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VALÉRIA APARECIDA BAQUETTI MOSCA

Patogenicidade de leveduras do gênero *Candida* em modelo de candidíase vulvovaginal experimental

Maringá
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Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Biociências e Fisiopatologia.
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Patogenicidade de leveduras do gênero *Candida* em modelo de candidíase vulvovaginal experimental

RESUMO

A candidíase vulvovaginal (CVV) é um distúrbio frequente e é considerada a segunda infecção vaginal mais comum em mulheres entre 18 e 40 anos. Nos Estados Unidos e Europa a prevalência de CVV varia de 5% a 8%. Apesar deste contexto, os fatores de risco para o desenvolvimento de CVV, bem como o potencial de patogenicidade entre as principais espécies de *Candida* ainda não são bem compreendidos. Dentre os fatores de risco para essa patologia, destaque tem-se dado ao Diabetes Mellitus, uma vez que essa patologia favorece o aumento nos níveis de glicogênio do tecido vaginal, além de causar alterações metabólicas favorecendo a CVV. *C. albicans* é considerada o principal agente causador da CVV, seguido de *C. glabrata* e *C. tropicalis*. Dados epidemiológicos nas últimas duas décadas revelaram uma notável mudança micológica em relação à variação geográfica em espécies etiológicas de CVV. Neste estudo nós avaliamos um modelo experimental de CVV em camundongos diabéticos e não diabéticos. Os níveis de glicose foram determinados no soro e na urina por um longo período experimental. Inoculamos *C. albicans, C. tropicalis* e *C. glabrata* intravaginalmente e os animais foram acompanhados por 10 dias. Em geral, os animais diabéticos mantiveram maior carga fúngica, independente da espécie, mas isso ocorreu de maneira diferente para cada espécie de levedura. Enquanto em *C. albicans* essa diferença foi significativa no dia 3, *C. tropicalis* no dia 7 e para *C. glabrata* não houve diferença estatística entre os dois grupos. A interação fungo-hospedeiro também foi diferente conforme a espécie, *C. albicans* produziu filamentação e induziu alta descamação, resposta inflamatória e induziu liberação de altos níveis de TNF e IL-6. *C. tropicalis* provocou quadro semelhante, mas com maior liberação de citocinas, nos dois grupos. Enquanto isso, *C. glabrata* induziu menor resposta tanto em camundongos diabéticos e não diabéticos. Portanto, concluímos que a infecção por *Candida* spp., bem como a arquitetura de fixação de leveduras nas células epiteliais animais são diferentes entre os três principais agentes de CVV. Entretanto, houve uma diferença sutil (não estatisticamente significativa) entre animais diabéticos e não diabéticos. Assim, obtivemos algumas respostas importantes no cenário complexo em etiopatogenia de CVV, mas são necessários mais estudos para melhor compreensão.

Palavras-chave: Candidíase vulvovaginal. *Candida* spp. Patogenicidade. Diabetes Mellitus.

Pathogenicity of yeasts of the genus *Candida* in experimental vulvovaginal candidiasis model

ABSTRACT

Vulvovaginal candidiasis (VVC) is a common disorder and is considered the second most common vaginal infection in women between 18 and 40 years of age. In the United States and Europe the prevalence of VVC ranges from 5% to 8%. Despite this context, the risk factors for the development of VVC as well as the potential for pathogenicity among the major *Candida* species are still not well understood. Among the risk factors for this pathology, it has been highlighted to Diabetes Mellitus, since this pathology favors the increase in glycogen levels of the vaginal tissue, besides causing metabolic alterations favoring VVC. *C. albicans* is considered the main causative agent of VVC, followed by *C. glabrata* and *C. tropicalis.* Epidemiological data in the last two decades have revealed a remarkable mycological change in relation to geographic variation in etiological species of VVC. In this study we evaluated an experimental model of VVC in diabetic and non-diabetic mice. Glucose levels were determined in serum and urine for a long experimental period. We inoculated *C. albicans*, *C. tropicalis* and *C. glabrata* intravaginally and the animals were followed for 10 days. In general, diabetic animals maintained a higher fungal load, independent of the species, but this occurred differently for each yeast species. While in *C. albicans* this difference was significant at day 3, *C. tropicalis a*t day 7 and for *C. glabrata* there was no statistical difference between the two groups. The fungus-host interaction was also different according to the species, *C. albicans p*roduced filamentation and induced high desquamation, inflammatory response and induced release of high levels of TNF and IL-6. *C. tropicalis* caused a similar picture, but with a greater release of cytokines, in both groups. Meanwhile, *C. glabrata* induced lower response in both diabetic and non-diabetic mice. Therefore, we conclude that *Candida* spp. Infection as well as the yeast fixation architecture in animal epithelial cells is different among the three major VVC agents. However, there was a subtle (non-statistically significant) difference between diabetic and non-diabetic animals. Thus, we obtained some important answers in the complex scenario in etiopathogeny of VVC, but more studies are needed for a better understanding.

Keywords: Vulvovaginal candidiasis. *Candida* spp. Patogenicity. Diabetes Mellitus.

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SUMÁRIO

1. CAPÍTULO I

1.1 Candidíase vulvovaginal

A candidíase vulvovaginal (CVV) é um distúrbio frequente e é considerada a segunda infecção vaginal mais comum em mulheres entre 18 e 40 anos (1, 2). Nos Estados Unidos e Europa a prevalência de CVV varia de 5% a 8% (3). Há associação estatisticamente significante entre CVV e nível de escolaridade, segundo Bitew A, et al., 2018, a infecção por *Candida* é mais frequente em mulheres analfabetas do que naquelas com ensino primário e superior. Além disso, outros fatores fisiográficos como melhoria nas condições de higiene pessoal e / ou na situação econômica resultante da educação pode explicar a diferença na taxa de infecção entre analfabetos e aquelas com melhor educação (4). A gama de pacientes com risco de infecção fúngica continua a se expandir, justificado principalmente pelo aumento da população imunocomprometida por diversas causas: síndrome da imunodeficiência adquirida; terapia para câncer e órgão transplantação; diabetes mellitus; pacientes submetidos a grandes cirurgias (5) entre outros. Especificamente em relação à CVV os fatores de risco mais conhecidos são o uso de anticoncepcionais orais, dieta rica em carboidratos, vestuário, além das alterações sistêmicas comuns a outras formas de candidíases. CVV acomete principalmente a vulva e a vagina, e os principais sintomas incluem secreção espessa semelhante a queijo coalhado, associada a prurido vaginal e vulvar, dor, queimação, eritema e edema. Disúria e dispareunia externas também podem ocorrer (6). Aproximadamente 22% das mulheres têm colonização assintomática da levedura vaginal por *Candida* spp (7).

Acredita-se que 75% das mulheres em idade fértil sofrem de pelo menos um episódio de CVV durante a vida e 40 a 50% dessas mulheres apresentam um segundo episódio, e que 5-10% das mulheres desenvolvam candidíase vulvovaginal recorrente (CVVR), também são classificadas como CVV complicada, é caracterizada por quatro ou mais episódios por ano na presença de fatores de risco, como gravidez, diabetes e imunossupressão. (8, 9). De acordo com Achkar&Fries, 2010 e Pappaset al., 2009, a CVV complicada inclui episódios graves causados principalmente por espécies de leveduras pertencentes ao gênero *Candida* que não da espécie *C. albicans*, as quais são conhecidas como *Candida* não-*C. albicans* (CNCA). Já CVV não-complicada, é caracterizada por menos de quatro episódios durante um ano com gravidade leve a moderada, causada por *C. albicans*, em mulheres aparentemente saudáveis. Além disso, um recente estudo de revisão destaca que a prevalência da CVV não-complicada provavelmente varia conforme idade das mulheres e área geográfica (10).

1.2 *Candida* spp.

As leveduras do gênero *Candida* pertencem à microbiota humana e podem colonizar as superfícies de mucosas do tratos genitais, urinários, respiratórios e gastrointestinais, cavidade oral, unhas, couro cabeludo e pele (3). As espécies de *Candida* são caracterizadas como organismos oportunistas, podendo causar patologia após a variação das condições do hospedeiro. As mulheres podem apresentar *Candida* spp. na microbiota vaginal sem apresentar qualquer sintoma de infecção (11).

Dados epidemiológicos nas últimas duas décadas revelaram uma notável mudança micológica em relação à variação geográfica em espécies etiológicas de CVV. Enquanto que *C. albicans* está diminuindo, apesar de manter sua posição como a espécie mais comum do gênero *Candida* spp., há uma crescente prevalência de infecções causadas por espécies de CNCA (12, 13). Entre outras, *C. tropicalis* e *C. glabrata* oscilam entre o primeiro e o segundo lugar com taxas de aproximadamente 66,3% (14), com ênfase nos casos de CVVR em pacientes com Diabetes Mellitus (DM), e neste cenário *C. glabrata* é uma das principais espécies envolvidas (15).

1.2.1 *Candida albicans*

Entre as espécies mais isoladas de *Candida*, dos casos de CVV, *C. albicans* é a principal espécie envolvida. Por se tratar de um fungo oportunista capaz de causar infecções mucosas e invasivas, possui rápida capacidade de adaptação diante de mudanças de ambientes dentro do hospedeiro (14). Durante uma infecção da mucosa, como a CVV, as hifas invadem as células epiteliais e endoteliais, causando danos, principalmente pela ação de enzimas hidrolíticas (2). Os fatores de virulência de *C. albicans* têm sido definidos como mecanismos que interagem diretamente com os componentes do hospedeiro, contribuindo para a adesão, penetração e invasão dos tecidos. Tanto as células blastoconídios quanto as hifas de *C. albicans* são encontradas em tecidos hospedeiros infectados, e acredita-se amplamente que a formação de hifas seja importante por suas propriedades invasivas e está associada à adesão e invasão de tecidos (16). A transição entre as formas de crescimento da levedura e da hifa é denominada dimorfismo e ambas são importantes para a patogenicidade (17). A forma de hifa demonstra ser mais invasiva que a forma de levedura (blastoconídio), porém, acredita-se que a forma de levedura represente a forma primariamente envolvida na disseminação (18). Além disso, *C. albicans* possui um conjunto especializado de proteínas (adesinas) que medeiam à sua aderência a outros microrganismos, a superfícies abióticas e a células hospedeiras (19)

Outro importante fator de virulência desta espécie é a formação de biofilme, pois são altamente estruturados; eles contêm células da forma de levedura, células pseudo-hifas e células da hifa envolvidas por uma matriz extracelular, e, por fim, dispersão de células de levedura do biofilme (20, 21).

Além de formar biofilmes em dispositivos médicos implantados (por exemplo, dispositivo intrauterino e anel vaginal), os biofilmes de *Candida* spp. também se formam nas superfícies do hospedeiro, como as mucosas, revestimentos de células epiteliais e órgãos parenquimatosos (22, 23). O biofilme é formado em diferentes estágios: Primeiro, as células de levedura se ligam a uma superfície, que pode ser abiótica ou biótica. Em segundo lugar, as células aderidas proliferam na superfície para formar micro colônias, depois a matriz extracelular é produzida. No estágio final, algumas células se desprendem do biofilme para se dispersar para outros locais do corpo. *C. albicans* não só é capaz de adaptar-se ao pH ambiental, mas também pode modular o pH extracelular, alcalinizando o ambiente circundante sob jejum de nutrientes e, assim, auto induzindo a formação de hifas (24, 25).

1.2.2 *C. glabrata*

C. glabrata é um comensal do trato intestinal em humanos, é a segunda causa mais comum de CVV (26). No entanto, a patogênese dessa espécie na infecção por CVV é pouco compreendida (3). Esta espécie não é polimórfica e só cresce como levedura em formação, ao contrário de *C. albicans*, em que a transição levedura-hifa mostrou ser um dos mais importantes fatores de virulência (27). Isso indica que a adesão e, portanto, os biofilmes, são importantes para a virulência em *C. glabrata* (28, 29). Como *C. glabrata* só cresce por brotamento, os biofilmes maduros são caracterizados por uma densa rede de células de levedura embutidas em uma matriz extracelular (29). Uma vez aderida a superfícies, a levedura continua a causar colonização e, consequentemente, pode causar sintomas de CVV. No entanto, devido à ausência de filamentação, o mecanismo de invasão parece ser prejudicado. Além disso, a ausência desse atributo de virulência parece favorecer a atividade dos neutrófilos sobre *C. glabrata* (28).

1.2.3 *C. tropicalis*

Dentre as CNCA, *C. tropicalis* é considerada uma das espécies mais importantes, em termos de virulência, no entanto, está na terceira posição no *ranking* epidemiológico de CVV (30), e é classificada como a terceira ou quarta espécie CNCA mais comumente isolada na prática clínica (31, 32). *C. tropicalis* pertence à microbiota humana normal e está presente na

pele, trato gastrointestinal, geniturinário e respiratório de humanos (33). Tem a capacidade de produzir hifas verdadeiras, e considerado altamente aderente às células epiteliais e um potente produtor de biofilme (34). A estrutura da parede celular é composta por proteínas hidrofóbicas embutidas em uma matriz celular que favorecem a interação inicial, pois essas tendem a se ligar a uma grande variedade de materiais plásticos e proteínas hospedeiras como laminina, fibrinogênio e fibronectina. Paiva et al. (2012) avaliaram a formação de biofilme *in vitro* por isolados de *C. tropicalis* obtidos de CVV e apesar de ser uma espécie altamente virulenta, não é possível explicar porque *C. tropicalis* é pouco isolada da infecção vaginal (36).

1.3 Fatores de risco

Os fatores de risco relacionados à levedura evidenciam que enzimas líticas, capacidade de formação de biofilme e resistência antifúngica estão intimamente relacionados à patogenicidade das espécies de *Candida*. Já em relação ao hospedeiro, mulheres com menos de 40 anos de idade e com contato sexual antes dos 20 anos de idade têm aumentado em até 2 e 4 vezes o risco de desenvolver a CVV (2). Recente estudo afirma que seis principais fatores de risco mais comuns dos pacientes com CVV são DSTs, ducha vaginal, relação sexual precoce, diabetes mellitus e gestação (37).

A condição sintomática da CVV causada por *C. albicans* está fortemente associada a uma resposta inflamatória mediada por neutrófilos polimorfonucleares (PMNs), ao invés de deficiências imunológicas capazes de definir a suscetibilidade à infecção (38). Assim, as células epiteliais vaginais são desencadeadas por *C. albicans*, a fim de produzir citocinas, promovendo o recrutamento de PMNs para a região vaginal (21). O principal desencadeador da resposta inflamatória é a morfogênese de *C. albicans* associada à sensibilidade das células epiteliais aos fungos. Os PMNs não são eficazes na redução da carga fúngica durante uma forte resposta inflamatória (21, 39).

Os hormônios da reprodução contribuem para alterações fisiológicas e teciduais e aumentam a suscetibilidade à infecção por *Candida*. Além disso, distúrbios na resposta imune celular sistêmica ou local podem contribuir para os casos de CVV. Um estudo mostrou que a CVV foi significativamente maior entre as mulheres diabéticas quando comparadas às não diabéticas (5). Nesse contexto, condições clínicas como DM descontrolada poderiam ser consideradas um dos principais fatores de risco para o desenvolvimento da CVV (40). Pacientes com DM geralmente passam por níveis elevados de açúcar no plasma e a dieta rica em açúcar pode contribuir para o risco de CVV (41).

1.4 Diabetes Mellitus (DM) e Candidíase Vulvovaginal (CVV)

DM é uma síndrome clínica associada à deficiência na secreção e/ ou ação da insulina. É considerado um problema de saúde emergente do século 21, com cerca de 422 milhões de pessoas afetadas (42). Além das complicações clínicas típicas, DM é descrita como uma condição clínica patológica que causa anormalidades funcionais em pacientes, como uma resposta imune insuficiente contra infecções bacterianas e fúngicas, diminuindo a resposta imune nas células T, neutrófilos, provocando alterações na imunidade humoral, e, consequentemente, aumentando a suscetibilidade à infecção e o desenvolvimento de mais doenças (43, 44). De fato, a defesa contra infecções fúngicas está marcadamente relacionada à resposta inflamatória Th1, com a estimulação de citocinas que ativam o recrutamento de neutrófilos e ativam essas células para matar a célula fúngica (45).

A interleucina TNF-α é um dos principais participantes do processo inflamatório agudo e também induz a iniciação de neutrófilos, levando a um aumento nas respostas dessas células e de outros mediadores (46). Já a IL-6 é capaz de induzir células Th17 a liberar a citocina IL-17 que tem a função de melhorar as defesas antifúngicas (45, 47). Entretanto, essa resposta é prejudicada em hospedeiro diabético, sendo possível que a ausência dessa resposta inflamatória tenha como consequência a persistência de infecções fúngicas e recorrentes ou crônica (48).

Muitas alterações imunológicas estão relacionadas aos níveis de hipoinsulinemia e hipoglicemia, alterando a função dos órgãos linfóides (44). Algumas infecções são muito prevalentes em pessoas com DM, tais como infecções respiratórias por *Streptococcus pneumonie*, pneumonia devido ao vírus *Influenza* e *Mycobacterium tuberculosis*. Indivíduos com DM também podem desenvolver sintomas mais graves e complicações metabólicas, como hipoglicemia, cetoacidose e coma (49). Pessoas com DM têm um risco aumentado no desenvolvimento de infecções cutâneas e nas mucosas, incluindo aquelas causadas por *Candida* spp. (50).

A prevalência de *C. glabrata* está aumentando em pacientes com CVV com diabetes mellitus descontrolado (51). Essa alta prevalência é um dado intrigante, pois essa espécie não sofre morfogénese, o que é considerado uma característica importante na virulência de *C. albicans*, pois os genes que regulam a levedura para a transição de hifas são necessários para desencadear as respostas das células epiteliais vaginais imunopatogênicas (52). Enquanto *C. glabrata* é considerada menos virulenta em comparação a *C. albicans*, é inatamente resistente aos antifúngicos azólicos e apresenta maior resistência a todos os azóis disponíveis em comparação com a maioria dos isolados de *C.albicans* (53).

5. Justificativa

A candidíase vulvovaginal (CVV) é uma doença causada pelo crescimento anormal de fungos do tipo leveduras na mucosa do trato genital feminino. Por acometer milhões de mulheres anualmente, causando grande desconforto, interferindo nas relações sexuais e afetivas e, ainda, prejudicando o desempenho laboral, a CVV tem sido considerado importante problema de saúde pública mundial. Apesar da espécie *Candida albicans* ser a mais frequente nos casos de CVV, os estudos epidemiológicos mostram o aumento da prevalência de outras espécies como *C. glabrata* e *C. tropicalis*.

Os fatores para o desenvolvimento da CVV não estão claros na literatura, mas estão atualmente ligados à imunossupressão do hospedeiro, com destaque às pacientes diabéticas ou ainda à virulência da levedura. Neste sentido, não há dados na literatura que mostrem a patofisiologia da CVV nas diferentes espécies de *Candida*. Desta forma, este projeto tem por objetivo avaliar a patogenicidade de leveduras do gênero *Candida* em modelo de candidíase vulvovaginal experimental, com animais (camundongos fêmeas Balb/c) diabéticos e não diabéticos infectados com as espécies *C. albicans, C. glabrata* e *C. tropicalis*.

1.6 Objetivos

1.6.1 Objetivo geral

Avaliar comparativamente a patogenicidade de leveduras do gênero *Candida* em modelo de candidíase vulvovaginal (CVV) experimental, em animais diabéticos e não diabéticos. Para isso, serão avaliadas as espécies mais frequentes responsáveis por casos de CVV, como *C. albicans, C. glabrata* e *C. tropicalis.*

1.6.2 Objetivos específicos

1. Padronizar modelo de CVV com diferentes espécies de leveduras do gênero *Candida*;
2. Estabelecer um modelo consistente de Diabetes em camundongos Balb/c fêmeas.
3. Padronizar o modelo de CVV experimental em animais diabéticos;
4. Avaliar a patogenicidade das três espécies, nos diferentes modelos de hospedeiro através dos seguintes protocolos:

    4.1. Avaliação da carga fúngica no tecido vaginal em cinco diferentes tempos (1, 3, 5, 7 e 10 dias pós-infecção);

    4.2. Avaliar a presença de leveduras na secreção vaginal dos animais infectados;

    4.3 Avaliar a presença de leveduras e alterações através da metodologia de Papanicolau.

    4.4 Avaliar a presença de leveduras e alterações epiteliais através da metodologia Histopatológica.

    4.5 Avaliar a presença de leveduras, filamentação e alterações epiteliais através da metodologia de Microscopia Eletrônica de Varredura (MEV).

    4.6 Avaliar a presença de citocinas no fluido vaginal de animais diabéticos e não diabéticos.

1.7 Referências

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2. CAPÍTULO II

Influence of diabetes on murine vulvovaginal candidiasis by three different C*andida* species

Maringá
2019

INFLUENCE OF DIABETES ON MURINE VULVOVAGINAL CANDIDIASIS BY THREE DIFFERENT C*andida* SPECIES

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Abstract

Vulvovaginal candidiasis (VVC) is the second most common vaginal infection in women in childbearing age. Despite this context, the risk factors for the development of VVC as well as the pathogenicity potential among the main *Candida* are still not well understood. Here, we have evaluated an experimental VVC model in diabetics and non-diabetics mice. Female Balb/c age six weeks, weighing 22 grams were induced for diabetes mellitus (DM) by an injection intravenously of Alloxan 80 mg/kg. Next, the estrogenic phase was induced in all animals by β-estradiol 17-valerate, injected subcutaneously weekly during the experimental period. The glucose levels were determined in serum and urine to long of experimental period. *C. albicans* ($2x10^6$ CFU/mL); *C. tropicalis* and *C. glabrata* ($2x10^7$ CFU/mL) were inoculated intravaginally (n= 4 to 8 mice/group) and animals were accompanied by 10 days. In general, the diabetic animals maintained greater fungal burden, regardless of species studied, but it occurs in different way for each yeast species. While in *C. albicans* it was significant on day 3, *C. tropicalis* on day 7 and for *C. glabrata* there is no statistic difference between two groups. *C. albicans* produced filamentation and induced high desquamation, inflammatory response and induced release of TNF-α, IL-6 and IL-17 levels. *C. tropicalis* provoked similar picture, but with more released of cytokines, in the two groups. Meanwhile, *C. glabrata* induced minor host response in both diabetic and non-diabetic mice. Therefore, we conclude VVC infection, as well as the architecture of attachment of yeasts on the epithelial cells are different among the three main agents, but the difference between diabetic and non-diabetic animals were discrete. Thus, some important answers on complex scenario on etiopathogeny of VVC were obtained but are necessary much more studies until it completely understood.

Introduction

Vulvovaginal candidiasis (VVC) is a frequent disorder and is the second most common vaginal infection in women between 18 and 40 years old (1, 2), affecting mainly the vulva and vagina. It has been accept that 75% of women in childbearing age have at least one episode of VVC during life and 40% to 50% of these women present a second episode, and that 5-10% of women develop recurrent vulvovaginal candidiasis (RVVC). Besides, a recent review study highlight that the its prevalence probably varies by women age and geographic area, but further studies are required to quantify these disorder (3).

Among the most isolated species of *Candida,* from VVC cases, *Candida albican*s is the main species involved (4). However, is increasing the number of VVC by *Candida* non-*C. albicans* species (CNCA). Among others, *C. tropicalis* and *C. glabrata* oscillate between first and second most common CNCA causing VVC, with rates of approximately 66.3% (4). Emphasizing the scenario on the cases of RVVC, in patients with Diabetes Mellitus (DM) *C. glabrata* is the main species involved (6), but it is not known exactly why it occurs.

Risk factors related to yeast and host are determinant to divert fungal colonization to the infectious process. In the first case, it is evidenced that lytic enzymes, ability to form biofilm and antifungal resistance are closely related to the pathogenicity of *Candida* species. In relation to the host, women less than 40 years old and with sexual contact before of 20 years has been two-fold and four-fold increased risk of developing the VVC (2). The reproductive hormones contribute for physiological and tissue changes and increase susceptibility to *Candida* infection. In addition, disturbances in the systemic or local cellular immune response may contribute to VVC cases. A study has showed VVC was significantly higher ($p = 0.004$) between the type 2 diabetic women versus non-diabetic ones (7). In this context, clinical conditions such uncontrolled DM could be considered as one of the main risk factors for the development of VVC (8). Despite few studies, there are indications that the hyperglycemic state may favor colonization and fungal infection (5).

There was a change in the understanding of the pathogenesis of vaginitis by *C. albicans.* The symptomatic condition is strongly associated with an acute inflammatory response mediated by polymorphonuclear neutrophils (PMNs), rather than immunological deficiencies capable of defining the susceptibility to infection (9). Thus, vaginal epithelial cells are triggered by *C. albicans* in order to produce cytokines by promoting the recruitment of PMNs to the vaginal region (10).

The main triggers of the inflammatory response are the morphogenesis of *C. albicans* associated with a sensitivity of epithelial cells to fungi. PMNs are not effective in reducing fungal load during a strong acute inflammatory response (10, 11).

Despite this context, the risk factors for the development of VVC are still not well understood, as well as the pathogenicity potential among the main *Candida* species which causes VVC. In this sense, this study aimed to characterize the VVC process in diabetics and non-diabetics in a reproducible murine model of the VVC by the three *Candida* spp. species most described in the literature as VVC agents, i.e. *C. albicans*, *C. tropicalis* and *C. glabrata*.

Results

Experimental model for study of vaginitis by *Candida* spp. in diabetic and non-diabetic mice

Aiming to better understand the etiopathogeny of VVC in diabetics, we used the experimental model of diabetes as a risk factor for VVC. For this, female mice were treated with a single dose of Alloxan to induce diabetic condition and mimetize to type 1 DM of human patients. Fig 1 shows a relationship between serum and urinary glucose levels. It is possible to observe that urinary glucose levels increase proportionally to the serum level. These data supported the follow-up of hyperglycemia through glycosuria levels, minimizing discomfort for animals.

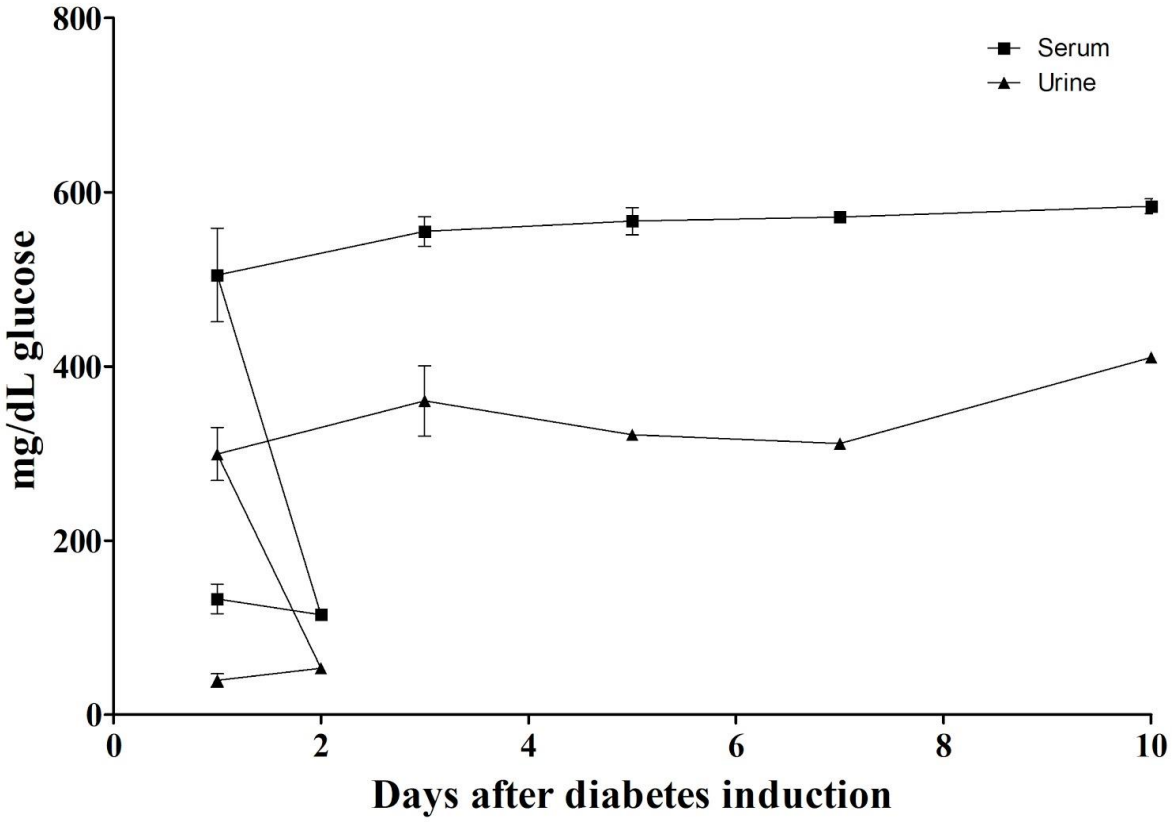

Fig 1.Establishment of type I diabetes mellitus in female Balb/C mice. Induced by 80 mg/kg of Alloxan. Documented by serum and urinary glucose dosages of mice (n=5) in different days after induction.

Table 1 shows the results of levels glucose in serum and vaginal fluid of diabetic and non-diabetic mice. It is possible to observe that serum glucose levels were always greater than 250 mg / dL. These results evidenced that the experimental model of diabetes was maintained at all evaluated times. In addition, table I shows that glucose levels in the vaginal fluid were

higher in diabetic animals, until the last analyzed time, independently of the yeast species evaluated.

Table 1.Accompaniment of levels glucose on serum and vaginal fluid of vulvovaginal candidiasis with three species of *Candida* in diabetic and non-diabetic hosts.

		Days after infection									
A		1		3		5		7		10	
DIABETICS		**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**
	C. albicans	489.2	45.2	567.0	10.9	441.8	29.4	324.3	19.5	638.8	23.7
	C.tropicalis	430.4	19.3	355.8	18.2	554.0	25.3	302.9	16.8	404.7	30.9
	C. grabrata	385.2	1.1	403.7	18.6	404.2	8.0	291.5	1.7	267.7	24.8
	Mean	434.9	21.9	442.2	15.9	466.7	20.9	306.2	12.7	446.7	26.5

		Days after infection									
B		1		3		5		7		10	
NON - DIABETICS		**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**	**Serum**	**Fluid**
	C. albicans	104.3	0.8	146.6	9.7	61.7	2.2	144.8	2.9	157.1	2.3
	C.tropicalis	64.2	5.6	136.2	1.2	126.5	1.0	121.5	1.0	108.2	0.5
	C. grabrata	124.4	8.7	99.8	0.0	108.4	0.0	102.8	0.0	146.2	0.9
	Mean	97.6	5.0	127.5	3.6	98.9	1.1	123.0	1.3	137.2	1.2

Each data presents the mean values (mg/dl) of glucose levels from at least five animals in at least two independent experiments. Animals were with food and water *ad libidum.* Normal values were considered 75 + - 128 mg / dl. The mice had no food restriction for serum glucose.

To establish a consistent experimental model of VVC, an estrogenic vaginal environment was induced with β-Estradiol 17-valerate, which was monitored by epithelial cells morphology. According Fig. 2 immature epithelium is constituted by rounded and nucleated cells (Fig 2A), while vaginal epithelium of mice has receipt the estrogen the matured cells were large and enucleated characterizing an epithelium favorable to *Candida* infection (Fig 2B).

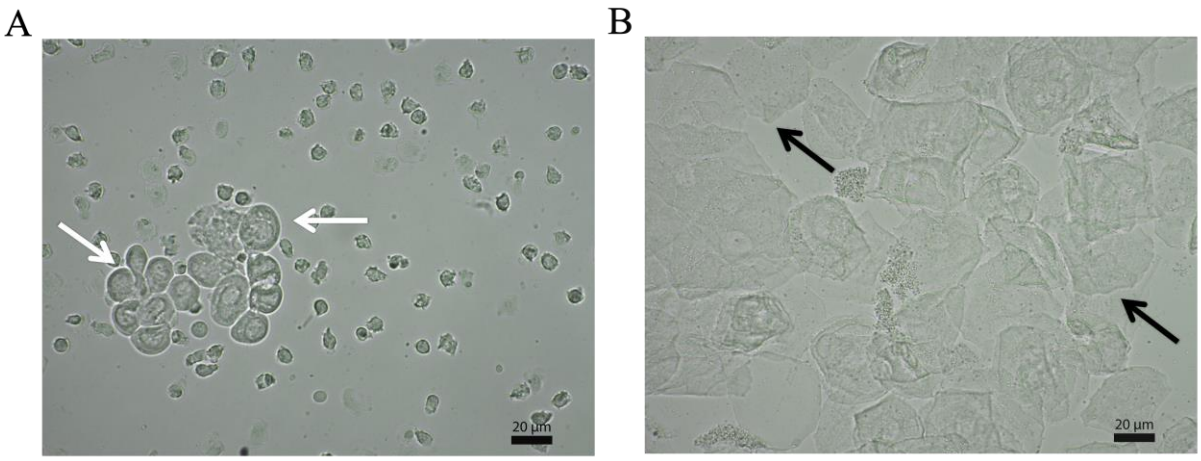

Fig 2.Standardization of hormonal concentration and analysis of cells in the vaginal fluid to check hormone activity. Effect of estrogenization on the murine vaginal epithelium observed in a fresh vaginal fluid.**A)** Vaginal epithelium of mice, without administration of estradiol, observed fresh by light microscopy. White arrows indicate immature, rounded and nucleated cells. **B)** Vaginal epithelium of mice after β-Estradiol 17-valerate at a concentration of 0.3mg/weekly/mice inoculation. This picture illustrates the observation from 3 to 10 days after estrogenization started. The black arrows indicate mature cells characterizing estrogenized epithelium. Vaginal fluid samples were analyzed with (40x objective).

Quantitative evaluation of fungal burden from experimental vaginitis in diabetic and non-diabetics mice

To know the pathophysiological aspects of VVC in diabetic and non-diabetic hosts, our first step was to evaluate the fungal burden on vaginal environment (fluid and tissue). This strategy resulted in highly variable fungal load in relation to the diabetic mice, among the *Candida* species and also the infection time (Fig. 3). In general diabetic mice have sustained higher fungal burden in the vaginal fluid, regardless of yeast specie, with statistical relevance in seven and 10 days after infection ($p=<0.05$). In experimental vaginal infection by *C. albicans*, the diabetics animals maintained fungal load in vaginal fluid ranging from 4 to 4.9 Log CFU/mL on all evaluated days. While non-diabetic animals were able to reduce the fungal burden more than 2 Log CFU/mL, from the seventh day of infection. This result showed a statistically significant difference between the groups over time ($p<0.05$). On the other hand, the vaginal tissue evidenced difference greater than 2 logarithmic units from the third day, $p<0.05$ in diabetic mice infected with *C. albicans*. Regarding to *C. tropicalis*, diabetics animals maintained fungal load around 5 Log CFU/mL in the vaginal tissue, allowing statistical difference from the seventh day with the non-diabetic animal, which reduced the fungal burden to approximately 3 Log. Similar behavior resulted in a significant difference in the vaginal fluid ($p<0.05$). Differently, mice infected with *C. glabrata* decreased the CFU number in vaginal tissue (3 to 5 Log CFU) at all times, for both groups, with no statistical difference. Nevertheless, the vaginal fluid maintained a similar fungal load

(approximately 4 Log) between the times, for diabetic animals, while there was a significant decrease for non-diabetic animals (1 to 4 Log) ($p<0.05$) on the seventh and tenth days.

Fungal-host relationship on morphological aspects of vaginal epithelium

The vaginal epithelium of the infected animal was illustrated by Figs. 4 - 7. Fig. 4 shows diabetic animals infected by *C. albicans*, have a strong interaction between vaginal pseudohyphal cells. In addition, it is possible to observe polymorphonuclear infiltrates (PMN), characteristics of inflammatory alteration in cells with nucleus alterations, all markers of inflammation. (Fig. 4A). For *C. tropicalis* on the fifth post-infection day, it is possible to observe inflammatory alteration characteristics in the cells with nucleus and PMN alterations. In addition, following the infection, we observed on the tenth day pseudohyphas with germination, indicating cell viability. (Fig. 4B). On the other hand, *C. glabrata* has a differentiated cellular pattern of the other species without evidence of inflammatory process and low colonization (Fig. 4C).

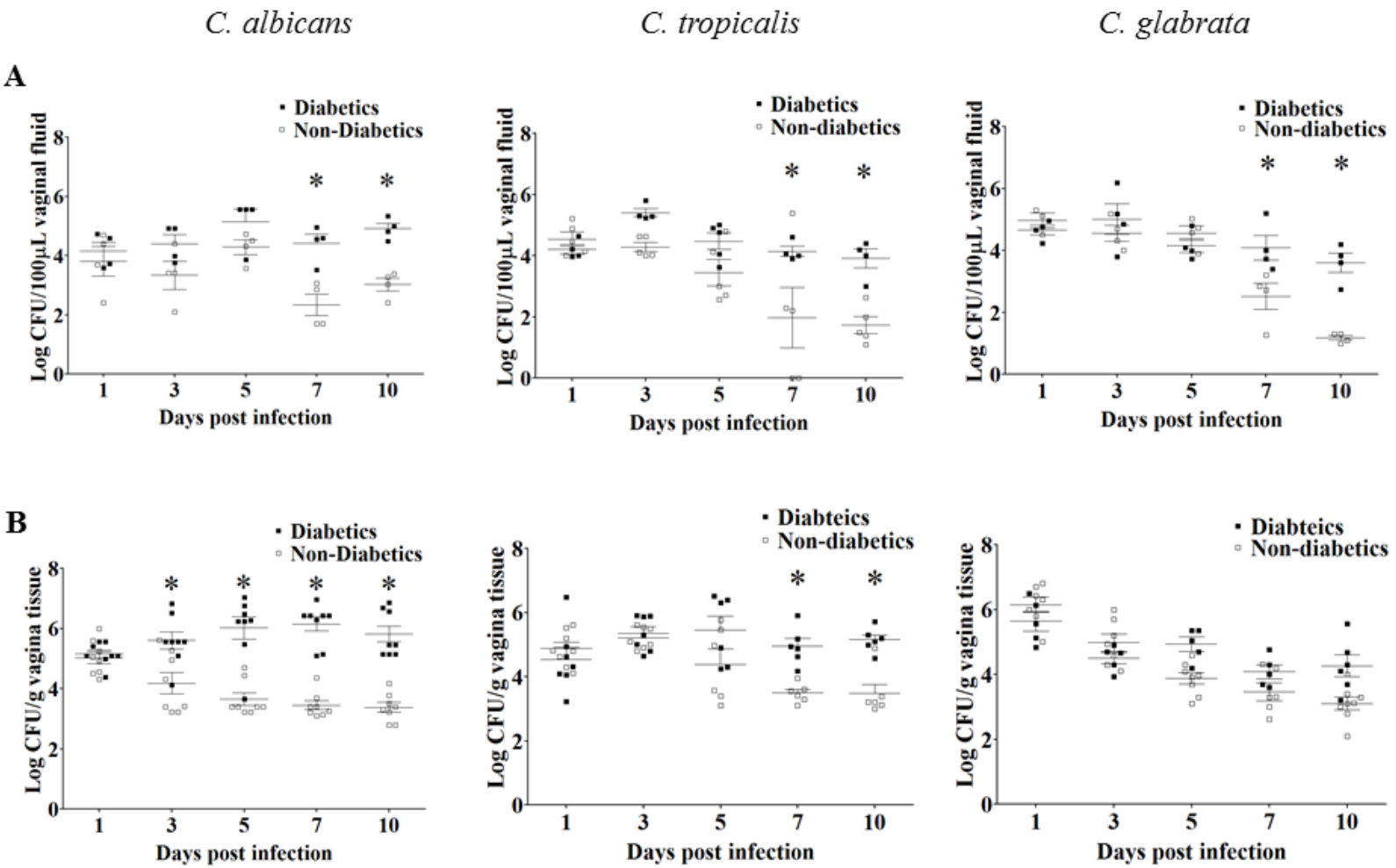

Fig 3.Effect of diabetic condition on fungal burden. Diabetes was induced in mice by an intravenous injection of Alloxan (80 mg / kg) (at least 5). Non-diabetic group (n = 5). All mice were estrogenized (0.3 mg in sesame oil subcutaneously 72 h before inoculation) and inoculated intravaginally with 30µL of a PBS suspension containing $1x10^6$ CFU of *Candida albicans* and $2x10^7$ CFU for *Candida tropicalis* or *Candida glabrata*. The fungal burden was evaluated longitudinally on days 1, 3, 5, 7 and 10 after infection in: A) vaginal fluid and B) vaginal tissue. The vaginal fluid was analyzed in groups with four animal (n = 4). All results were expressed as median CFU among the animal, and from two independent experiments. CFU: colony forming unit. *Values statistically significant, $p<0.05$ between diabetic and non-diabetic groups.

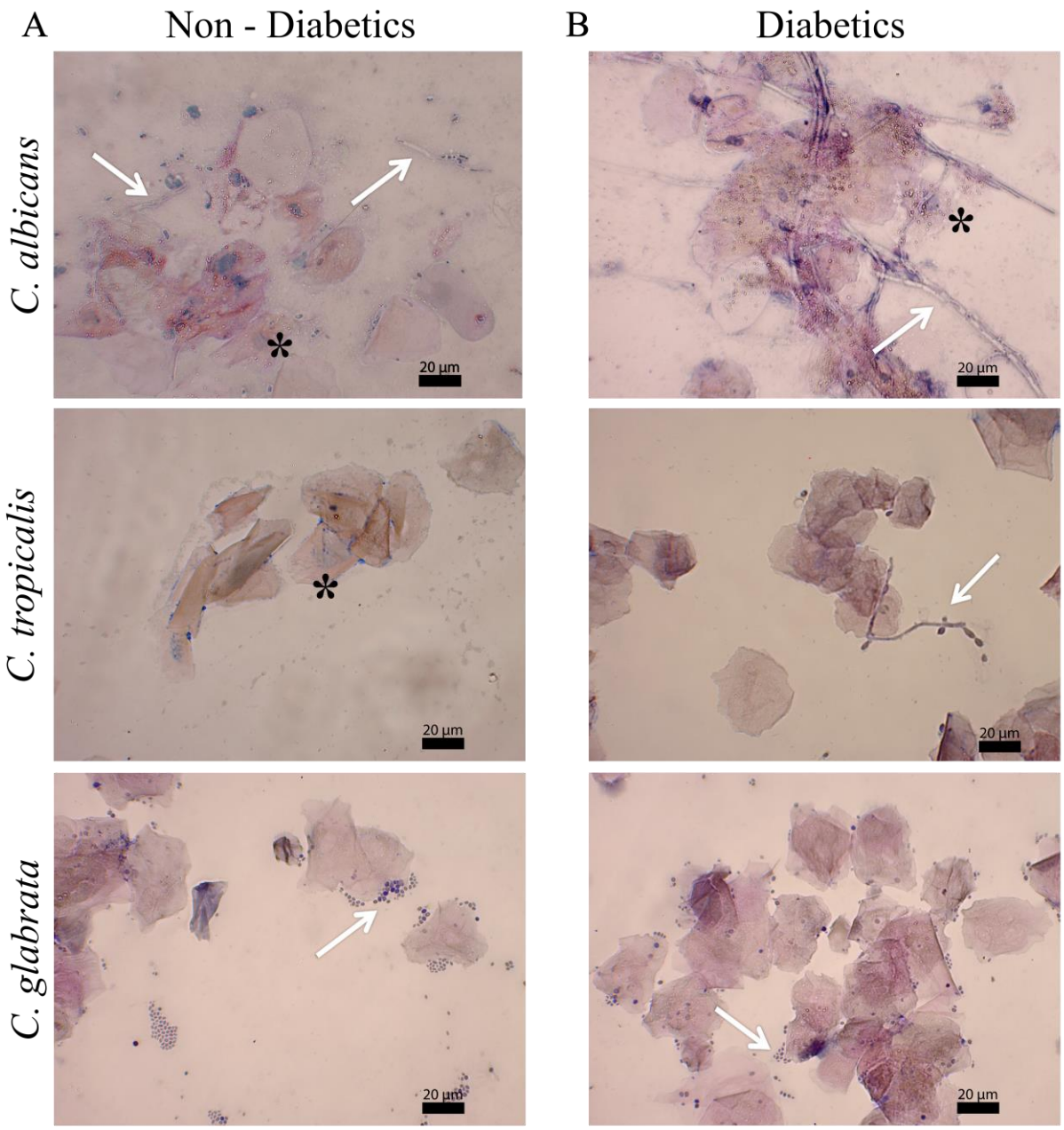

Fig 4. Fungal-host relationship on the cytopathologic view. At the concentration of 0.3 mg β-Estradiol 17-valerate/ week/ mice, we observed predominance of anucleated cells representative of pseudo-estrus. In general in the mucosa of diabetic mice infected with *C. albicans*, *C. tropicalis* and *C. glabrata*, non-diabetic A) and diabetic B) groups. it is possible to observe vaginal epithelium with more established infection for *C. albicans* and *C. tropicalis*, the black arrows indicate fungal structures, the black asterisks indicate inflammatory alteration characteristics in the cells with nucleus and the white arrows indicate PMNs. For *C. glabrata* appeared in minor concentration and also induced minor epithelial changes C). 400x magnification for all images.

The fungal-host interaction is more explicit by HE analysis, since that is possible to evidence the yeasts in closed contact with the epithelium. In addition, tissue congestion and PMN presence are easily observed in some cases. *C. albicans* has a very expressive pattern of filamentation, independent of the host. It was observed congestion in the connective tissue, suggesting a more expressive eosinophilic pattern in diabetics; *C. tropicalis* shows a similar pattern to *C. albicans,* but with a lower concentration of yeasts as well as a larger PMNs infiltrate on diabetic vaginal tissues. *C. glabrata* does not appear to induce alterations on the vaginal tissue. The Fig. 5 illustrate this event observed by day 10 only.

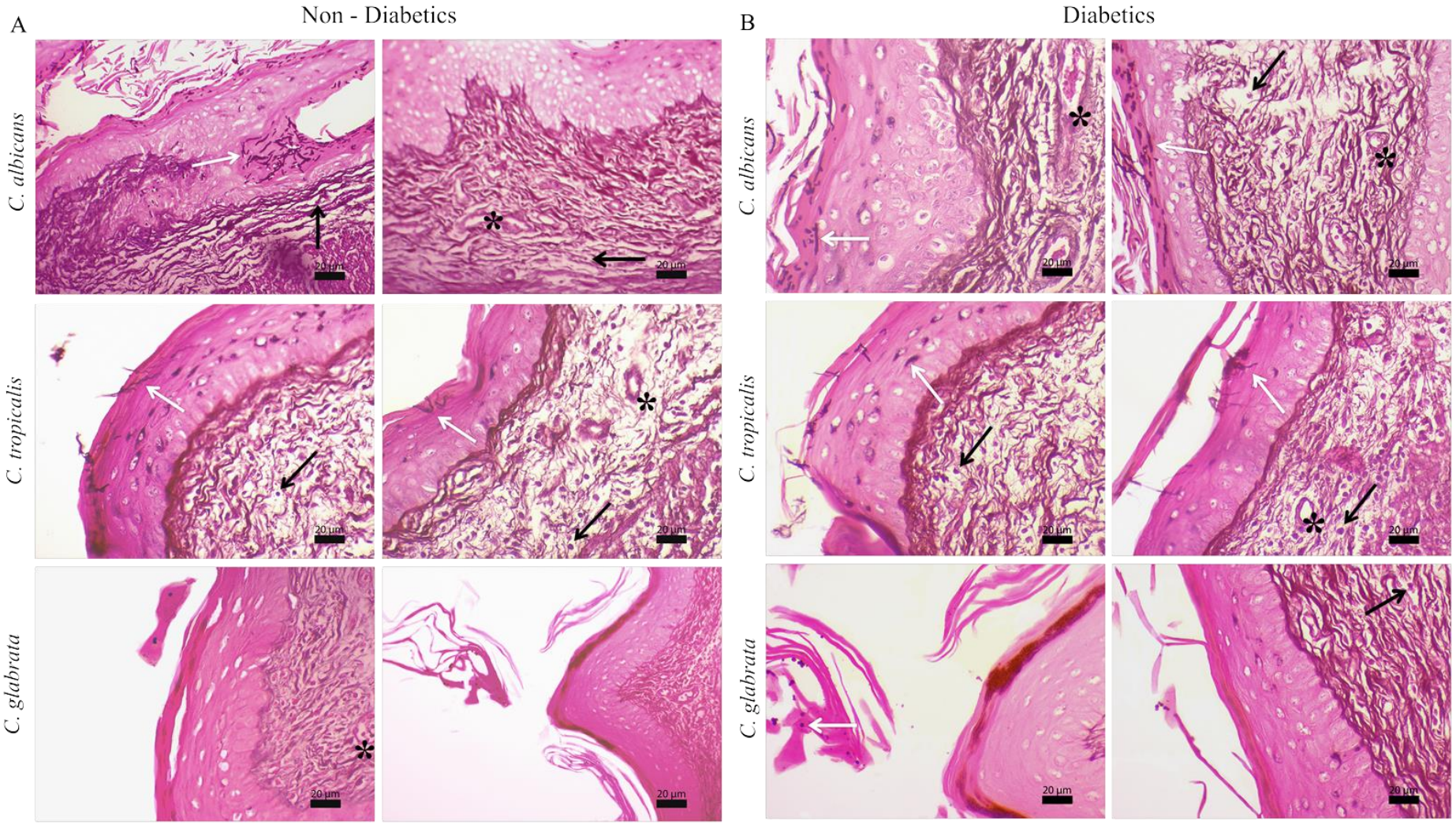

Fig 5. Histopathological examination of the vaginal tissue of mice infected with *C. albicans*, *C. tropicalis* and *C. glabrat*a, non-diabetic A) and diabetic B) groups. Representative micrographs showing tissue morphology with invasion of fungal structures, indicated by white arrows, presence of PMNs indicated by black arrows and tissue congestion indicated by black asterisks. 400x magnification for all images.

Some changes observed in SEM images were in agreement with the observation of stained vaginal smears by Papanicolaou and histological findings (Fig. 4).

Fig. 6 allows, by SEM, a detailed comparison, of the vaginal tissue between diabetic and non-diabetic infected with three of the main VVC's agents. In general way, while diabetic mice showed a vaginal epithelium with increased disorganization and fungal colonization, the non-diabetic group presented epithelium with less alterations, lower desquamation and colonization, regardless of the yeast specie evaluated.

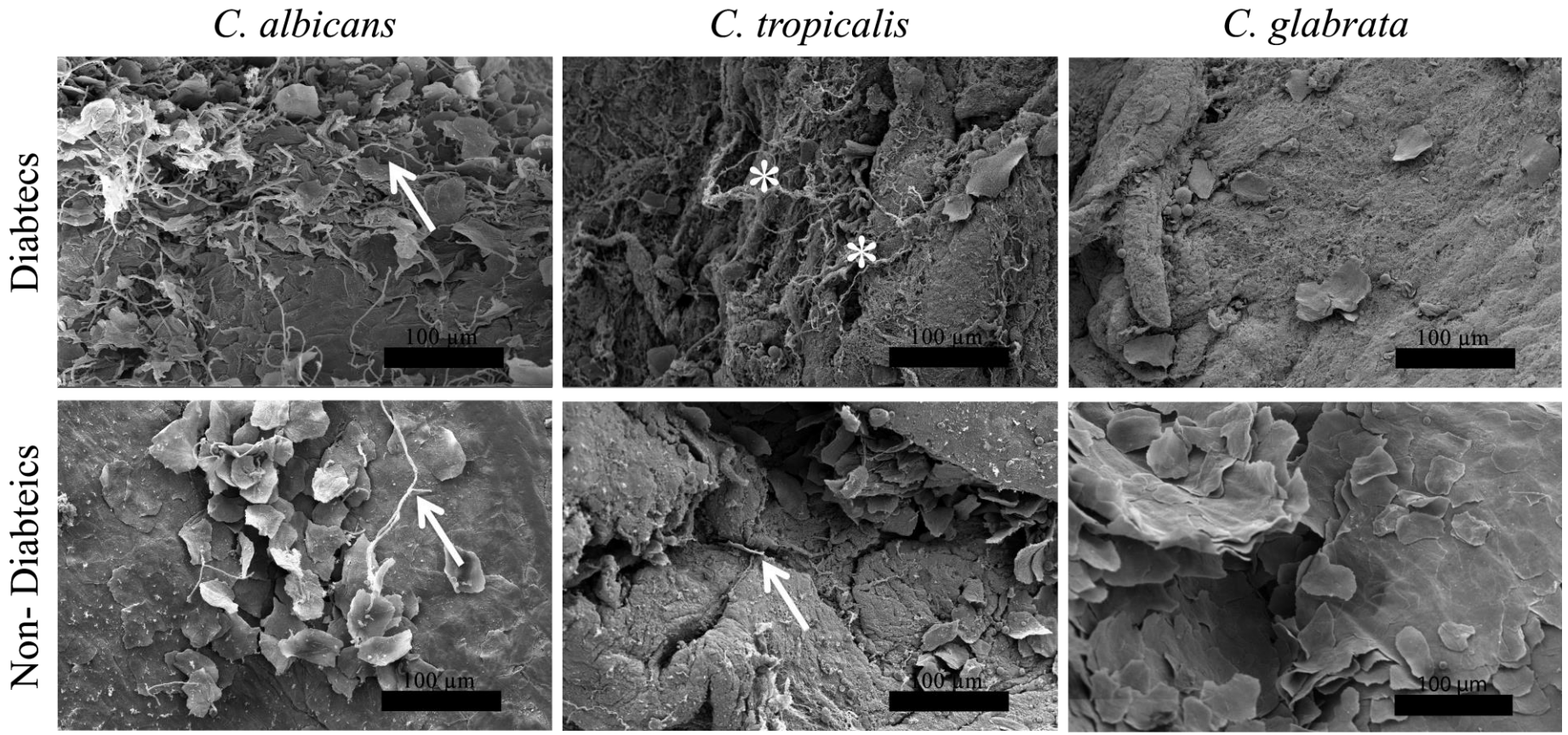

Fig 6.Electron microscopy of the vaginal epithelium of diabetic and non-diabetic mice. Vaginal tissue were submitted to electron microscopy after administration of β-Estradiol 17-valerate and confirmation of the diabetogenic state and infected by *Candida albicans*, *Candida tropicalis* and *Candida glabrata*. At day 5 post-infection (intermediate period), a 1000x magnification shows the difference in colonization of the vaginal epithelium of diabetic and non-diabetic mice, with vaginal tissue and colonization standard differentiated for infected and diabetic animals, independent of the species analysed. The white arrows indicate fungal structures and white asterisks indicate extracellular matrix.

As was visualized a greater alteration in the diabetic group, then we decided to analyze this group more detail during the infection period evaluated (day 1, 5 and 10 after infection). These data are shown by (Fig. 7) exclusively regarding the diabetic mice infected with one of the three yeasts species. It is clear that there is a different pattern interspecies, which is maintained during the evaluation time. *C. albicans* and *C. tropicalis* present exclusively the filamentous form, and as expected, *C. glabrata* was in the form of blastoconidia. Additionally, *C. tropicalis* and *C. glabrata* showed a distinct epithelial pattern. Analyzing all the fields, we observed that the most frequent event was the presence of a structure in the cellular organization, suggesting the presence of extracellular matrix.

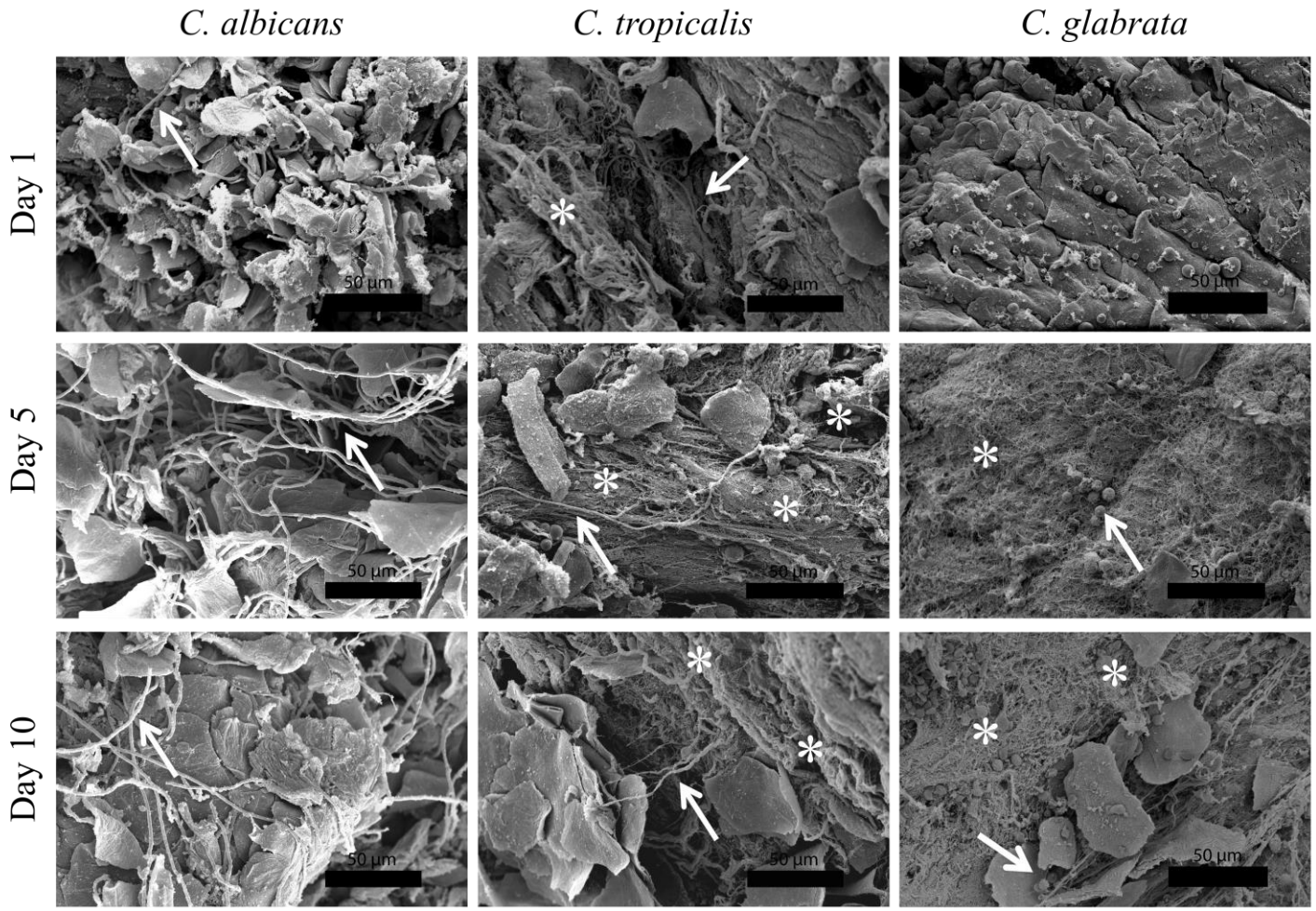

Fig 7. Electron microscopy of vaginal epithelium of diabetic mice submitted to infection by *Candida albicans*, *Candida tropicalis* and *Candida glabrata*. On days 1, 5 and 10 after infection, an increase of 2.000x shows *Candida albicans* with budding and filamentation, in animal desquamated cells, indicating strong cellular interaction with yeast; *Candida tropicalis* infection, has resulted in dispersed cells, low cell adhesion, lower fungal adhesion and presence of configured extracellular matrix. While *Candida glabrata* appear to induce minor epithelial changes, the surface of the mucosa was preserved with lower colonization. The white arrows indicate fungal structures and white asterisks indicate extracellular matrix.

Animal diabetics-infected by *C. albicans*, have strong vaginal cell-pseudohyphae interaction. In addition, it is possible to observe PMN infiltrates,perinuclear), characteristics of inflammatory alteration in cells with nucleus alterations, all markers of inflammation. (Fig. 5B). For *C. tropicalis* on the fifth day post infection, it is possible to observe), characteristics of inflammatory alteration in cells with nucleus alterations and PMN, in addition, in the follow-up of the infection, we observed on the tenth day pseudohyphae with germination, indicating cellular viability (Fig. 5B**)**. *C. glabrata* has a differentiated cellular pattern of the other species without evidence of inflammatory process and low colonization (Fig. 5B). It was possible to confirm the presence of anucleated epithelial cells observed by optical microscopy in the fresh vaginal fluid (Fig. 2), characterizing the state of pseudo-estrus. In addition, the SEM images (Figs. 6 - 7) also showed that the vaginal epithelium presented proliferation and maturation, probably in response to the hormonal concentration administered, confirmed by abundance of enucleated cells.

Local production of three protective cytokines, in each group, during vaginal candidiasis

The cytokine release analysis showed that only Th1 and Th17 were activated by vulvovaginal candidiasis, independent of the species evaluated. Detection was obtained for: TNF-α, IL-6 and IL-17. Other cytokines were not detected or were to values below the sensitivity of the assay.

In animals infected with *C. albicans*, we observed a pro-inflammatory response linked to the release of TNF-α and less marked by IL-6 and IL-17, Fig. 8A and B. For TNF-α, the non-diabetic group (Fig. 8A) presented stable values (126.78, 158.92, 117.28, respectively), while the diabetic group (Fig 8B) increased significantly in the last evaluated time (277.77, 151.37, 579.78, respectively). Similarly, in relation to IL-6, a small production was observed on the tenth day evaluated in the non-diabetic group (Fig. 8A) (2.94). On the other hand, in the diabetic group (Figure 8B), there was higher production in the first and, mainly, in the tenth day evaluated (26,62, 674,21, respectively). However, in the intermediate period the values were not detected (ND). IL-17 had production at the beginning and mainly at the tenth day evaluated (0.84, 19.65, respectively), and in the intermediate period values were not detected (ND) in the non-diabetic group. On the other hand, the diabetic group had production at the beginning and during the infection (8.45, 4.21, respectively) and at the end of the infection the values were not detected (ND).

In the animals infected with *C. tropicalis* there was an increase of TNF-α in the two groups on the first day, intermediate period and on the tenth day evaluated (66.35, 4234.49, 321.84, respectively) in non-diabetics (Fig. 8C). and in the diabetic group (Fig. 8D) the values were (301.34, 739.59, 4112.08, respectively). IL-6 showed high production in the intermediate period and on the tenth day in the non-diabetic group (Fig. 8C) (ND 3475.63 and 179.72, respectively), and in the diabetic group (Fig. 8D) (22.14, 91.47, 3.9, respectively). IL-17 had production on the tenth day and the intermediate period of infection (168.38, 33.86, respectively) and on the first day the values were not detected (ND) in the non-diabetic group. On the other hand, in the diabetic group values were not detected (ND).

Groups of animals infected with *C. glabrata* showed an increase in TNF-α for both groups on the first day, intermediate and tenth day (1434.12, 762.25, 321.43, respectively) in the non-diabetic group (Fig. 8E). In the diabetic group (Fig 8F), it presented values (446.82, 116.89, 845.16, respectively). IL-6 presented values in the intermediate period for the non-diabetic group (Fig. 8E) (176.48) and for the diabetic group (Fig. 8F) presented production on the first day, intermediate period and on the tenth day (1, 31, 162.72). and 154.00, respectively). For non-diabetic animals, IL-17 had constant release in the intermediate period (mean of 7.52). On the other hand, the diabetic group was not detected (ND) the release of IL-17.

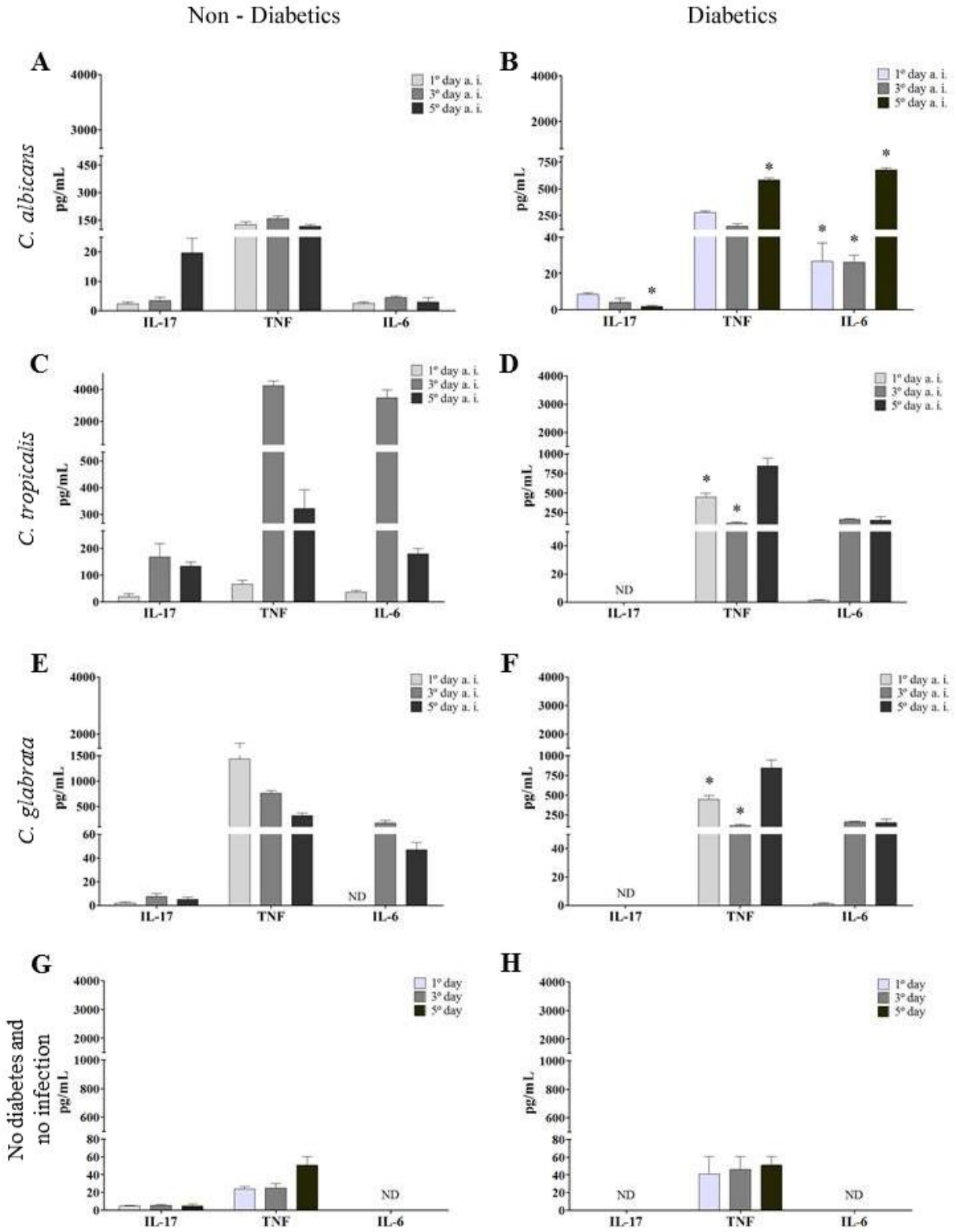

Fig 8. Pro-inflammatory cytokines IL-17, TNF-α and IL-6 in non-diabetic (A, C and E) and diabetic (B, D and F) mice infected with *C. albicans* (A and B) *C. tropicalis* (C and D) and *C. glabrata* (E and F). Control group without diabetes and without infection (G) and diabetic control group without infection (H). Cytokines were evaluated in at least three animals per group at key times during the experiment. The absence of bars represents undetected (non-detectable - ND) levels of the respective cytokine at the time evaluated. Dosages were performed in duplicate. a.i.: after infection. *statistically significant values, $p < 0.05$ compared to diabetic and non-diabetic groups.

4. DISCUSSION

VVC mainly affects women of childbearing age; however, its development depends of factors associated to the fungus and also to the host, such as DM, that has been considered a factor related to this infection. Although the association between diabetes and risk of developing VVC has been studied (6, 12), still is not clear how the diabetic condition contributes to promoting colonization. Thus, our study provides an extensive investigation about the physiopathology of VVC caused by *C. albicans*, *C. glabrata* and *C. tropicalis* associated with the diabetic host.

According to (Fig 1) we have shown, for the first time, a strategy for accompanying the diabetic mice, during the experimental time. As observed, serum and urinary glucose levels were high and constant from the second to the tenth day post-Alloxan injection. Most research uses just the serum dosage, but it has negative points as a method of collection and discomfort for the animal (6, 13). We chose to use serum dosage only after euthanasia, and the results are shown in Table 1. These data make sure that the Alloxan-induced animals maintained the diabetes status until the last evaluated day.

Although there are no consensus in the literature, research of DM and infectious states has been investigated and often correlated to the sugar-rich environment that favors the growth of microorganisms, with emphasis on fungal agents (6,14). In this sense, we for the first time in the literature, investigated the glucose dosage in the vaginal fluid of diabetic and non-diabetic animals, infected with one of the three *Candida* species. Table 1 clearly shows that the glucose quantification in vaginal fluid not only can be detected, as may be an important marker for the colonization of *Candida* species. Thus, we understand that this is our first clue to the understanding of VVC in a diabetic model.

For the development of a robust VVC model, the most important aspect and which demonstrates confidence is the maintenance of high and constant levels of estrogen during the experimental period (15). We submitted all animals, diabetics and non-diabetics, to exogenous estrogen-therapy weekly during the experimental period. Fig 2 summarizes the vaginal fluid analysis of the estrogenized animals. The cells show the typical pattern of the estrogenic phase, with abundant desquamation of cells anucleated, characterizing a mature and keratinized epithelium, susceptible to *Candida* infection. According to Nash et al., 2016, the estrogenized mice exhibit high fungal colonization when compared to non-estrogenized mice, which present low colonization.

In agreement, the Fig 3 shows our results regarding the diabetic state, which was generally associated with a higher number of CFUs in the vaginal fluid for the three species

evaluated, compared to the non-diabetic group. In addition, it is possible observe that statistical relevance occurred in the last days evaluated (7 and 10 days). On the other hand, in the vaginal tissue was possible to evidence a greater variability among the species evaluated. While in mice infected with *C. albicans* it was significative highest from the third day after infection, for *C. tropicalis* it occurred just from the seventh day, and for *C. glabrata* there was no statistical difference between diabetic and non-diabetic animals. Apparently, the species which are able to filamentation (*C. albicans* and *C. tropicalis*) were more efficient to vaginal tissue invasion, on the diabetic animal. In fact, this virulence attribute is an important factor related to the invasive process (6, 7). The yeast-hyphae transition stimulates the production of adhesions and proteolytic enzymes able of adhering and invading the epithelial tissue (16). It is known that the presence of glucose is an essential stimulus for the formation of pseudohiphae (17). In this sense, our findings of higher glucose concentration in the vaginal fluid of diabetic animals (Table 1) support the possibility of this carbohydrate stimulating the yeast-hyphae transition favoring the ability to adhere to the epithelial tissue.

Really, evidences about DM show an important change regarding available nutrients, and for a microorganism to colonize any environment, in diabetic host, it is necessary to adapt to these alterations (14, 18). The nutrients available in the host tissue have a total connection with as well to host as microbial metabolisms. In this sense, species which are able to filamentation, seem have more ability in this adaptation.

Our study shows, for the first time, the simultaneous evaluation of fungal burden between vaginal fluid and tissue. This experimental design, confirmed that *C. albicans* and *C. tropicalis* were more effective in invade vaginal tissue, since the CFU remained practically constant at all evaluated times, for diabetic group. On the other hand, in the non-diabetic group, the decrease in the fungal burden in vaginal tissue was 1.5-fold, 1.2-fold and 1.7-fold, for *C. albicans*, *C. tropicalis* and *C. glabrata*, respectively. This findings reinforced that the presence of higher levels of glucose in the vaginal fluid may be the first clue that DM is a risk factor for VVC.

Knowing the differentiated pattern of fungal load between the *Candida* species and between diabetic and non-diabetic groups, our next step was to investigate the relationship between vaginal cells and *Candida* by papanicolaou methodology. Fig 4 presents mature, large and anucleated epithelial cells, throughout the experimental period, for both groups and the three species evaluated. In addition, we observed a high adhesion of hyphae and yeasts on epithelial cells also at all times and species evaluated independent of diabetic state. On the other hand, diabetic group infected with *C. albicans* and *C. tropicalis*, showed clear

inflammation, marked by the greater presence of PMN, others inflammatory characteristics and hyphae formation. This profile is typical of a classic vaginal smear of women with active VVC, agreeing with other study (19).

In order to better understand the alterations in the vaginal epithelium, we performed the histopathological tests of the vaginal tissues. Overall, the diabetic animals showed higher histological findings related to inflammation and it was different among the yeasts species. Although the analysis showed that the three species were able to adhere to the vaginal epithelium, a differentiated infection pattern was observed. *C. albicans* had a greater capacity for colonization and epithelial invasion, followed by *C. tropicalis* and *C. glabrata*, which was present only in keratinized desquamative epithelium.

Indeed, *C. albican*s is an opportunistic fungus capable of causing mucosal and invasive infections, this species is well known regarding to ability for rapid adaptation in front of changing environments within the host (18). The morphological plasticity of *C. albicans* is an important virulence factor and plays a key role in mucocutaneous infections (20). During mucosal infection, such as VVC, hyphae invade the epithelial and endothelial cells, causing damage by the presence of hydrolytic enzymes (21). This ability to remain in the vaginal epithelium is probably the key point for this species to be more frequent in VVC cases, and support our fungal burden data. Ours results showed infiltration of PMNs and tissue congestion were evident in loose connective tissue, similarly to a recent study (22). It is important to note that, PMNs are indispensable components of the innate immune system. These cells play an important role in the host defense mechanism against several infections, especially in the initial period of response to infection, by a series of functions, such as chemotaxis, phagocytosis and generation of reactive oxygen species (16, 23).

Although *C. tropicalis* and *C. albicans* had been similar behaviour regarding the vaginal tissue invasion (Fig 3 and Fig 5), it seems *C. tropicalis* to have induced a greater inflammatory response in connective tissue, indicated by the presence of PMN cells. This specie is one of the most important species of *Candida* in terms of virulence, however, it is not very frequent in the epidemiological ranking for VVC (24). *C. tropicalis* has the ability to produce true hyphae, and considered to be highly adherent to epithelial cells and a potent biofilm producer (25). Despite, of our interesting results (Fig 3 and 4), they cannot explicit why *C. tropicalis* is few isolated from vaginal infection (18). The pronunciate fungal burden and visible inflammatory response in histological analysis, show the necessity of more studies relating *C. tropicalis* and VVC. In addition, it is possible to emphasize that to our knowledge, this is the first reproducible-VVC model by *C. tropicalis*.

C. glabrata is a commensal of the intestinal tract in humans, it is the second most common cause of VVC (16). Nevertheless, the pathogenesis of this specie in VVC infection is poorly understood (5). According to Nakamura-Vasconcelos, et al., 2017, *C. glabrata* is effective in adhering and forming biofilm, an essential virulence factor. Once adhered to biotic or abiotic surfaces, yeast continues to cause colonization and, consequently, may cause VVC symptoms. However, due to the absence of filamentation, the invasion mechanism appears impaired. Additionally, the absence of this virulence attribute seems to favor the activity of neutrophils on *C. glabrata* (27). In concordance with the literature, in current study, this species of *Candida* showed colonization restricted to the surface of the vaginal epithelium and low inflammatory infiltrate (Fig 5).

In addition, our results confirm an important link between diabetes and *C. glabrata* colonization (6), but it is still unclear how DM contributes to *Candida* colonization. Reinforcing this discussion, SEM images (Fig 6) show *C. glabrata* as being unable to filamentation and it is not easily visualized on vaginal tissue, even in the diabetic animal. Thus, seems this specie is able to cause VVC without to invade the vaginal tissue. These findings suggest *C. glabrata* have others virulence attributes instead inability to filament. This would explain the reason why this species has increased in human diabetic patients (6). Nevertheless this potential is not known yet.

Specifically, in the diabetic group, the murine VVC infection exhibit particular pattern for each one of *Candida* species evaluated (Fig 7). SEM images from vaginal tissue revealed different pictures regarding to vaginal mucosa. Mice infected with *C. albicans* showed an important desquamation of epithelial cells, indicating a closed yeast interaction, in addition, a great filamentation ability was observed in yeasts. Meanwhile, *C. tropicalis* was associated with dispersed epithelial cells, low cellular adhesion and lower colonization than *C. albicans* and showed a configured filamentous extracellular matrix, reinforcing a high invasive ability. According to Nett et al., 2015, this extracellular matrix is especially evidenced during biofilm formation *in vivo.* Examination of a biofilm formed on the surface of a vascular catheter revealed a dense febrillar coating on the yeasts and hyphae, suppostly host proteins may contribute to the biofilm maturation process *in vivo.*

Our results show that the diabetic host seems to favor infection by the different species of *Candida*, with a possible embrittlement of vaginal tissue, which could favor the colonization by species with filamentation attributes. The hyperglycemic state plays an important role in the inflammatory response. Since, the presence of glucose leads to glycosylation of intra and extracellular proteins, the products generated are deleterious to the

extracellular matrix leading to endothelial complications in diabetic people (6). We hypothesized that diabetic animals were left with the vaginal epithelium weakened by the presence of systemic and local glucose.

The literature shows that the state of diabetes is a metabolic condition that can lead to abnormalities in the immune response against different types of infections, including fungal infections (17). In this sense, we analyzed the release of cytokines into the vaginal fluid of diabetic and non-diabetic animals intravaginally infected with one of the three *Candida* species studied.

The local response in all animals analyzed was marked by a pro-inflammatory cytokines with the release of IL-6, TNF-α and IL-17 (Fig 8). In fact, fungal infections are markedly related to the Th1 inflammatory response, with the stimulation of cytokines that activate neutrophil recruitment and activate these cells to kill the fungal cell (29). TNF-α was the interleukin detected in all species analyzed and in both host groups. This interleukin is one of the main participants in the acute inflammatory process. Its most important actions in the endothelium induce adhesion molecules and chemical mediators such as the production of enzymes associated with matrix remodeling. In addition, TNF-α also induces the priming of neutrophils leading to an increase in the responses of these cells and other mediators (30). In this sense, we observed that this cytokine was present at high levels in all groups analyzed, including diabetic animals. It is understandable that this cytokine associated with IL-6 was a good helper to the elimination of yeasts in the non-diabetic group. On the other hand, in the diabetic group these interleukins were not sufficient to completely eliminate the microorganisms. A study has shown that, inflammatory cytokines TNF-α, IL-β and IFN-γ, as well as iNOS, are overexpressed in the diabetic kidneys (31), without efficiency in the elimination of the aggressor agent. Studies show that the sugar-rich environment triggers two strands that favor fungal infection. Indeed, glucose favors fungal colonization, inclusive data shown in this study. On the other hand, hyperglycemia impairs host defense, as it makes PMNs less responsive by showing the virulence aspects of fungal agents. The failure of the PMNs activity recruited by pro-inflammatory cytokines has already been attributed to different metabolic factors, but there is important evidence for the fact that hyperglycemia deviates intracellular metabolism via polio through the aldose reductase enzyme (31). This enzyme is key to antioxidant mechanisms and the reduction of its levels increases the susceptibility to oxidative stress. These findings contribute to the understanding that diabetic animals intravaginally infected with *C. albicans* and *C. tropicalis* have high fungal load even with the presence of inflammatory infiltrate seen in papanicolaou images and histology.

It should be noted that IL-17 was detected with greater evidence in non-diabetic animals. In fact, diabetic animals generally have low IL-17 (29, 32) values and it is known that this cytokine is important for maintaining the mucosal barrier effect. In this study, the absence or decrease of the expression of this cytokine could explain higher CFU of yeasts in the vaginal tissue, attributed greater fragility of the mucosa of diabetic animals. More studies would be needed to understand why diabetics negatively regulate IL-17. It is known that IL-6 is capable of inducing Th17 cells to release the cytokine IL-17 which has the function of enhancing the antifungal defenses (29, 32). Thus, it is evident that this cytokine may have contributed to prevent the colonization or to the clearance of yeasts in non-diabetic animals. However, this response is impaired in diabetics host, it is possible the absence of this inflammatory response had as consequence a persistence of fungus and recurrent or chronic infections (33).

Altogether, this study showed that the three species of *Candida* studied have a different pattern of infection. *C. albicans* and *C. tropicalis* have pathophysiological characteristics that keep them longer in the vaginal environment. In addition, diabetic animals are more susceptible to these species, probably due to failure in the immune response of major defense cells. On the other hand, *C. glabrata* has a light infection potential, independent of the host studied. Furthermore, this work evidenced important points which made the reason that *C. albicans* is the most frequent and most virulent agent during the VVC process. *C. glabrata* does not have a persistence in the infectious process. And finally, this investigation unveiled an insight about VVC by *C. tropicalis*, that its infectious pattern is similar to *C. albicans*.

Material and Methods

Ethics Statement

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by Institutional Ethics Committee for Animal Experimentation, State University of Maringá, Brazil (CEUA nº 3624120717). Mice were created in conditions free of specific pathogens in the animal facilities of the State University of Maringá, Brazil. Animals were treated according to the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize pain and discomfort in animals.

Mice

Balb / c female mice at approximately six weeks, mean weight of 23 grams, of age were used for this study,

***Candida* isolates**.

Three standard yeast strains of the genus *Candida* were used, *C. albicans* ATCC 90028, *C. glabrata* ATCC 90050 and *C. tropicalis* ATCC 750. All of them were stored at -80ºC at the Laboratory of Medical Mycology of the State of Maringá State. The inoculation will be performed 72h after estrogenization. For the purpose of interpreting the vaginal infection, was considered a period of 10 days after vaginal inoculation of the yeasts. Academically we assign day 1 as the beginning, day 5 representing the intermediate period (during) the infectious process and the 10th day as the end of the infection in the analyzed period.

Estrogenization and induction of females to the state of pseudo-estrus

The pseudo-estrus was induced by the estrogenization of the animals. An estrogen solution with a concentration of 3mg/mL was prepared from the solubilization of β-Estradiol 17-valerate (Sigma®, USA) in LIPO® sesame oil, also known as sesame oil. This solution was sterilized by filtration in a 0.22 μm microporous membrane and conditioned under aseptic conditions in a sterile vessel. Each mice received 0.3 mL of the solution, corresponding to a dose of 0.1 mg/animal, injected subcutaneously weekly, throughout the experimental period.

Type 1 diabetic murine model of *C. albicans, C. glabrata* and *C. tropicalis* vaginitis

To induce type 1 diabetes in mice, 80 mg / kg Alloxan (Sigma Aldrich Co., St. Louis, MO, USA) were injected once into the caudal vein from a new solution in sterile PBS (Phosphate Buffered Saline, 0.1M, pH 7.4, sterilized by autoclaving). To confirm hyperglycaemia, the urinary glucose level of mice were evaluated 48 hours after the administration of Alloxan, and it was considered rates higher than 180 mg/dL. The seric glucose levels were determined during the euthanasia process, and when exceed 120 mg/dL have confirmed the diabetogenic state. Estrogen-treated all mice were intravaginally inoculated by introducing 30 μL of PBS containing $1x10^6$*C. albicans*, $2x10^7$*C. glabrata* and *C. tropicalis.*

Assessment of vaginal infections

Estrogen-treated mice were divided in three groups with DM and three groups without DM (non-DM), with at least 5 animals each. Suspension of *C. albicans* ($1x10^6$ yeast/mL); *C.*

glabrata ($2x10^7$ yeast/mL) and *C. tropicalis* ($2x10^7$ yeast/mL) in 30 µL of PBS were inoculated intravaginally in mice from two groups (one DM and one non-DM). The inoculated mice were evaluated and submitted to euthanasia with isoflurane by asphyxia at 1, 3, 5, 7 and 10 days after infection, and the material was obtained for the following tests:

Vaginal fluid

100µL of lavage fluid was collected and used to quantify the fungal and recruitment load. The remaining fluid was centrifuged at 10.000 rpm (Mini Spin, Eppendorf AG 22331 Hamburg) for 5 min stored at - 80° C for further analysis of cytokines. For quantification of the fungal load, a 2 serial dilutions was performed using PBS and plated on Sabouraud Dextrose Agar (SDA), Kasvi, Italy, plates with Chloramphenicol. Colony forming units (CFU) were enumerated after incubation at 35° C for 24 h and expressed in CFU/ 100 µL of fluid. Lavage fluid was also spread on microscope slides (10 µL) for analysis of PMNs, filamentation and cells.

Papanicolaou stain

Vaginal cells were collected using a cytobrush before removed vaginal tissue and smears in glass slide with immediate fixed with ethanol. All smears were stained with Papanicolaou and qualitative evaluation in optical microscopy for detection of: cell inflammatory changes and presence of leukocytes. Analyses were evaluated in at least 20 different fields in 400x magnification.

Vagina

The vagina was removed and part of it was macerated with 1000µL of organ lysis buffer pH 8.3 to assess fungal load (CFU/mL) and plated, pure organ with buffer, and plus 2 serial dilutions 1:10 and 1:100 on SDA plates, incubated at 35 ° C for 24h.

Histology

After the development of experimental vulvovaginal candidiasis in mice, the vaginal tissue was fixed in 4% paraformaldehyde, paraffin-embedded and cut into thin sections (5 µm). The sections were stained by Grocott-Gomorimethenamine silver (GMS) to visualize fungi, and counterstaining with hematoxylin and eosin (H & E) for characterization of host cells. Slides were observed and photographed using a binocular light microscope (Motic BA310), with a camera (Moticam 5) coupled to a computer using Motic Images Plus 2.0 software.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed with vaginal tissue infected with *C. albicans* ATCC 10028, *C. glabrata* ATCC and *C. tropicalis* ATCC) were fixed by immersion in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M Sodium Cacodylatebuffer (pH 7,2), post-fixed in 1% O_sO_4 and dehydrated in the ethanol series (30, 50, 70, 90 and 100% ethanol). The samples were critical-point dried with CO2 (Baltec CPD 030 critical-point Dryer) and coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a scanning electron microscope (FEI Quanta 200) at x 1000, x 2000 and x 5000 enlargement.

Quantification of cytokines from vaginal fluid

The lavage fluids homogenate from each animal was collected and stored at -80ºC. The Th1 / Th2 / Th17 cytokine kit (BD Cytometric Bead Array (CBA)) was performed according to the manufacturer's instructions. TNF-α and IL-6 were tested by FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA). The results were analyzed according to a standard cytokine curve using the software FCAP Array 3.0. Only the detected cytokines and statistically different results were represented in graphics.

Statistical analysis

One-way ANOVA with Bonferroni's post-test were performed using GraphPad Prism version 5.0. Values of $p<0.05$ were considered statistically significant. The experiments were performed in triplicate and repeated in three separate assays.

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3. CAPÍTULO III

3.1 Conclusões

1. Neste estudo foi estabelecido um modelo consistente de Diabetes em camundongos Balb/c fêmeas. Além disso, a quantificação de glicose no fluido vaginal é possível e representa uma ferramenta útil para o estabelecimento do modelo diabético em camundongos, destinado a estudos sobre candidíase vulvovaginal.

2. Foi estabelecido modelo de CVV com as três espécies de leveduras do gênero *Candida* mais frequentes como agente de CVV humana, tanto em animais diabéticos quanto não diabéticos.

3. Nos animais diabéticos, *C. albicans* e *C. tropicalis* (espécies que são capazes de filamentar) foram mais eficientes quanto a invasão do tecido vaginal, confirmando que esse atributo de virulência é um fator importante no processo invasivo.

4. Nossos resultados de maior concentração de glicose no líquido vaginal de animais diabéticos sustentam a possibilidade desse carboidrato estimular a transição levedura-hifa, favorecendo a capacidade de adesão ao tecido epitelial.

5. No grupo de animais diabéticos, a carga fúngica no tecido vaginal foi maior em 1,5 vezes, 1,2 vezes e 1,7 vezes, para *C. albicans*, *C. tropicalis* e *C. glabrata*, respectivamente. Esses achados reforçam que a presença de níveis mais elevados de glicose no fluido vaginal pode ser o primeiro indício de que DM é um fator de risco para CVV.

6. *C. tropicalis* mostrou capacidade de colonizar e invadir o epitélio semelhantemente a *C. albicans*, o principal agente de CVV, porém parece ter induzido maior resposta inflamatória no tecido conjuntivo, indicado pela presença de células PMN.

7. Na Microscopia Eletrônica de Varredura (MEV) foi possível analisar interação entre leveduras e células epiteliais, principalmente no grupo dos animais diabéticos, mostrado que o hospedeiro diabético parece favorecer a infecção pelas diferentes espécies de *Candida*, com uma possível fragilização do tecido vaginal, o que poderia favorecer a colonização por espécies com atributos de filamentação.

8. As três espécies de leveduras são capazes de induzir resposta inflamatória independente do hospedeiro. Animais diabéticos parecem não induzir a resposta de células Th17, e apesar de TNF-α e IL-6 estarem presentes, não foram suficientes para eliminarem o patógeno.

3.2 Perspectivas futuras

Frente aos promissores resultados obtidos com o desenvolvimento de CVV em animais diabéticos com objetivo de estabelecer e padronizar um modelo consistente de Diabetes em camundongos Balb/c fêmeas análises adicionais também podem trazer resultados promissores em relação à patogenicidade de leveduras do gênero *Candida* em animais diabéticos.

C. glabrata por ser uma espécie com comportamento diferente de *C. albicans* e *C. tropicalis*, devido à incapacidade de filamentação, mas é a segunda espécie mais isolada em casos de CVV, merece ser motivo de estudos mais aprofundados. Além disso, investigar se ocorre ascendência de CVV da vagina para o útero, seriam bons objetos para a continuidade deste estudo, cujos resultados poderiam auxiliar na compreensão da patogenicidade e possíveis alterações epiteliais e imunológicas em hospedeiros diabéticos e não diabéticos.

Outra contribuição interessante seria avaliar a resposta imune sistêmica neste modelo, realizando dosagens de citocinas tanto no soro quanto no fluido vaginal de animais diabéticos e não diabéticos para análise e comparação de resultados.