The role of metronidazole on the establishment and persistence of oral candidosis

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Abstract
The aim of this study was to study the effects of metronidazole on the establishment of oral candidosis and *Candida albicans* colonization in the oral cavity of rats. Forty-eight male rats, negative for yeasts in the oral cavity, were used in the study. The rats were inoculated with a suspension of *Candida albicans* and treated with metronidazole or plain water (control group). The rats of the candidosis experimental group were sacrificed 7, 15, or 30 days after inoculation and their tongues were analyzed by light microscopy. Colonization by *Candida albicans* was evaluated 1, 2, 5 and 7 days after inoculation and progressively at 15-day intervals, with a total of 18 collections. The results demonstrated the development of candidosis on the tongue dorsum was similar between the Control and Metronidazole groups for each sacrifice period. However, the colonization results showed that yeasts were recovered in the Metronidazole group in greater numbers than in the Control group after the 37th day of the experiment (6th collection). According this, the long term metronidazole therapy favored the colonization of *C. albicans* in the oral cavity of rats.

Key Words:
Candida albicans, candidosis, metronidazole, rats

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Introduction

The long-term use of antibiotics can lead to an imbalance in the oral microbiota, which can increase yeast colonization in the oral cavity, including *Candida albicans*[1-3].

A majority of researchers have observed an exacerbation and persistence of clinical and histological lesions on the tongue dorsum of rats treated with tetracycline, after the inoculation of *C. albicans*[1,4-5]. Russell and Jones[3] studied the effect of both tetracycline-laced drinking water and a carbohydrate-rich diet (CRD) on candidal infection. The persistence of *Candida* in the mouth of each rat was examined by swabbing the tongue and mucosal surfaces throughout the experimental period. The oral yeast carriage was monitored semi-quantitatively by counting the number of oral swabs positive for *C. albicans*. Tetracycline administration resulted in the oral persistence of *C. albicans* in all rats over a period of 24 days. The prolonged carriage induced by a tetracycline-laced diet was far superior to that achieved by feeding with CRD alone. Furthermore, an increased frequency and severity of infection were seen in tetracycline-fed rats compared with the control.

The relation between oral candidosis and the use of antibiotics is explained through the imbalance in the ecosystem that the medicament can cause. Fungi present in the normal microbiota are not destroyed by antibacterial drugs, but their competitors are. Therefore, the survivors can undergo an increase in their population and become opportunistic pathogens, to the point of originating a superinfection. Some researches have shown that the oral microbiota can show alterations within the first 24 hours of antibiotic therapy[6-8].

Metronidazole, a broad spectrum antibiotic with bactericidal action, is a synthetic 5-nitroimidazole. The mechanism of metronidazole activity and other 5-nitroimidazoles, appears to depend on the ferredoxin-mediated reduction of their nitro group, with the generation of a reactive metabolite or metabolites which interact with DNA, leading to the subsequent inhibition of nucleic acid and protein synthesis[9]. It is active only against obligate anaerobic bacteria; however, showing little or no effect against most aerobic bacteria. It is also recommended for the treatment of trichomoniasis, vaginitis by *Gardenerella vaginalis*, giardiasis and amebiasis[10-11].

In the area of dentistry, recent studies have suggested the use of metronidazole, specially combined with amoxicillin, for the treatment of patients with juvenile and refractive periodontitis, where it is an effective adjunct to conventional mechanical therapy[10,12].

During the systemic use of metronidazole, it reaches high concentration in the gingival fluid. Its long-term use is commonly associated to the development of pilous tongue and stomatitis, although superinfections by *Candida* are rarely reported[2,13].

The evidence of the many works performed to study experimental candidosis in rats treated with tetracycline, makes it clear that few studies have been realized to verify the role of metronidazole in this process. Therefore, the purpose of the present work was to evaluate the effects of metronidazole in the development of oral candidosis on the tongue dorsum of rats and in the colonization of *C. albicans* in the oral cavity.

Materials and Methods

Animals: Fifty-four male rats (*Rattus norvegicus*, Albinus, Wistar) initially weighing 250g, at 12 weeks old, were used. This study was approved by the Research Ethics Committee of the School of Dentistry of São José dos Campos/UNESP, under protocol number 002/2003-PA/CEP.

Research on natural colonization by Candida spp.: All the rats were submitted to an examination to verify previous natural colonization by *Candida* spp. For this purpose, material was collected from the oral cavity with a sterile swab, which was then grown on Sabouraud dextrose agar plates (Difco, Detroit, USA), with chloramphenicol (0.1mg/mL, Itafarma, SP, Brazil) in duplicate. The plates were incubated for 48 hours at 37°C. Colonies of *Candida* were isolated and identified according to Williams and Lewis[14]: germinative tube formation, production of chlamydoconidium and hyphae in microculture, and the fermentation and assimilation of carbohydrates.

Out of the fifty-four rats analyzed, six animals presented natural colonization by yeasts in the oral cavity and were excluded from the study. Only forty-eight rats, negative for *Candida* spp., were used in the subsequent experiments, thirty-six for the experimental candidosis study and twelve for colonization by *C. albicans* (Table 1).

Table 1 - Number of rats used for experimental candidosis and colonization by *C. albicans*

	Groups	n	Groups	n	Total
Candidosis Oral	Control 7 days	6	Metronidazole 7 days	6	12
	Control 15 days	6	Metronidazole 15 days	6	12
	Control 30 days	6	Metronidazole 30 days	6	12
Colonization	Control	6	Metronidazole	6	12
Total		24		24	48

Inoculation: A suspension of *C. albicans*, containing 5 x 10^8 viable cells/mL was prepared according to Reed et al.[15] (1990). A strain of *C. albicans* originating from the Microbiology Laboratory of the School of Dentistry of São José dos Campos/UNESP was used, isolated from a patient who presented stomatitis due to prosthesis use. This strain presented high lethality for mice and produces proteinase, hyaluronidase, chondroitin sulfatase and phosphatase[16]. For the inoculation, the rats were sedated with a Rompun (Bayer, SP, Brazil) and Dopalen (Vetbrands, SP, Brazil) solution in the proportion of 1/0.5mL, at a dose of 0.05mL/100g of body weight, intramuscularly. The suspension of *C. albicans* (0.2mL) was dripped into the rat's mouths with the aid of an insulin syringe. The material was uniformly spread on the mucosa of the tongue dorsum with a sterile swab soaked in the suspension. This procedure was repeated for three consecutive days.

Treatment with metronidazole: The metronidazole (Aventis, SP, Brazil) was administrated at a dose of 22.5mg/Kg/day[11] in the rat's drinking water.

The rats of the experimental candidosis began treatment with metronidazole on the first day of *C. albicans* inoculation, and were treated for 7, 15 or 30 days. The rats used for the study of *C. albicans* colonization began treatment with metronidazole four days after the first inoculation of the yeast, and were treated for a total of one hundred fifty seven days.

Experimental candidosis: After 7, 15, or 30 days of treatment with metronidazole, the rats were sacrificed and their tongues were removed and submitted for macroscopic analysis using a stereoscopic microscope (Zeiss, Germany).

For the histopathological examine, the tongues were fixed in 10% formalin for 24 hours and sectioned in two parts in direction of the sagittal. After being paraffinated, a series of cuts of 7µm thickness in microtome were obtained, which were stained with Hematoxylin Eosin (HE) and Periodic Acid Schiff (PAS).

To determine the degree of *C. albicans* colonization, a semiquantitative analysis was performed based on the methodology proposed by Junqueira et al.[17]. Twenty-eight histological fields of the tongue dorsum from each cut were analyzed, in anteroposterior direction , at 400X magnification. Each histological field was attributed a score (score 0: absence of colonization; score 1: one to five hyphae; score 2: six to fifteen hyphae; score 3: sixteen to fifty hyphae; and score 4: over fifty hyphae). Thus, 28 scores were attributed to each histological cut. From each rat, two randomly chosen histological cuts were analyzed, totaling fifty-six scores per rat. For the realization of statistical analysis, the mean between the fifty-six scores was determined.

Colonization by C. albicans: Oral samples were collected on days 1, 2, 5, and 7 after the last inoculation of *C. albicans* and after that, every fifteen days until eighteen samples were collected.

The oral samples were collected from the oral cavity by using a swab for 60 seconds, immersing it in a tube with 0.95mL sterile physiologic solution and agitating for 60 seconds (one hundred times diluted) according to Jorge et al.[4], Freire Garabal et al.[18] and Junqueira et al.[19]. 100µL samples from this suspension were dropped in duplicate, after serial four fold dilution, on Sabouraud agar plates containing chloramphenicol (0.1mg/mL, Itafarma, SP, Brazil). The plates were incubated for 48 hours at 37°C. After the incubation period, the colony forming units per milliliter of saliva (cfu/mL) were calculated.

Statistical analysis: The results were submitted to the Mann-Whitney test and Student´s *t* test at a significance level of 5%.

Results

Research on natural colonization by Candida spp.: Out of the fifty-four rats analyzed at the beginning of the study, six (11.1%) presented *Candida* spp. in their oral cavity and were excluded. In relation to their identification, three samples were *C. guilliermondii* (50%); two were *C. tropicalis* (33.3%) and one was *C. lusitaniae* (16.6%).

Experimental Candidosis: The macroscopic analysis showed that 21 (58.33%) out of the 36 animals in the experimental candidosis study, presented clinically visible lesions on the tongue dorsum (Table 2).

Table 2 – Number of rats of the Control and Metronidazole groups with clinical lesions on the tongue dorsum in the periods of observation

Periods of observation	Control (n= 6)		Metronidazole (n = 6)	
7 days	3	50%	5	83.3%
15 days	3	50%	4	66.6%
30 days	3	50%	3	50%

The clinical lesions were erythematous and nonpapillated (Figure 1), well delimited and situated in the simple conic papillae, near the giant papillae. The true papillae were the least affected. The following characteristics were observed in the microscopic analysis:

Control Group: out of the 18 rats analyzed, 9 (50%) presented lesions constituted by yeasts and hyphae inside the corneal layer, predominantly in the region of simple conic papillae, near the giant papillae (Figure 2). The epithelium presented acanthosis and discrete hydrophic degeneration. In the subjacent connective tissue congested vases were observed, as well as the presence of a local discrete inflammatory infiltrate. In only one of the histological cuts of this group, an extensive candidosis lesion and the formation of intraepithelial microabscesses were observed.
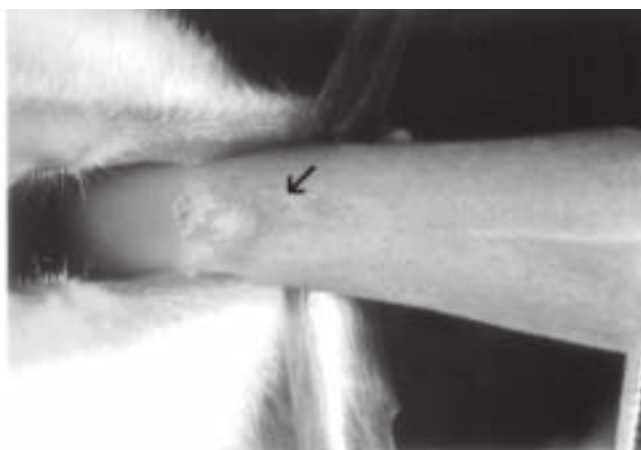
Fig. 1 - *Metronidazole group, sacrifice at 15 days: non-papillary lesion in the region of the simple conic and giant papillae of the tongue (→).*
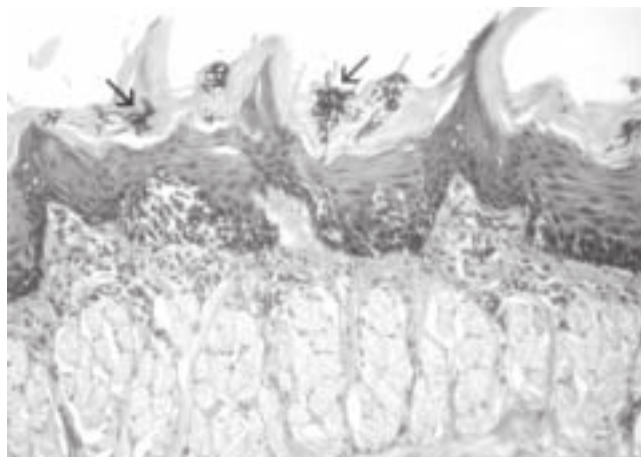

Fig. 2 - *Control group, sacrifice at 7 days: invasion of hyphae and yeasts in the corneal layer (→) of the epithelium in the simple conic and giant papillae regions.*
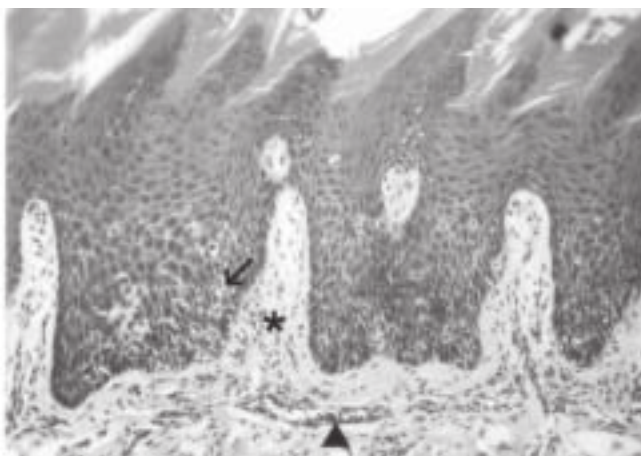

Fig. 3 - *Metronidazole group, sacrifice at 15 days: region of the giant papillae presenting spongiosis, acanthosis of the prickle layer of the epithelium (→), inflammatory infiltrate (*) and congested vases (▲) in the underlying connective tissue.*

Fig. 4 - *Metronidazole group, sacrifice at 30 days: region between the simple conic papillae and giant papillae; observe the formation of intra-epithelial microabscess (→), basal layer acanthosis (▲) and intense inflammatory infiltrate (*) in the underlying connective tissue.*

Metronidazole Group: out of the 18 rats studied, 13 (71%) presented foci of hyphae and yeasts invasion, most being well situated affecting some simple and giant conic papillae. The epithelium displayed acanthosis of the prickle layer, exocytosis, spongiosis and epithelial disorganization. The subjacent connective tissue showed many congested vases and discrete inflammatory infiltrate (Figure 3). Some cuts of the 30-day period presented hyperparakeratosis, hydropic degeneration, epithelium hyperplasia, loss of the basal cell layer stratification, in one of them papillary destruction with the formation of intraepithelial microabscesses were observed (Figure 4).

In relation to the semiquantitative analysis of epithelium colonization by *Candida*, there was no statistically significant difference between the Control and Metronidazole groups (Table 3).

Table 3 - Mean scores obtained in the semiquantitative analysis of the colonization of the oral epithelium by *C. albicans*

Rats	7 days		15 days		30 days	
	Control	Metro	Control	Metro	Control	Metro
1	0.00	0.07	0.23	0.17	0.00	0.01
2	0.00	0.00	0.08	0.03	0.00	0.00
3	0.00	0.17	0.00	0.00	0.16	0.05
4	0.00	0.28	0.00	0.07	0.17	0.01
5	0.25	0.00	0.10	0.07	0.00	0.08
6	0.10	0.01	0.03	0.37	0.17	0.00
Median	0.00	0.04	0.05	0.07	0.08	0.01

*Mann-Whitney's test: 7 days p = 0.44; 15 days p = 0.74; 30 days p = 0.67 Metro: metronidazole*

Colonization by C. albicans: Yeasts were recovered in all rats of the Control group in the periods of 1, 5, 7, 22 and 37 days after inoculation. After that, the number of rats positive for yeasts decreased to 4 (66.66%) rats after 52 days and to 2 (33.33%) rats after 67 days. On the 142nd and 157th days, *C. albicans* was no observed.

In the metronidazole group, the *C. albicans* colonization occurred in all rats in the periods of 1, 5, 7, 22, 37 and 52 days after inoculation. After the 67th day, the number of rats positive for *Candida* dropped to 3 (50%), remaining that way up to the 172nd day. On the 187th day of *C. albicans* colonization, another rat was negative, and on the 202nd day *C. albicans* was recovered from only one rat; this result was repeated up to the 217th day of recovery, when it was sacrificed. The candidosis observed in this rat was nonpapillated and extended through the simple conic papillae, giant papillae and true papillae, very similar to median rhomboid glossitis (Figure 5).

The logarithm of the cfu/mL of *C. albicans* colonization from the oral cavity of the rats up to the 22nd day was similar between the Control and Metronidazole groups.

Fig. 5 - *Metronidazole group of the colonization by* C. albicans*, sacrifice 217 days: non-papillated lesion (→), extended trough the simple conic papillae, giant papillae and true papillae of the tongue.*
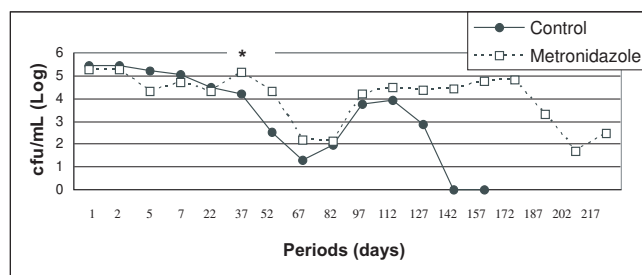
* statistically significant difference (p= 0.01) Student's t *test*

Graph 1 - Means of cfu/mL (Log) of *C. albicans* observed in samples from the oral cavity of the Control group and Metronidazole group in the all periods studied.

After the 37th day, the mean log of cfu/ml was higher in the Metronidazole group then in the Control group (Graph 1).

Discussion

Out of the fifty-four animals examined, six rats (11.1%) were positive for the *Candida* spp. in the oral cavity. This result was inferior to those reported by Jorge et al.[4] (17%) and Totti et al.[20] (42%). On the other hand, this result is similar to that reported by Junqueira[17], who found 9.48% of rats colonized by *Candida* at the beginning of their work.

These isolates were identified as *C. guilliermondii* (50%), *C. tropicalis* (33.3%) and *C. lusitaniae* (16.6%). These data corroborated with the results of Junqueira[17], who found 44.4% of *C. guillermondii*, 5.55% of *C. tropicalis* and 38.8% of *C. lusitaniae*. These species were also found by Jorge et al.[21] in the following percentages 2.5%, 8.13%, and 1.25%, respectively.

In relation to *C. albicans* colonization, out of the six control rats, 100% were positive up to the 37th day of observation. These results are superior to those found by Jorge et al.[4], who recovered *Candida* in only 50% of the control rats 35 days after inoculation and to the results of Junqueira et al.[19], who observed colonization by yeasts in 80% of the control animals up to the 37th day of recovery. These data demonstrate that the inoculations of *C. albicans* realized in the current work were able to induce a strong *Candida* infection in the oral cavity of rats.

Therapy and prophylaxis with broad spectrum antibiotics are known to be major risk factors for the development of fungal infections[22]. Oral administration of antibiotics may induce alterations in the normal oropharyngeal, intestinal and vaginal microbiota. These alterations depend on the dosage and schedule, the spectrum of activity and the pharmacokinetic properties of the antimicrobial agent used[23]. Up until the present, numerous studies have been developed to investigate the effect of tetracycline in oral candidosis in rats, demonstrating that this antibiotic predisposes the host to *C. albicans* colonization and the development of persistent infections[1,3-4]. However, many other antibacterial medications are prescribed in clinical odontology and their potential effects on the establishment of candidosis need to be studied.

Several studies have documented that drugs active against anaerobic microbiota such as vancomycin, clindamycin or metronidazole are significantly more associated with fungal superinfections than are other antimicrobials[22]. For these reasons, this work studied the effects of metronidazole in the development of candidosis and *C. albicans* colonization in the oral cavity of rats.

In the experimental candidosis study the control rats developed candidosis lesions on the tongue dorsum similar to the treated group with metronidazole in all periods of

observation (7, 15, and 30 days). In the study of *C. albicans* colonization, the number of yeasts recovered from the oral cavity of the rats up to the 22nd day (5th collection) was similar between the control and metronidazole groups. However, after the 37th day (6th collection), the number of cfu/ml was higher in the metronidazole group than in the control group. These results show that the metronidazole influenced the oral colonization by C*andida* when administered for long periods (over 37 days). However, Pultz et al.[24] verified an increase in intestinal colonization by *Candida glabrata* in mice after subcutaneous administration of metronidazole for 8 days. The differences between the current results and the results of Pultz et al.[24] are probably due to a combination of factors; the way metronidazole was administered, the species of *Candida* studied, the colonization site in the organism and experimental animal model.

Besides Pultz et al. (2005), other authors have also studied the influence of metronidazole on *C. albicans* colonization of the gastrointestinal tract of mice[25-26]. Samonis et al.[25] reported that mice fed on a diet containing *C. albicans* (concentration of *C. albicans* in the food was 7.48 log10 cfu/g) and treated with antibiotics for ten days presented substantially higher *Candida* counts in their stools than control mice fed *C. albicans* and treated with saline. The concentrations of *Candida* in the stools of mice treated with tetracycline were much higher when compared to those of mice treated with metronidazole and norfloxacin. However, Kinsman and Pitblado[26] verified that the treatment with metronidazole, gentamicin and vancomycin for three days did not affect the gastrointestinal *Candida* levels. Only ceftizoxime, augmentin and cefoperazone promoted *Candida* overgrowth.

The lower predisposition of metronidazole to the development of candidosis in relation to other antimicrobials could be due to the slight inhibition of anaerobic microbiota induced by this medication. This can be explained by the fact that the *in vivo* susceptibility of anaerobic microorganisms to metronidazole is considerably less than the *in vitro* susceptibility, and that high doses of the drug are needed to suppress oropharyngeal and intestinal microbiota[23]. The effects of metronidazole on candidosis have already been studied in humans[23,27]. Maraki et al.[23] demonstrated that treatment with metronidazole for 10 days increased human gastrointestinal, oropharyngeal and vaginal colonization by the *Candida* species, though not significantly. Trenschel et al.[27] found that the combined use of ciprofloxacin, metronidazole and fluconazole, as an antifungal prophylaxis, increased intestinal yeast colonization when compared to ciprofloxacin and fluconazole alone in patients undergoing allogenic bone marrow transplantation. Although an evaluation of experimental candidosis for longer periods was not performed, a higher incidence of yeasts in the oral cavity of rats in the metronidazole group was observed in the study of *C. albicans* colonization after the 37th day (6th collection), indicating that the long-term use of metronidazole favored the *C. albicans* colonization in the oral cavity of treated rats.

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