Experimental candidosis on rat's tongue

Candidose experimental em língua de rato

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Abstract

The purpose of this study was to evaluate the development of candidosis in rat's tongue after intraepithelial injections of Candida albicans. Fifty rats (Rattus norvegicus, Albinus, Wistar), originally negative for the Candida spp. received ten intraepithelial injections of C. albicans on the dorsal tongue. Groups of five animals were killed after 1, 2, 4, 6, 8, and 12 hours and 1, 2, 7, and 15 days after the injection. The rat's tongues were surgically removed and then macroscopic and microscopic analyses were performed. The development of candidosis lesions was observed in all the rats studied. One hour after the injection, the development of germ tubes from the yeast cells could be observed. After 4 hours, Candida spp. pseudohyphae penetrated the epithelial cells with the formation of microabscesses. After 24 to 48 hours, the epithelial areas with pseudohyphae invasion presented desquamation, hyperplasia of the basal layer and discrete inflammation of the connective tissue. After seven days, few pseudohyphae could be observed. The epithelium presented acanthosis, hyperkeratosis and loss of filiform papillae. After 15 days, neither yeasts nor pseudohyphae were found. In some areas, the epithelium presented acanthosis and loss of filiform papillae. It can be concluded that the intraepithelial injection of Candida albicans on the dorsal rat tongue caused candidosis lesions in all the animals studied. C. albicans was present until seven days after the injections.

Uniterms

Candida albicans; candida; rats; tongue

Introduction

Candida albicans is the most common and potentially invasive fungus of the human oral cavity. *C. albicans* colonization can result in the development of a saprophytic association with the tissues, cause superficial localized lesions of the mucosa or systemic infections[12, 23]. The interest on the study of this opportunistic pathogen has been increased in recent years, mainly due to the high incidence of this infection in AIDS patients[18].

Several animals, including rats, have been largely used to study the colonization and pathogenicity of the *Candida albicans* in the oral cavity[4, 7, 9, 10, 14, 19, 21, 22]. The first experimental model developed with the purpose of examining *Candida* colonization on rat's tongue concluded that 55% of the animals studied presented infection by this fungus

after the inoculation. Moreover, these studies showed that the mycelium forms did not penetrate underneath the stratum corneum and were associated with the loss of lingual papillae, presence of hyperkeratosis in the epithelium, accumulation of mononuclear cells and alterations on the most superficial muscle layer[10].

The most frequent places for the infection by *Candida albicans* in the oral cavity of rats are the marginal gum, interpapillar areas of the dorsal tongue and mucosa of the vestibular and lingual sulcus[8]. The tongue of these animals is easily colonized by *Candida*, demonstrating conditions such as median rhomboid glossitis and atrophic candidosis[1]. Some authors believe that the low cellular cohesion and the abundant intracellular spaces between the keratinized cells of the dorsal tongue facilitate the penetration and colonization of the hyphae[15].

During the development of chronic candidosis in rat's tongues, clinically evident lesions and inflammatory alterations of the connective tissue were observed, respectively after two and four weeks of the inoculation of *Candida albicans* in the oral cavity[6].

After an extended inoculation of *C. albicans* in rat's mouth, yeasts and pseudohyphae could be observed only in the keratin layer of the tongue. In the area of the conic and true lingual papillae the presence of *C. albicans* did not cause significant alterations of the epithelium and connective tissue. However, the candidosis in the region of the giant papillae caused loss of lingual papillae, hyperkeratosis, basal layer hyperplasia and an inflammatory cell infiltrate, consisting mainly of neutrophils, in the epithelium and subjacent connective tissue[11].

In all the experimental models mentioned, the inoculation of *C. albicans* was performed by the introduction of a yeast suspension in the oral cavity of the animals. The purpose of this study was to observe the development of candidosis induced by intraepithelial injections of *C. albicans* in the dorsal rat tongues.

Materials and Methods

This study was performed according to the Ethical Principles for Animal Research, avowed by the Brazilian School of Animal Research (COBEA) and approved by the Research Ethics Committee of the São José dos Campos School of Dentistry/UNESP.

Fifty rats (*Rattus norvegicus,* Albinus, Wistar), originally negative for the presence of *Candida* spp. in the oral cavity and with an initial weight of 170-200 grams, were studied. The *C. albicans* strain isolated from a patient with chronic oral candidosis was used for the suspension preparation. *C. albicans* sample was cultured on Saboraud agar for 48 hours at 37º C.

The growth was suspended in 5ml sterile saline solution (NaCl 0.85%) and centrifuged at 1300 X*g* for 10 minutes, disregarding the supernatant. This procedure was repeated once more and the sediment suspended again in 5mL sterile saline solution (NaCl 0.85%). The count of number of viable cells from the suspension was obtained using a Neubauer chamber after previous dyeing with 0.05% methylene blue[17]. The suspension was standardized in order to obtain 5 x 10^8 viable cells/mL.

The animals were anesthetized with Rompun solution (Bayer, São Paulo, SP, Brazil) and Francotar (Virbac, Roseira, SP, Brazil) intramuscularly in a 1/0.5mL proportion - dose of 0.1mL/100 of body weight. Ten intraepithelial injections containing 25 _L of the *C.* a*lbicans* suspension were made after the anesthesia by means of a 1mL sterile syringe and 13 X 3.8 sterile needle. Eight injections were made in the central region of the dorsal tongue immediately before the giant papilla and two additional injections were made in the intermolar tubercle region.

Groups of 5 rats were killed after 1, 2, 4, 6, 8, and 12 hours and 1, 2, 7, and 15 days after the injections. The tongues were extracted surgically, observed and analyzed macroscopically, using a stereoscopic loupe (Carl Zeiss, Jena, Germany) with magnifications of 10 and 15X.

For the microscopic analysis, the tongue was fixed in 10% formaldehyde for 24 hours. Then, the pieces were sectioned in the sagittal form, divided into four parts and included in paraffin. From each animal, sixteen 7µm cuts of the tongue were obtained and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Gomori-Grocott.

Results

Macroscopic aspects

After an hour of the intraepithelial injections in the dorsal tongue, only the inoculation points

were visible, as discrete lines, slightly projecting and whitish. After two hours, the injection places were almost imperceptible.

The inoculated areas became visible again after 6 hours, as whitish regions, forming projections in the epithelium, generally as straight lines, approximately 5 to 7mm long. After 8 hours, the macroscopic aspect was similar to the one observed after 6 hours .

After 12 hours, the dorsal tongue lesions were more evident, with reddish and shinny central depression, surrounded by elevated and whitish borders (Figure 1). During the first day, the dorsal tongue presented small areas with loss of filiform papillae and erymathous aspect in the injections places. After seven days, these areas of papillae loss aggregated, forming larger erymathous areas (Figure 2). After 15 days, the tongue mucosa was almost normal, presenting only small areas of papillae loss.

Microscopic aspects

1 hour:

The intraepithelial injection of *C. albicans* disrupted the epithelium in the region of the spinous layer, forming a split inside it. The epithelium did not present morphological changes, besides the ones from the mechanical trauma caused by the needle, with the presence of fibrin, erythrocytes, and a few polymorphonuclear leukocytes.

C. albicans cells were in the form of yeasts adhered to the epithelial cells or isolated in the interior of the fissure (Figure 3). Germinative tubes formation could be observed.

2 hours:

Yeasts, pseudohyphae and polymorphonuclear cells were observed inside the split caused by the injection. Many yeasts showed larger germinative tubes than the group killed after 1 hour of the inoculation (Figure 4). A small number of pseudohyphae invading the keratin could be observed. Yeasts could also be observed along the epithelial surface of the dorsal tongue.

4 hours:

Great number of yeasts, pseudohyphae and polymorphonuclear leukocytes could be observed in the fissure after 4 hours. The pseudohyphae showed a progressive development and invaded the keratin layer (Figure 5).

Intense infiltration of polymorphonuclear cells in the basal layer with tissue disorganization and formation of intraepithelial microabscesses could be observed in different sites. The connective tissue underneath these regions presented inflammatory infiltrate with the prevalence of polymorphonuclear leukocytes.

6 hours:

In some areas, yeasts adhered to the epithelial cells, with the pseudohyphae penetrating the keratin and the most superficial cells of the spinous layer, but without the formation of microabscesses could be observed. In other sites, an intense infiltrate of polymorphonuclear cells with degenerated leukocytes and rests of epithelial cells, characterizing microabscesses could be seen (Figure 6). The subepithelial connective tissue presented sparse infiltrate of polymorphonuclear and few mononuclear cells.

8 hours:

Intraepithelial abscesses containing yeasts, many pseudohyphae, epithelial rests and great quantity of polymorphonuclear cells could be observed (Figure 7). The surrounding regions to the abscess showed pseudohyphae penetration in the keratin or between the epithelial cells of the spinous layer.

In some sites the basal layer of the epithelium presented integrity, but in others, it was disorganized with the presence of leukocyte infiltrate. The adjacent connective tissue was moderately infiltrated by polymorphonuclear and some mononuclear cells.

12 hours:

Intraepithelial abscesses with greater quantity of yeasts and pseudohyphae than that observed at 8 hours were seen. The pseudohyphae were big and more numerous than the yeasts. Some microabscesses were projecting from the epithelial surface, with the keratin layer detached in the epithelium surface (Figure 8).

In the majority of the preparations, the epithelium basal layer remained intact and in the subepithelial connective tissue there was discrete infiltrate of polymorphonuclear and some mononuclear cells.

1 day:

Lower quantity of yeasts and pseudohyphae in the keratin in relation to the observed after 12 hours

was seen. The pseudohyphae were very long and the epithelium presented desquamation regions. Fragments of keratin with agglomerated pseudohyphae detaching from the surface could be seen (Figure 9). The basal layer of these areas presented hyperplasia and the connective tissue presented discrete inflammatory infiltrate, with polymorphonuclear and some mononuclear cells.

2 days:

Yeasts were rarely found and there were some pseudohyphae in the keratin layer. Moreover, some desquamation areas of the keratin, with pseudohyphae and yeasts, acanthosis, loss of filiform papillae, hyperkeratosis, and hyperplasia of the basal layer were observed (Figure 10). The connective tissue showed moderate mononuclear inflammatory infiltrate.

7 days:

In some animals, the presence of a few pseudohyphae and yeasts in the keratin was still observed. The epithelium showed loss of filiform papillae, hyperkeratosis, acanthosis, and in some cases, neutrophils in the stratum corneum. In the subepithelial connective tissue, an intense mononuclear inflammatory infiltrate was predominant (Figure 11).

15 days:

No yeasts or pseudohyphae were found in this period. In some areas, the epithelium presented acanthosis, hyperplasia and loss of filiform papillae (Figure 12). The subepithelial connective tissue showed discrete mononuclear inflammatory infiltrate.

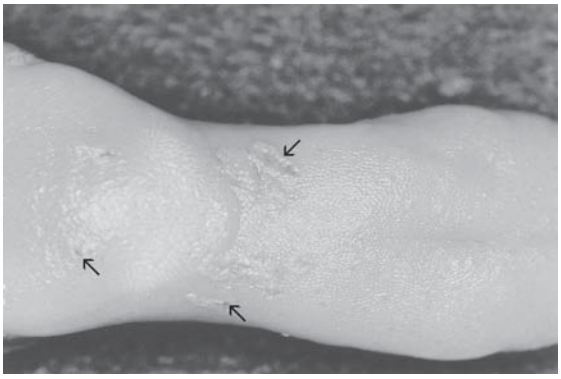

FIGURE 1 - Dorsal rat tongue, 12 hours after the intraepithelial injection of *C. albicans*. The lesions are evident with central depression and elevated borders (➔). Magnification: X5.

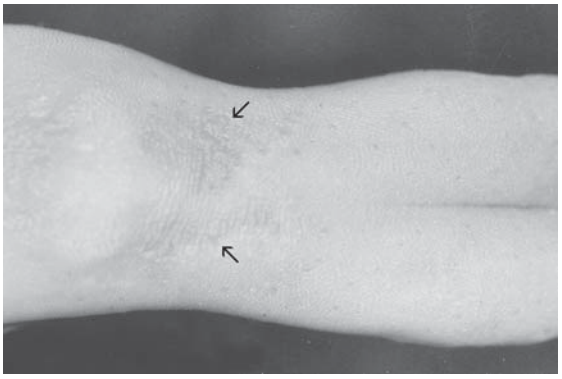

FIGURE 2 - Dorsal rat tongue, 7 days after the intraepithelial injection of *C. albicans*. Areas with loss of filiform papillae can be observed (➔). Magnification: X5.

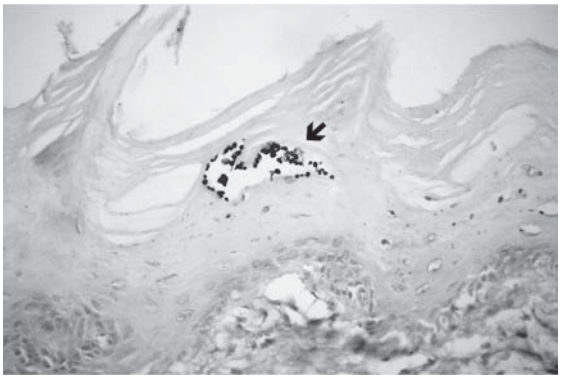

FIGURE 3 - Sagittal cut of the dorsal rat tongue, 1 hour after the intraepithelial injection of *C. albicans*. Yeasts inside the split (➔) produced by the epithelial injection can be observed. PAS, X400.

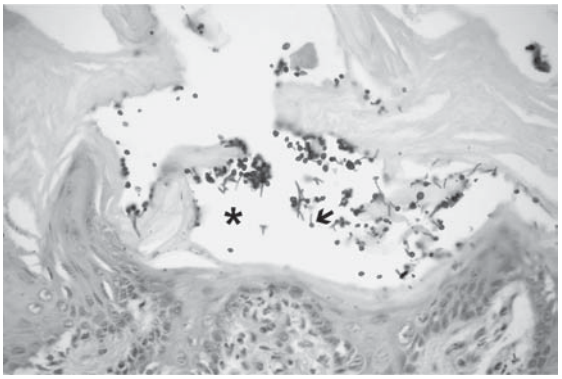

FIGURE 4 - Sagittal cut of the dorsal rat tongue, 2 hours after the intraepithelial injection of *C. albicans*. In the place of the injection (*) isolated yeasts or adhered to the epithelial cells can be observed. Some yeasts showed formation of germinative tube (➔). PAS, X400.

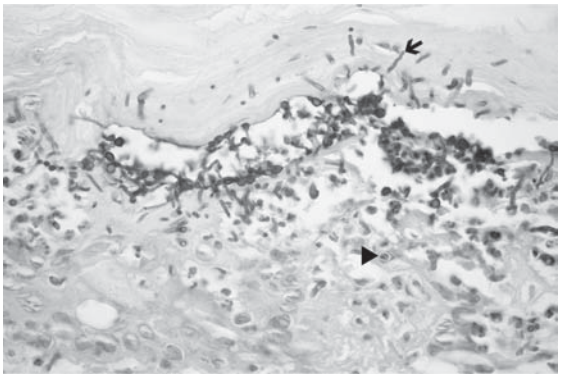

FIGURE 5 - Sagittal cut of the dorsal rat tongue, 4 hours after the intraepithelial injection of *C. albicans*. The split caused by the injection contains yeasts and pseudohyphae (➔), which had penetrated the epithelium. The presence of several polymorphonuclear leukocytes can also be observed (▸). PAS, X400.

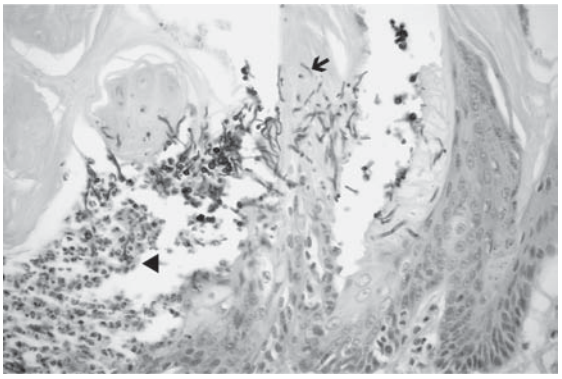

FIGURE 6 - Sagittal cut of the dorsal rat tongue, 6 hours after the intraepithelial injection of *C. albicans*. Yeasts, pseudohyphae (➔), and accumulation polymorphonuclear leucocytes forming microabscesses are observed. (▸). PAS, X400.

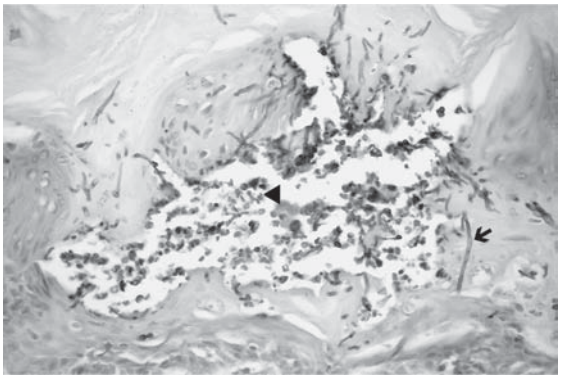

FIGURE 7 - Sagittal cut of the dorsal rat tongue, 8 hours after the intraepithelial injection of *C. albicans*. Microabcesses (▲) and pseudohyphae (➔), which penetrate the keratin, are present in the split caused by the injection. PAS, X400.

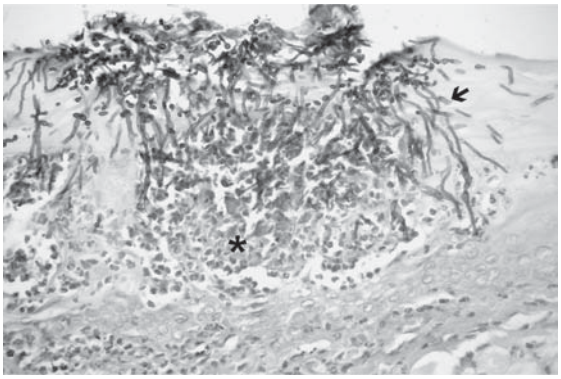

FIGURE 8 - Sagittal cut of the dorsal rat tongue, 12 hours after the intraepithelial injection of *C. albicans*. It can be observed intraepithelial microabscesses (*) underneath the intense proliferation of pseudohyphae (➔). PAS, X400.

FIGURE 9 - Sagittal cut of the dorsal rat tongue, 1 day after the intraepithelial injection of *C. albicans*. It can be observed proliferation of pseudohyphae (➔) in the keratin and intraepithelial microabscesses (*). PAS, X200.

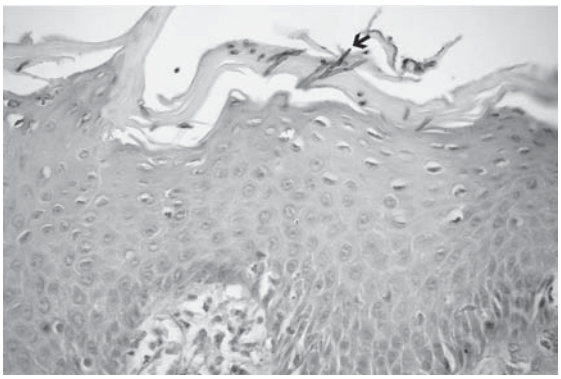

FIGURE 10 - Sagittal cut of the dorsal tongue, 2 days after intraepithelial injection of *C. albicans*. Desquamation of the keratin, with pseudohyphae and yeasts (➔) can be observed. The epithelium presents acanthosis and hyperplasia of the basal layer. PAS, X400.

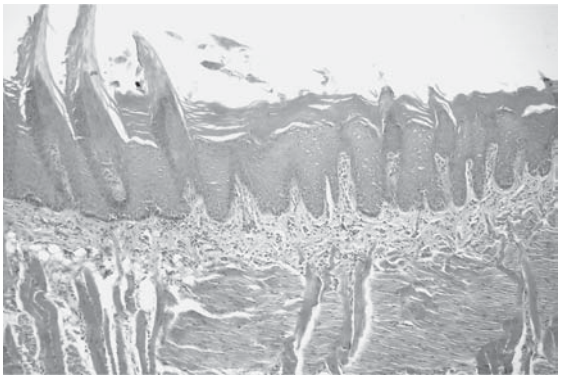

FIGURE 11 - Sagittal cut of the dorsal rat tongue, 7 days after the intraepithelial injection of *C. albicans*. The epithelium presents loss of filiform papillae, hyperkeratosis, acanthosis, and hyperplasia of the basal layer. H&E, X100.

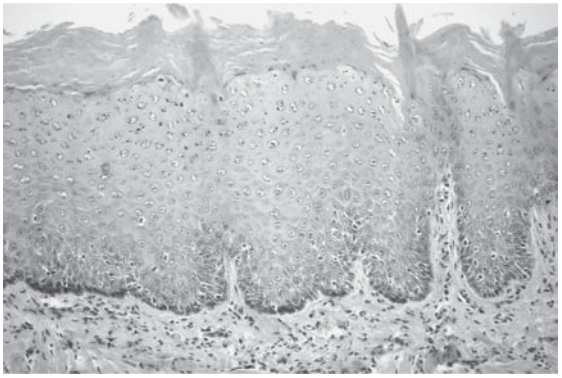

FIGURE 12 - Sagittal cut of the dorsal rat tongue, 15 days after the inoculation of *C. albicans*. It can be observed the region with loss of filiform papillae and the epithelium presents acanthosis, and hyperplasia of the basal layer, but yeasts and pseudohyphae are not visible anymore. H&E, X100.

Discussion

In this study, the intraepithelial injection of a suspension of *C. albicans* cells in the dorsal rat tongue induced macroscopic lesions and microscopic alterations in the epithelium as well as in the connective tissue subjacent to the inoculated area. These experiment findings are in accordance to previous studies that proved that the oral inoculation of *C. albicans* is capable of producing lesions in the mucosa of rats[5, 6, 11, 12, 17]. These lesions were characterized by tissue destruction that, generally, were initiated in the giant papillae region and gradually involved bigger areas, also destroying the filiform papillae and the conic papillae[2,3,5].

The intraepithelial injection of *C. albicans* produced, macroscopically, after 6 hours, whitish regions with projections in the epithelium, which corresponded microscopically to the formation of microabscesses with pseudohyphae and yeasts in the epithelium. After 12 hours, the macroscopic lesions were more evident and corresponded to the extensive formation of pseudohyphae and intraepithelial microabscesses, with desquamation of the keratin in some areas. After 24 hours, areas with loss of filiform papillae of smooth and erythematous aspect were visible in the places of the injection, probably due to the fast regeneration of the epithelium. After seven days, the erythematous area was visible macroscopically, corresponding to the papillae loss, which reduced after 15 days of the injection, and the aspect of the tongue was similar to normal.

Allen et al.[6] introduced *C. albicans* suspension into mouths of rats by instillation with a syringe and Freire-Garabal et al.[9] (1999) inoculated the same suspension through the application of a swab in the tongue of these animals. These two authors observed evident clinical lesions only after 15 days of the inoculation by *C. albicans*, however in our work the clinical lesions were visible after 6 hours of the intraepithelial injection. Our results disagree with these works, probably because we introduced *Candida* suspension inside the epithelium and these authors just inoculated *C. albicans* into oral cavity of rats.

The microscopic analysis showed that 1 hour after the *C. albicans* injection there was formation of germinative tubes in approximately 25% of the yeasts, which was increased to more than 50% after 2 hours of the inoculation. MacKenzie[13] (1964), introduced *C. albicans* subcutaneously into mice. One hour after the injection, 60% of the yeasts presented germinative tube, and this number was increased by 90% after two hours. The difference in the quantity of germinative tubes formed by the

yeasts in the present study must be due to the sample of *C. albicans* used, since different samples produce different quantities of these structures *in vitro*[13, 20]. The place of the injection must have interfered also, since MacKenzie[13] injected it in the subcutaneous of mice skin, while we used intraepithelial injections in rat tongues. However, the size and form of the germinative tubes were as described by MacKenzie[13](1964).

Four hours after the intraepithelial injection of *C. albicans*, the results revealed the formation of pseudohyphae and presence of polymorphonuclear cells, characterizing acute inflammation. MacKenzie[13] (1964), obtained similar data on mice skin. The pseudohyphae present on rat tongues invaded the keratin from the yeasts filament near the epithelial cells and the inflammatory infiltrate was found in the basal layer of the epithelium and connective tissue. The penetration of the pseudohyphae in the epithelium layer seems to be related to the initial stimulus to the inflammatory response, thus justifying the histological findings described above[16].

After 6 hours, the pseudohyphae penetrated not only the stratum corneum, but also reached more superficial cells of the spinous layer causing the formation of microabscesses in some regions that were characterized by degenerated leucocytes and rests of epithelial cells. The subjacent connective tissue presented acute inflammatory infiltrate with presence of few mononuclear cells. After 8 hours, the quantity of polymorphonuclear and mononuclear cells in the interior of the connective tissue was equivalent. In some cases, these cells were mixed with the cells of the epithelium basal layer in areas that did not preserve its integrity. The presence and absence of microabscesses was observed occasionally in the same animal. We suppose this fact can be because of the depth of the epithelial injection, which explains the variation in stimulation of the leucocytes response.

The quantity of yeasts and pseudohyphae was the higher after 12 hours of the injection in relation to the other periods studied. However, Lacasse et al.[12] (1990) observed the most colonization by *C. albicans* in the oral mucosa of rats only after two days of the topic application of this fungus.

After one day, the basal layer of the epithelium correspondent to the areas with pseudohyphae and desquamation of keratin presented hyperplasia and after two days showed also acanthosis, hyperkeratosis, and loss of filiform papillae. Allen et al.[2] observed these epithelial alterations only after three weeks of the inoculation of *C. albicans* in the oral cavity of rats.

In this work, the intraepithelial injections of *C. albicans* on the dorsal rat tongue caused a quick candidosis development. In previous works, a single oral inoculation of *Candida* or an inoculation by the topical application caused slower development of candidosis, probably because the microorganisms had to get into the epithelium to cause candidosis lesions.

One of the characteristics of experimental candidosis in animals is the increase in the mitotic epithelial activity, which leads to the proliferation of the epithelium and the fast desquamation of the oral mucosa[18]. According to Reed et al.[17] (1990), *C. albicans* can produce a broad variety of enzymes, such as proteinases, phospholipases and some hydrolytic enzymes that possibly produce destructive defects in the epithelial structures and components.

In the results of this work, after the intraepithelial injection in the rat tongues, *C. albicans* showed ability to invade the keratin and basal layer, producing the destruction of the latter in some sites. On the other hand, *C. albicans* did not invade the connective tissue possibly due to the host's immunological reaction. This reaction was very fast, with intraepithelial microabscesses formation only 4 hours after the inoculation, preventing the penetration of *C. albicans* pseudohyphae in the connective tissue.

One can conclude that the intraepithelial injection of *C. albicans* in the rat's dorsal tongue was able to produce candidosis lesions in all the studied animals. One hour after the injection the formation of germinative tubes could be observed. After 4 hours, pseudohyphae penetrated the epithelial cells with the formation of microabscesses. After 24 and 48 hours, the areas of the epithelium that presented pseudohyphae, showed desquamation, hyperplasia of the basal layer and discrete inflammation of the connective tissue. After seven days, few pseudohyphae could be observed and the epithelium presented acanthosis, hyperkeratosis and loss of filiform papillae. After 15 days, no yeasts and pseudohyphae were found. In some areas, the epithelium presented acanthosis and loss of filiform papillae.

Resumo

O objetivo desse estudo foi observar o desenvolvimento de candidose em língua de rato após injeções intraepiteliais de *Candida albicans*. Foram utilizados cinqüenta ratos (*Rattus norvegicus,* Albinus, Wistar) negativos para o gênero *Candida*, que receberam dez injeções intraepiteliais de *C. albicans* no dorso da língua. Grupos de cinco animais foram sacrificados após 1, 2, 4, 6, 8 e 12 horas e 1, 2, 7 e 15 dias após as injeções. As línguas foram removidas cirurgicamente e submetidas à análise macroscópica e de microscopia óptica. Houve desenvolvimento de candidose em todos os ratos, sendo que após 1 hora da injeção as leveduras mostravam brotamentos de tubo germinativo e decorridas 4 horas, pseudohifas penetravam nas células epiteliais com formação de microabscessos. Depois de 24 a 48 horas as áreas do epitélio com pseudohifas apresentaram descamação, hiperplasia da camada basal e discreta inflamação no tecido conjuntivo. Aos sete dias haviam poucas pseudohifas e o epitélio exibia acantose, hiperqueratose e perda das papilas filiformes. Após 15 dias, leveduras e pseudohifas não foram mais encontradas e o epitélio apresentava áreas com acantose e perda das papilas filiformes. A injeção intraepitelial de *C. albicans* no dorso da língua de ratos provocou candidose em todos os animais, com presença de *Candida* até sete dias após as injeções.

Unitermos

Candida albicans; Candida; ratos; língua

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