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Microbiology

In vitro minocycline activity on superinfecting microorganisms isolated from chronic periodontitis patients

Atividade in vitro *de minociclina sobre microrganismos superinfectantes isolados de pacientes com periodontite crônica*

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ABSTRACT: Chronic periodontitis is the most common type of periodontitis and it is associated with various species of microorganisms. Enteric rods, *Pseudomonas, Staphyloccocus* and *Candida* have been retrieved from periodontal pockets of patients with chronic periodontitis and correlated to cases of superinfection. Local or systemic antibiotic therapy is indicated to reinforce the effects of the conventional mechanical therapy. Minocycline has been suggested as one of the most effective drugs against periodontal pathogens. The aim of this work was to evaluate the minimal inhibitory concentration (MIC) of minocycline on superinfecting microorganisms isolated from the periodontal pocket and the oral cavity of individuals with chronic periodontitis. Isolates of Enterobacteriaceae (n = 25), *Staphylococcus* spp. (n = 25), *Pseudomonas aeruginosa* (n = 9) and *Candida* spp. (n = 25) were included in the study. Minimal inhibitory concentrations (MIC) of minocycline were determined using the Müeller-Hinton agar dilution method. *Staphylococcus* spp. isolates were the most sensitive to minocycline with a MIC of 8 μg/mL, followed by Enterobacteriaceae with a MIC of 16 μg/mL. The concentration of 16 μg/mL inhibited 96% of *Candida* spp. isolates. The MIC for 88.8% of the isolates of *Pseudomonas aeruginosa* was 128 μg/mL. A concentration of 1,000 μg/mL was not enough to inhibit 100% of the tested isolates.

DESCRIPTORS: Periodontitis; Minocycline; Minimum inhibitory concentration.

RESUMO: Periodontite crônica é a forma mais comum de periodontite e está associada a diversas espécies de microrganismos. Enterobactérias, *Pseudomonas*, *Staphyloccocus* e *Candida* têm sido recuperados de bolsas periodontais de indivíduos com periodontite crônica e implicados em casos de superinfecção. A terapia antimicrobiana local ou sistêmica pode ser utilizada para reforçar os efeitos da terapia mecânica convencional, e a minociclina tem sido sugerida como antimicrobiano eficaz frente a periodontopatógenos. O objetivo deste trabalho foi avaliar a concentração inibitória mínima (CIM) de minociclina sobre microrganismos superinfectantes isolados de bolsas periodontais e cavidade bucal de indivíduos com periodontite crônica. Foram utilizadas 84 cepas de microrganismos, incluindo Enterobacteriaceae (n = 25), *Staphylococcus* spp. (n = 25), *Pseudomonas aeruginosa* (n = 9) e *Candida* spp. (n = 25). A CIM foi determinada pelo método de diluição em ágar Müeller-Hinton. *Staphylococcus* foram os microrganismos mais sensíveis a minociclina com CIM de 8 μg/mL, seguidos por Enterobacteriaceae com CIM de 16 μg/mL. Entre as espécies de *Candida,* 96% foram inibidas na concentração de 16 μg/mL. Para 88,8% das cepas de *Pseudomonas aeruginosa* a CIM foi de 128 μg/mL. A concentração de 1.000 μg/mL não foi suficiente para inibir 100% das cepas testadas.

DESCRITORES: Periodontite; Minociclina; Concentração inibitória mínima.

INTRODUCTION

Periodontitis is a multi-factorial disease and dental biofilm is considered the initiator of periodontal disease[4,7,10,24]. However, the manifestation and progression of periodontitis are influenced by a wide variety of factors, including subject characteristics, social and behavioral factors, systemic factors, genetic factors, microbial composition of dental biofilm and other emerging risk factors[14]. Several hundred recognized species of microorganisms, and many more that have yet to be identified, inhabit the gingival crevice. However, it has been shown that only few species play a significant role in the etiology of periodontal dieseases. This evidence is largely based on epidemiologic data, the

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ability of a microorganism to produce disease when inoculated in animals, and the capacity to produce virulence factors. Therefore, the mere presence of putative periodontopathogens in the gingival crevice is not sufficient to initiate or cause periodontal inflammation[11].

Enterobacteria, *Pseudomonas, Staphylococcus* and *Candida* have been collected from periodontal pockets of chronic periodontitis subjects[23] and are described as superinfectious microorganisms[19]. Such microorganisms present virulence factors that contribute to their action on the periodontal tissues, as leukotoxins and colagenases produced by *Staphylococcus,* the production of endotoxin (LPS) by enteric bacilli, and exotoxin A produced by *Pseudomonas*[15,17,25].

Minocycline is an antimicrobial drug with large spectrum of activity that has been used as adjunct to periodontal treatment, through systemic or local administration, due to its efficacy on periodontopathogens[1,29]. Its antifungal effect has been already referred to when used alone or in association with other antimicrobial drugs[8,18,22,28]. However, an antibiotic therapy of large spectrum can promote the development of resistant or opportunistic pathogens, and consequent superinfection[27].

The purpose of this *in vitro* study was to assess the minimum inhibitory concentration (MIC) of minocycline on Enterobacteriaceae, *Pseudomonas, Staphylococcus* and *Candida* isolates from periodontal pockets and oral cavities of chronic periodontitis patients.

MATERIALS AND METHODS

All subjects included in the present study signed an Informed Consent which was previously approved by the Institutional Committee on Research Involving Humans (Protocol of the University of Taubaté Ethics Committee #035/02).

The microorganisms included in the study belonged to the Culture Collection of the University of Taubaté (CCUT) and were isolated during previous studies from periodontal pockets and oral cavities of chronic periodontitis patients[9,12,20]. Eighty-four (84) strains were included, Enterobacteriaceae (n = 25), *Staphylococcus* spp. (n = 25), *Pseudomonas aeruginosa* (n = 9), *Candida* spp. (n = 25).

Initially the microorganisms were transferred to Brain Heart Infusion broth (BHI, Difco, Detroit, USA) and inoculated in selective media. MacConkey agar (Difco, Detroit, USA) was used for Enterobacteriaceae and *Pseudomonas aeruginosa*; Baird-Parker agar (Difco, Detroit, USA), for *Staphylococcus* spp.; and Sabouraud agar (Difco, Detroit, USA), for *Candida* spp. The plates were incubated at 37°C for 24 h.

The minimum inhibitory concentration (MIC) of minocycline was determined using the method of dilution in Müeller-Hinton Agar (Difco, Detroit, USA), in duplicate[16].

The antimicrobial agent (minocycline, Deg import, Italy, batch 0202009CX2) was sterilized by filtration, using 0.22 μm Millipore membrane and 0.1 ml of each dilution was added to the Müeller-Hinton Agar media at 50°C. Plate series were prepared containing from 0.25 to 256 μg/mL of minocycline in sequential dilutions multiple of two, plus 340, 500 and 1,000 μg/mL concentrations.

Each microbial sample was suspended in 10 ml of saline (0.9% NaCl) until a density corresponding to 3×10^8 cells/mL was obtained (tube #1 of McFarland scale), inoculated with the aid of Steers replicator, and the plates were incubated at 37°C for 24 h. Readings were performed every 24 h. Media without the addition of minocycline was used as positive control.

Readings were performed by observing the presence or absence of microbial growth on the agar surface[16]. Descriptive statistical analysis was used to interpret the results.

RESULTS

All tested microorganisms developed in the media without addition of minocycline (control group) after incubation for 24 h at 37°C.

Enterobacteriaceae (n = 25) presented a MIC between 4 and 16 μg/mL and strains of *Staphylococcus* spp. (n = 25), between 0.25 and 8 μg/mL. Among the tested *Pseudomonas aeruginosa* (n = 9) isolates, 88.89% showed a MIC between 64 and 128 μg/mL, whereas 55.56% (cumulated percentage) were inhibited at 64 μg/mL and one strain was resistant at the 1,000 μg/mL concentration level. Fungi of the *Candida* genus (n = 25) showed a MIC between 0.25 and 16 μg/mL after 24 h of incubation at 37°C, except for one strain that grew at the 1,000 μg/mL concentration level.

MIC absolute and cumulated frequencies, as well as the cumulated percentages, for all tested microorganisms are shown on Table 1. The absolute frequency corresponds to the number of strains inhibited in each concentration.

TABLE 1 - MIC frequency for superinfecting microorganism after incubation for 24 h at 37°C.

Microorganisms	MIC μg/mL	Absolut frequency	Cumulated frequency	Cumulated %
Enterobacteriaceae	4	3	3	12
	8	2	5	20
	16	20	25	100
Staphylococcus	0.25	1	1	4
	1	23	24	96
	8	1	25	100
Pseudomonas aeruginosa	64	5	5	55.56
	128	3	8	88.89
	> 1,000	1	9	100
Candida	0.25	9	9	36
	2	3	12	48
	4	1	13	52
	8	3	16	64
	16	8	24	96
	> 1,000	1	25	100

DISCUSSION

The results obtained in the present study for *Staphylococcu*s spp. agree with those presented previously by Trzcinski *et al.*[26] (2000), who described MIC_{50} and MIC_{90} variation of minocycline for *Staphylococcus aureus* between 0.25 and 8 μg/mL. Gales, Jones[3] (2000) found that the MIC variation for minocycline for *Staphylococcus aureus* was 0.06 to 8 μg/mL, and Fluit *et al.*[2] (2001), between 1 and 4 μg/mL. However, Wilson *et al.*[30] (1991) presented different results with MIC varying from 6 to 128 μg/mL. This fact could be justified by Trzcinski *et al.*[26] (2000) who found TetM proteins (known to confer resistance to all tetracyclines including minocycline) in *Staphylococcus aureus* samples, explaining the development of resistance to minocycline by these isolates.

Our data showed that the MIC for Enterobacteriaceae varied between 4 and 16 μg/mL while Gales, Jones[3] (2000) observed a variation between 0.25 and 8 μg/mL. The presentation of data by concentrations > (greater than) or < (smaller than) prevents a clear comparison of the MIC obtained in the present study with that of other results, because > 8 μg/mL may represent a great variety of results. According to Ikeda *et al.*[6] (1999) the MIC for *E. coli* was 0.78 μg/mL. Although strains of the same species demonstrate variation concerning sensitivity to minocycline, 16 μg/mL was enough to eliminate all isolates belonging to the Enterobacteriaceae family tested in the present study as well as in those mentioned in the literature[3,6].

Pseudomonas aeruginosa is one of the most resistant bacteria to antimicrobial agents and is one of the main causes of hospital infections[13]. Ichimiya *et al.*[5] (1994) found MIC values for *Pseudomonas* between 1.56 μg/mL and 3.13 μg/mL. Gales, Jones[3] (2000) observed MIC variation between 0.25 μg/mL and > 8 μg/mL. The present work's results demonstrated greater resistance to minocycline by these microorganisms, with MIC variation between 64 μg/mL and > 1,000 μg/mL. However, the incubation period in the study by Ichimiya *et al.*[5] (1994) was 16 h, which may have caused the variation in the results. Although the antibiotic therapy may eliminate microorganisms, these authors described that sub-inhibitory concentrations of minocycline may suppress the adhesion and expression of *Pseudomonas* spp. virulence factors.

Satomi[21] (1987) observed that after minocycline gel application (1,000 μg), a 130 μg/mL subgingival concentration was reached in the first hour, replaced by a 3.4 μg/mL concentration after 72 hours; such concentration would inhibit between 42.8 and 47.6% of the superinfectious microorganisms tested in this research. Fourteen days after the 1,000 μg minocycline application by a slow release device, a 340 μg/mL[29] concentration was detected, representing, in the present study, a MIC able to inhibit 97% of the superinfectious microorganisms in the first 24 hours.

In spite of minocycline being identified as an antibacterial substance, its antifungal action was observed in several studies[8,18,22,30]. The MIC of minocycline for *Candida* spp. observed by Schierholz *et al.*[22] (1999) was 256 to 512 µg/mL, after 18 h of incubation at 37°C. Wilson *et al.*[30] (1991) reported a MIC of 128 to 256 µg/mL after ten days of incubation. In the present work, *Candida* showed a MIC of 0.25 to > 1,000 µg/mL (MIC_{50} 4 µg/mL), after 24 h of incubation at 37°C.

The literature shows that minocycline is effective against the majority of periodontopathogens in low concentrations, but for superinfecting microorganisms such efficacy could not be clearly shown by the present work, because such microorganisms presented a MIC variation from 0.25 to > 1,000 µg/mL, and even the higher concentrations tested were not enough to inhibit 100% of the strains.

CONCLUSIONS

After analysis of the results, we concluded that:

a) *Staphylococcus* was the microorganism most sensitive to minocycline, presenting a MIC value of 8 µg/mL, followed by Enterobacteriaceae, with a MIC of 16 µg/mL.
b) Ninety six percent of the *Candida* spp. isolates were inhibited by concentrations up to 16 µg/mL.
c) For 88.8% of the *Pseudomonas aeruginosa* strains, the MIC was 128 µg/mL.
d) The 1,000 µg/mL concentration was not enough to inhibit 100% of the strains tested.

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REFERENCES

1. Ciancio SG, Slots J, Reynolds HS, Zambon JJ, McKenna JD. The effect of short-term administration of minocycline HCl on gingival inflammation and subgingival microflora. J Periodontol 1982;53(9):557-61.
2. Fluit AC, Verhoef J, Schmitz FJ. Frequency of isolation and antimicrobial resistance of Gram-negative and Gram-positive bacteria from patients in intensive care units of 25 European university hospitals participating in the European arm of the SENTRY antimicrobial surveillance program 1997-1998. Eur J Clin Microbiol Infect Dis 2001;20(9):617-25.
3. Gales AC, Jones RN. Antimicrobial activity and spectrum of the new glycylcycline, GAR-936 test against 1,203 recent clinical bacterial isolates. Diagn Microbiol Infect Dis 2000;36(1):19-36.
4. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994,5:78-111.
5. Ichimiya T, Yamasaki T, Nasu M. *In-vitro* effects of antimicrobial agents on *Pseudomonas aeruginosa* biofilm formation. J Antimicrob Chemoter 1994;34(3):331-41.
6. Ikeda T, Suegara N, Abe S, Yamaguchi H. Efficacy of antibacterial drugs in mice with complex infection by *Candida albicans* and *Escherichia coli*. J Antibiotics 1999;52(6):552-8.
7. Kinane DF. Periodontitis modified by systemic factors. Ann Periodontol 1999;4(1):54-63.
8. Lew MA, Beckett KM, Levin MJ. Antifungal activity of four tetracycline analogues against *Candida albicans in vitro*: Potentiation by amphotericin B. J Infect Dis 1977;136(2):263-70.
9. Loberto JCS, Martins CAPP, Santos SSF, Cortelli JR, Jorge AOC. *Staphylococcus* spp. in the oral cavity and periodontal pockets of chronic periodontitis patients. Braz J Microbiol 2004;35(1-2):64-8.
10. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. J Periodontol 1965;36:177-87.
11. Loomer PM. Microbiological diagnostic testing in the treatment of periodontal diseases. Periodontology 2000 2004,34:49-56.
12. Martins CAP, Santos SSF, Loberto JCS, Koga-Ito CY, Jorge AOC. Presença de *Candida* spp. em pacientes com periodontite crônica. Cienc Odontol Bras 2002;5(3):75-83.
13. Mimica LMJ, Martino MDV, Mimica MI, Barreto C, Pol AS, Sasagawa S *et al.* Alerta: *Pseudomonas aeruginosa* resistente a todos os antimicrobianos testados. Rev Contr de Infect Hosp 1994;1(1):12-4.
14. Nunn ME. Understanding the etiology of periodontitis: an overview of periodontal risk factors. Periodontology 2000 2003;32:11-23.
15. Oliveira EE, Silva SC, Soares AJ, Attux C, Cruvinel B, Silva MR. Toxinas killer e produção de enzimas por *Candida albicans* isoladas da mucosa bucal de pacientes com câncer. Rev Soc Bras Med Trop Brasília 1998;31(6):523-7.
16. Oplustil CP, Zoccoli CM, Tobouti NR, Sinto SI. Testes de avaliação da resistência aos antimicrobianos. *In*: Procedimentos básicos em microbiologia clínica. São Paulo: Sarvier, 2000. cap. 26. p. 165-80.
17. Pannuti CM, Lotufo RFM, Cai S, Freitas N, Ferraro AQ. Prevalência de microrganismos superinfectantes na placa

supragengival de deficientes mentais institucionalizados. Rev Pós Grad 2001;8:35-9.
18. Raad I, Darouiche R, Hachem R, Sacilowski M, Bodey GP. Antibiotics and prevention of microbial colonization of catheters. Antimicrob Agents Chemother 1995;39(11):2397-400.
19. Rams TE, Slots J. *Candida* biotypes in human adult periodontitis. J Oral Microbiol Immunol 1991;6(3):191-2.
20. Santos SSF, Loberto JCS, Martins CAP, Jorge AOC. Prevalência e sensibilidade *in vitro* de Enterobacteriaceae e *Pseudomonas* isoladas da cavidade bucal e bolsa periodontal de pacientes com periodontite crônica. Pós-Grad Rev Fac Odontol São José dos Campos 2002;5(2):74-83.
21. Satomi A. Minocycline HCl concentration in periodontal pocket after administration of LS-007. J Jap Assoc Periodontol 1987;29:937-43.
22. Schierholz JM, Pulverer G, Bach A, Wachol-Drebeck Z. *In vitro* activity of rifampin-minocyclin coating to *Candida albicans*. Crit Care Med 1999;27:1691-3.
23. Slots J, Feik D, Rams TE. Age and sex relationship of superinfecting microorganisms in periodontitis patients. Oral Microbiol Immunol 1990;5:305-8.
24. Socransky SS, Haffajee SD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol 1992;63(4 Suppl):322-31.
25. Tortora GJ, Funke BR, Case CL. Microbiologia. 6ª ed. Porto Alegre: Artmed; 2000.
26. Trzcinski K, Cooper BS, Hryniewicz W, Dowson CG. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother 2000;45:763-70.
27. van Winkelhoff AJ, Rams TE, Slots J. Systemic antibiotic therapy in periodontics. Periodontol 2000 1996;10:45-78.
28. Waterworth PM. The effect of minocycline on *Candida albicans*. J Clin Path 1974;27(4):269-72.
29. Williams RC, Paquette DW, Offenbacher S, Adams DF, Armitage GC, Bray K *et al.* Treatment of periodontitis by local administration of minocycline microspheres: a controlled trial. J Periodontol 2001;72(11):1535-44.
30. Wilson M, O'Connor B, Newman HN. Isolation and identification of bacteria from subgingival plaque, with low susceptibility to minocycline. J Periodontol 1991;28(1):71-8.

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