

***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*, *Staphylococcus* 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 Müller-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*, *Staphylococcus* 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 Müller-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 factors¹⁴. 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 diseases. 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 inflammation¹¹.

Enterobacteria, *Pseudomonas*, *Staphylococcus* and *Candida* have been collected from periodontal pockets of chronic periodontitis subjects²³ and are described as superinfectious microorganisms¹⁹. Such microorganisms present virulence factors that contribute to their action on the periodontal tissues, as leukotoxins and collagenases 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 superinfection²⁷.

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. MacCo-

nkey 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 duplicate¹⁶.

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 surface¹⁶. 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
<i>Staphylococcus</i>	0.25	1	1	4
	1	23	24	96
	8	1	25	100
<i>Pseudomonas aeruginosa</i>	64	5	5	55.56
	128	3	8	88.89
	> 1,000	1	9	100
<i>Candida</i>	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 *Staphylococcus* spp. agree with those presented previously by Trzcinski *et al.*²⁶ (2000), who described MIC₅₀ and MIC₉₀ variation of minocycline for *Staphylococcus aureus* between 0.25 and 8 µg/mL. Gales, Jones³ (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, Jones³ (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.*⁵ (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 infections¹³. Ichimiya *et al.*⁵ (1994) found MIC values for *Pseudomonas* between 1.56 µg/mL and 3.13 µg/mL. Gales, Jones³ (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.

Satomi²¹ (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/mL²⁹ 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 (MIC₅₀ 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.

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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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