0.303±0.14
E	0.95	1	129.2±2.60	0.220±0.06

LMC: Low-molecular-weight chitosan, STPP: Sodium tripolyphosphate

Triplicate readings of each individual value were carried out and their mean values are taken into the consideration.

Morphological evaluation of nanoparticles

As prescribed earlier,[36] transmission electron microscopy is a novel application for characterization of nanoparticles. Hence, using transmission electron microscopy (TEM; Tecnai 20, Philips, Holland), morphological evaluation of caffeine-loaded nanoparticles was carried out. Samples of the caffeine-loaded nanoparticles containing suspension (5–8 µl) were dropped onto copper grids. After complete drying, by utilization of 2% w/v phosphotungstic acid, the samples were stained. To perform the image capture and analysis, S-TWIN objective lens with high resolution was used.

In vitro drug release study

By utilizing a dialysis method,[37] the *in vitro* release profile of caffeine from the caffeine-loaded nanoparticles was investigated. In this method, phosphate buffer solution (pH 6.8) was used as a release medium. In brief, 0.02% w/v of caffeine solution or caffeine-loaded nanoparticles suspension in phosphate buffer solution (containing approx. 2 mg of caffeine) was introduced into a dialysis bag (avg. flat width = 10 mm, Typical MW cutoff = 14,000, Sigma-Aldrich, Bengaluru, India). At 37°C on mechanical shaking bath (100 cycles/min), this bag was incubated in 50 ml release medium containing glass beaker. At predetermined time intervals, 5 ml sample was withdrawn and it was replaced through an equal quantity of freshly prepared phosphate buffer, pH 6.8. Then, appropriate dilution of sample was done and quantitative measurement was carried out using a UV spectrophotometer at 282 nm.

Patient's saliva sample collection

Patient suffering with periodontal disease was identified at Parul Sevashram Hospital, Dental Department, Limda, Gujarat, India. In aseptic area, sterile paraffin was applied on patient's mucosal membrane with brush for stimulation of salivary flow. Approximately 3–5 ml stimulated saliva was collected in sterile glass tube and stored at −5°C ± 3 temperature for further use.

Ex vivo antibacterial activity

Patient's salivary sample was used in agar well diffusion method to check out potentiating effect of caffeine nanoparticles. For evaluation and confirmation of antimicrobial activity, sterilized nutrient agar (NA) growth medium was used.

A 0.1 ml of patient's saliva sample was poured with 20 ml NA in Petri plates. With the help of sterilized metal, borer wells were created. As shown in Table 2, in Petri plates, 1 ml of different concentrations of amoxicillin with or without 1 mg caffeine-containing nanoparticles were carefully poured into the wells. All plates were incubated in incubator at temperature of 37°C ± 0.5 for 24 h. The developments of clear zone of inhibition in diameter were determined by taking mean of four equivalent circular diameters. All experiments were carried out for 3 times.

RESULTS

FT-IR spectroscopy

As shown in Figure 1A, total seven bands for LMC can be attributed to N-H and O-H stretching at 3416 cm^{-1}, C-H stretching at 2923 cm^{-1}, the primary amide at 1658 cm^{-1}, the N-H bending from amine and secondary amide at 1598 cm^{-1}, $-CH_2$ bending at 1426 cm^{-1}, the CH_3 groups (a symmetric deformation) at 1383 cm^{-1}, and antisymmetric stretching of C-O-C and C-H stretching at 1154 cm^{-1}. In the FTIR spectrum [Figure 1B] of caffeine, we observe nine main bands which can be attributed to the stretching vibration region of > C=O at 1024 cm^{-1} and C-N stretching at 1128 cm^{-1}, combined contribution of > C=O and C-N stretching at 1238 cm^{-1}, a minor peak observed due to the stretching vibration of C-N at 1403 cm^{-1}, C=C stretching, C-H bending, and C=N stretching at 1455 cm^{-1}, C=N stretching at 1598 cm^{-1}, major peaks observed because of carbonyl groups (> C=O), C=C and C=N stretching at 1658 cm^{-1} and 1699 cm^{-1}, and C-H stretching vibration of methyl ($-CH_3$) groups at 2954 cm^{-1}.

The FT-IR data provided information of the compatibility between drug and excipients as well as caffeine-loaded nanoparticles formation. In the FTIR spectrum [Figure 1C] of LMC and caffeine, the N-H and O-H bands shifted to 3398 cm^{-1}, the $-CH_2$ band shifted to 1430 cm^{-1}, and C-O-C and C-H bands shifted to 1188 cm^{-1}. While in caffeine spectrum, the C-N band shifted to 1188 cm^{-1} and C-H band of CH_3 group stretching shifted to 2954 cm^{-1}. As shown in FTIR spectrum of prepared nanoparticles [Figure 1D], in case of LMC, the N-H and O-H bands shifted to 3410 cm^{-1}, the $-CH_2$ band shifted to 1430 cm^{-1}, and C-O-C and C-H bands shifted to 1187 cm^{-1}. On the other hand, in caffeine spectrum, the C-N band shifted to 1187 cm^{-1} and C-H band of CH_3 group stretching shifted to 2924 cm^{-1}.

Table 2: *Ex vivo* antibacterial activity

Plate no.	Growth medium	Zone of inhibition (cm)±SD
1	NA+0.5 µg/ml AMX	1.50±0.21
2	NA+0.5 µg/ml AMX+1 mg Cf-loaded NPs	1.81±0.24
3	NA+0.3 µg/ml AMX+1 mg Cf-loaded NPs	1.54±0.15

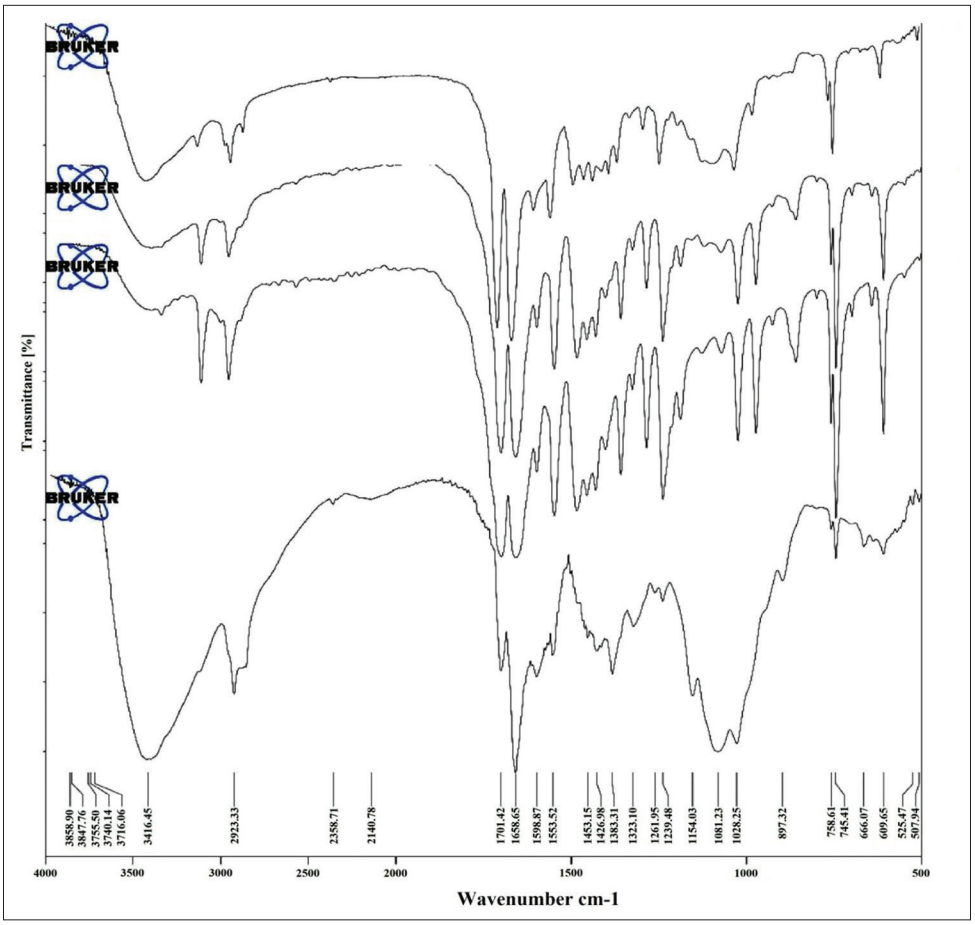

Figure 1: FT-IR spectra (A) Low-molecular-weight chitosan (LMC); (B) caffeine; (C) LMC + Caffeine; and (D) caffeine-loaded LMC carrying nanoparticles

Mean particle size and zeta potential analysis

As shown in Figure 2, batch E with least concentration of chitosan contain lowest particle size (129.2 ± 2.60 nm, $n = 3$) of prepared nanoparticles. On the other hand, chitosan concentration has positive effect on particle size of nanoparticles. With the increase in concentration of chitosan, the particle size was also increased (batch A to D, 281.3 ± 3.82 to 677.0 ± 17.98 nm, $n = 3$).

TEM analysis

The TEM results show [Figure 3] that the caffeine-loaded nanoparticles (batch E) have average size of 65.25 nm, which was shown similarity to previously reported nanoparticles by chitosan-sodium tripolyphosphate ionic gelation methodology.[38] Significant improvement in the antimicrobial activity can be achieved by the utilization of nanoparticles, as they are able to penetrate into underlying connective tissue, the periodontal pocket areas below the gum, and even the alveolar bone trabeculae.[37] All prepared nanoparticles have well spherical shape, which is necessary for their utilization in subgingival applications. The drug incorporation was found to be 97% for optimized batch-E, which was similar to previously reported results for chitosan-sodium tripolyphosphate nanoparticles.[39-43]

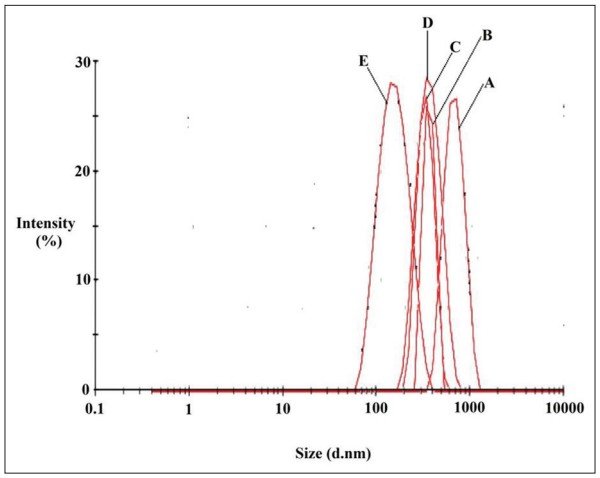

Figure 2: Nanoparticles size distribution by intensity (Batch – A, B, C, D, and E)

In vitro drug release

The *in vitro* cumulative release profiles of caffeine-loaded nanoparticles (batch E) and caffeine solution (control preparation) in phosphate buffer, pH 6.8, are shown in Figure 4. In the opening phase, both formulations demonstrated rapid release rate with maintenance of loading dose. The caffeine

solution was almost 100% released within 24 h, while the slower drug release of caffeine from nanoparticles was approximately 98.16% after 10 days. In effective periodontal treatment, demonstrated biphasic release rates from the developed LMC carrying caffeine-loaded nanoparticles may be significant formulation. The %cumulative release of LMC loaded nanoparticles and the time of release were applied with an equation (In(1-Q) = -Kt) of the first-order drug releasing, equation ($Q = Kt^{1/2}$) of Higuchi model, and equation ($Q = Kt^{n}$) of Ritger–Peppas model. In the result, Ritger–Peppas equation was best fitted ($Q = 0.46t^{0.26}$, r=0.997), demonstrating the release of caffeine mainly on diffusion based.

Ex vivo antibacterial activity

Ex vivo antimicrobial activity was carried out with batch E nanoparticles. From the zone of inhibition studies against periodontitis patient's saliva, it can be observed that 1 mg caffeine nanoparticles with low amoxicillin concentration (0.3 µg/ml) gave batter zone of inhibition [Figure 5c] as compared to high amoxicillin concentration (0.5 µg/ml) alone [Figure 5a]. In that also, 1 mg caffeine nanoparticles with high amoxicillin concentration (0.5 µg/ml) showed the maximum antibacterial activity [Figure 5b]. It was observed that the caffeine-loaded nanoparticles were potentiating the antibacterial action of amoxicillin [Table 2].

DISCUSSION

Caffeine diffuse from the nanoparticles may increase the concentration of certain cells of immunocompetent and strengthen the first-line host defense in opposition to microbial invaders.[44] The results of this experimental work exhibit that those caffeine nanoparticles have a direct potentiating antibacterial effect. We observed that the similar caffeine nanoparticles concentration affects the growth of periodontitis microorganisms with a different concentration of amoxicillin. Our findings show that caffeine nanoparticles with low concentration of amoxicillin are potentiating antibacterial effect to periodontitis microorganisms than amoxicillin alone. A significant inhibitory effect of caffeine nanoparticles on periodontitis microorganisms was observed with a 0.5 µg/ml amoxicillin concentration; below this amoxicillin concentration (i.e., 0.3 µg/ml) with caffeine nanoparticles had better inhibitory effect on the growth of microorganisms, whereas a low inhibitory effect of amoxicillin (0.5 µg/ml) alone on periodontitis microorganisms was observed. Caffeine has been also identified to reduce tissue damage and inflammation in different animal models.[45] In contrast, high doses of caffeine were demonstrated increase alveolar bone loss in periodontitis induced rats.[46] However, the caffeine doses given were very high. It was reported that excessive consumption of green tea will lead to higher concentration of caffeine administration[47] and it has been considered as a risk factor for periodontal disease.[48]

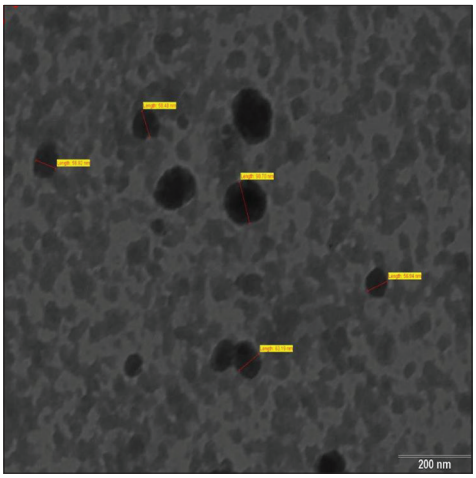

Figure 3: Transmission electron microscopy image of caffeine-loaded nanoparticles

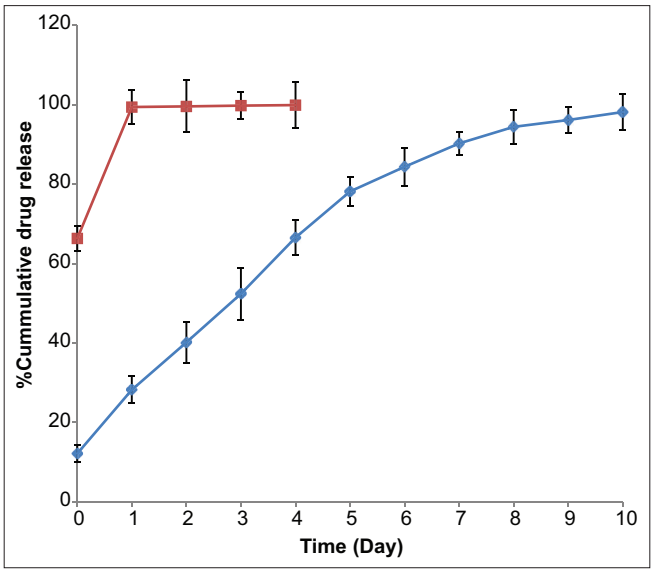

Figure 4: Release profile – (□) caffeine solution; (◊) caffeine-loaded nanoparticles

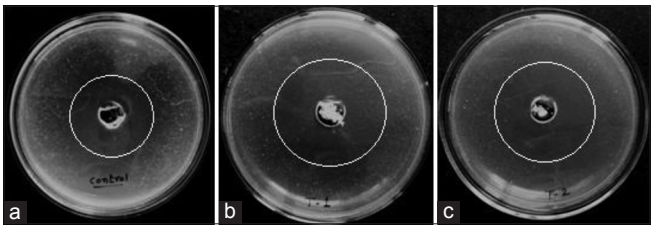

Figure 5: Zone of inhibition (a) amoxicillin 0.5 µg/ml; (b) amoxicillin 0.5 µg/ml + 1 mg caffeine nanoparticles; (c) amoxicillin 0.3 µg/ml + 1 mg caffeine nanoparticles

As previously studied,[49] caffeine-containing chewing gum demonstrated faster caffeine absorption through the mucosa. In addition, nanoparticles are able to adhere the mucosal membrane may also increase the absorption of caffeine. Caffeine exhibits the antibacterial effect and on human health, they have a negative or a positive impact. On the other hand, misuse or site-specific subinhibitory concentration of

antibacterial in systemic treatment of periodontitis, local delivery of caffeine nanoparticles can have a curative and positive effect.

CONCLUSIONS

Our findings demonstrate a clear inhibitory action of caffeine nanoparticles. The sensitivity of periodontitis microorganism to antibacterial agent with caffeine nanoparticles can vary greatly depending on concentration and potency of antibacterial agent. Periodontitis microorganisms are more sensitive to amoxicillin with caffeine nanoparticles than amoxicillin alone. This research could be considered into account of the periodontal treatments.

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REFERENCES

1. Teles R, Teles F, Frias-Lopez J, Paster B, Haffajee A. Lessons learned and unlearned in periodontal microbiology. Periodontol 2000 2013;62:95-162.
2. Popova C, Dosseva-Panova V, Panov V. Microbiology of periodontal diseases. Biotechnol Biotechnol EQ 2013;27:3754-9.
3. Castro MM, Duarte NN, Nascimento PC, MagnoMB, Fagundes NC, Flores-Mir C, *et al*. Antioxidants as adjuvants in periodontitis treatment: A systematic review and meta-analysis. Oxid Med Cell Longev 2019;2019:9187978.
4. Tripathi P, Blaggana V, Upadhyay P, Jindal M, Gupta S, Nishat S. Antioxidant therapy (lycopene and green tea extract) in periodontal disease: A promising paradigm. J Indian Soc Periodontol 2019;23:25-30.
5. Muniz FW, Nogueira SB, Mendes FL, Rösing CK, Moreira MM, de Andrade GM, *et al*. The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and periodontal outcomes: A systematic review. Arch Oral Biol 2015;60:1203-14.
6. Al-Janabi AA. Potential activity of the purine compounds caffeine and aminophylline on bacteria. J Glob Infect Dis 2011;3:133-7.
7. Netter KJ. Caffeine coffee and health. Toxicology 1993;85:215-6.
8. Zhou BB, Chaturvedi P, Spring K, Scott SP, Johanson RA, Mishra R, *et al*. Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J Biol Chem 2000;275:10342-8.
9. Murnane JP. Cell cycle regulation in response to DNA damage in mammalian cells: A historical perspective. Cavgncer Metastasis Rev 1995;14:253-54.
10. Sakurai H, Mitsuhashi N, Tamaki Y, Akimoto T, Murata O, Kitamoto Y, *et al*. Interaction between low dose-rate irradiation, mild hyperthermia and low-dose caffeine in a human lung cancer cell line. Int J Radiat Biol 1999;75:739-45.
11. Musk SR. Reduction of radiation-induced cell cycle blocks by caffeine does not necessarily lead to increased cell killing. Radiat Res 1991;125:262-6.
12. Kapoor A, Malhotra R, Grover V, Grover D. Systemic antibiotic therapy in periodontics. Dent Res J (Isfahan) 2012;9:505-15.
13. Slots J, Research, Science and Therapy Committee. Systemic antibiotics in periodontics. J Periodontol 2004;75:1553-65.
14. van Winkelhoff AJ, Rams TE, Slots J. Systemic antibiotic therapy in periodontics. Periodontol 2000 1996;10:45-78.
15. Barca E, Cifcibasi E, Cintan S. Adjunctive use of antibiotics in periodontal therapy. J Istanb Univ Fac Dent 2015;49:55-62.
16. Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP. Fighting biofilms with lantibiotics and other groups of bacteriocins. NPJ Biofilms Microbiomes 2018;4:9.
17. Patil V, Mali R, Mali A. Systemic anti-microbial agents used in periodontal therapy. J Indian Soc Periodontol 2013;17:162-8.
18. Sweeney LC, Dave J, Chambers PA, Heritage J. Antibiotic resistance in general dental practice--a cause for concern? J Antimicrob Chemother 2004;53:567-76.
19. Loesche WJ. The antimicrobial treatment of periodontal disease: Changing the treatment paradigm. Crit Rev Oral Biol Med 1999;10:245-75.
20. Goodson JM, Tanner A. Antibiotic resistance of the subgingival microbiota following local tetracycline therapy. Oral Microbiol Immunol 1992;7:113-7.
21. Liang J, Peng X, Zhou X, Zou J, Cheng L. Emerging applications of drug delivery systems in oral infectious diseases prevention and treatment. Molecules 2020;25:E516.
22. Zambrano LM, Brandao DA, Rocha FR, Marsiglio RP, Longo IB, Primo FL, *et al*. Local administration of curcumin-loaded nanoparticles effectively inhibits inflammation and bone resorption associated with experimental periodontal disease. Sci Rep 2018;8:6652.
23. Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. Int J Nanomedicine 2017;12:1227-49.
24. Fernando S, Gunasekara T, Holton J. Antimicrobial nanoparticles: Applications and mechanisms of action. Sri Lankan J Infect Dis 2018;8:2-11.
25. Baptista PV, McCusker MP, Carvalho A, Ferreira DA, Mohan NM, Martins M, *et al*. Nano-strategies to fight

multidrug resistant bacteria-"a battle of the titans". Front Microbiol 2018;9:1441.
26. Hegde MN, Attavar SH, Shetty N, Hegde ND, Hegde NN. Saliva as a biomarker for dental caries: A systematic review. J Conserv Dent 2019;22:2-6.
27. Hemadi AS, Huang R, Zhou Y, Zou J. Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment. Int J Oral Sci 2017;9:e1.
28. Vitorino R, Lobo MJ, Duarte JR, Ferrer-Correia AJ, Domingues PM, Amado FM. The role of salivary peptides in dental caries. Biomed Chromatogr 2005;19:214-22.
29. Faveri M, Mayer MP, Feres M, de Figueiredo LC, Dewhirst FE, Paster BJ. Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. Oral Microbiol Immunol 2008;23:112-8.
30. Gomar-Vercher S, Simón-Soro A, Montiel-Company JM, Almerich-Silla JM, Mira A. Stimulated and unstimulated saliva samples have significantly different bacterial profiles. PLoS One 2018;13:e0198021.
31. Othman N, Masarudin MJ, Kuen CY, Dasuan NA, Abdullah LC, Md Jamil SN. Synthesis and optimization of chitosan nanoparticles loaded with L-ascorbic acid and thymoquinone. Nanomaterials (Basel) 2018;8:920.
32. Desai KG. Chitosan nanoparticles prepared by ionotropic gelation: An overview of recent advances. Crit Rev Ther Drug Carrier Syst 2016;33:107-58.
33. Debnath S, Kumar RS, Babu MN. Ionotropic gelation a novel method to prepare chitosan nanoparticles. Res J Pharm Tech 2011;4:492-95.
34. Nafiu A, Sanjula B, Pramod K, Manisha S, Shweta D, Shahid HA, *et al*. Development and evaluation of triclosan loaded poly-?-caprolactone nanoparticles system for the treatment of periodontal infections. J Nanopart Res 2013;15:1-5.
35. Dora CP, Singh SK, Kumar S, Datusalia AK, Deep A. Development and characterization of nanoparticles of glibenclamide by solvent displacement method. Acta Pol Pharm 2010;67:283-90.
36. Petrushevska M, Pavlovska K, Laskova J, Zdravkovski P, Dodov MG. Transmission electron microscopy: Novel application of established technique in characterization of nanoparticles as drug delivery systems. Pril (Makedon Akad Nauk Umet Odd Med Nauki) 2019;40:67-72.
37. Yao W, Xu P, Pang Z, Zhao J, Chai Z, Li X, *et al*. Local delivery of minocycline-loaded PEG-PLA nanoparticles for the enhanced treatment of periodontitis in dogs. Int J Nanomedicine 2014;9:3963-70.
38. Hassan AN, Sahudin S, Hussain Z, Hussain M, Hussain M. Self-assembled chitosan nanoparticles for percutaneous delivery of caffeine: Preparation, characterization and *in vitro* release studies. Int J App Pharm 2018;10:172-85.
39. Csaba N, Köping-Höggård M, Alonso MJ. Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery. Int J Pharm 2009;382:205-14.
40. Dudhani AR, Kosaraju SL. Bioadhesive chitosan nanoparticles: Preparation and characterization. Carbohydr Polym 2010;81:243-51.
41. Hashad RA, Ishak RA, Fahmy S, Mansour S, Geneidi AS. Chitosan-tripolyphosphate nanoparticles: Optimization of formulation parameters for improving process yield at a novel pH using artificial neural networks. Int J Biol Macromol 2016;86:50-8.
42. Preparation of bioactive interferon alpha-loaded polysaccharide nanoparticles using a new approach of temperature-induced water phase/water-phase emulsion [Retraction]. Int J Nanomedicine 2018;13:2277.
43. Ragelle H, Vanvarenberg K, Vandermeulen G, Préat V. Chitosan nanoparticles for SiRNA delivery *in vitro*. Methods Mol Biol 2016;1364:143-50.
44. Ramanaviciene A, Acaite J, Ramanavicius A. Chronic caffeine intake affects lysozyme activity and immune cells in mice. J Pharm Pharmacol 2004;56:671-6.
45. Lv X, Chen Z, Li J, Zhang L, Liu H, Huang C, *et al*. Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress. Inflamm Res 2010;59:635-45.
46. Bezerra JP, da Silva LR, de Alvarenga Lemos VA, Duarte PM, Bastos MF. Administration of high doses of caffeine increases alveolar bone loss in ligature-induced periodontitis in rats. J Periodontol 2008;79:2356-60.
47. Bae J, Park PS, Chun BY, Choi BY, Kim MK, Shin MH, *et al*. The effect of coffee, tea, and caffeine consumption on serum uric acid and the risk of hyperuricemia in Korean multi-rural communities cohort. Rheumatol Int 2015;35:327-36.
48. Han K, Hwang E, Park JB. Excessive consumption of green tea as a risk factor for periodontal disease among Korean adults. Nutrients 2016;8:408.
49. Kamimori GH, Karyekar CS, Otterstetter R, Cox DS, Balkin TJ, Belenky GL, *et al*. The rate of absorption and relative bioavailability of caffeine administered in chewing gum versus capsules to normal healthy volunteers. Int J Pharm 2002;234:159-67.

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