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Feasibility of Histological Scoring and Colony Count for Evaluating Infective Severity in Mouse Vaginal Candidiasis

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Abstract: Qualitative measurement of the infective level is relatively difficult in experimental vaginal candidiasis. Female BALB/c mice aged 8 to 10 weeks were randomly divided into E1, E2 and E0 groups, which received subcutaneous injection of 0.05 mg, 0.1 mg of estradiol benzoate or 0.1 ml soybean oil 3 days before vaginal inoculation, respectively, and hormone treatment continued every other day thereafter. Each group was further divided into infected and noninfected subgroups. The infected mice were inoculated intravaginally with 10 μl (5×10^4 conidia) of *Candida albicans* suspension, while the noninfected mice were inoculated with 10 μl phosphate-buffered saline. Direct microscopic examination, colony count and vaginal histopathology including infection degree and inflammation extent were performed at 3, 7 and 14 days post inoculation. Estrogen treatment increased the vaginal fungal burden and extent of infection and inflammation compared with the control group, and 0.3 mg/week estrogen generally induced more severe infection and inflammation than 0.15 mg/week estrogen did. Colony count peaked on day 3 and decreased remarkably after 7 days. Infection score increased gradually during the first 7 days and decreased on day 14, while inflammation extent exacerbated progressively over the course of 14 days. This study demonstrates that the modified histological scoring system might be more feasible than colony count for evaluation of infectivity and dynamic change in experimental vaginal candidiasis.

Key words: *Candida albicans*, estrogen, mice, vaginal candidiasis

Introduction

Vaginal candidiasis (VC) is a common infection caused by *Candida albicans* in women of childbearing age. The pathogenesis of this infection is not fully understood, but may be related to the initial adherence of the yeasts to the vaginal mucosa, asymptomatic colonization and symptomatic infection [3]. The predisposing factors include immunosuppression, antibiotic misuse, diabetes mellitus, pregnancy, estrogen therapy, etc [3, 8]. Not only can sex hormones including estrogen and progesterone play an important role in regulating the physiology and functions of the female reproductive tract, but they can also determine both susceptibility and immune responses to sexually transmitted pathogens [9]. The responses of the vaginal epithelia to sexual steroids in rodents appear the same as in humans, so the murine models of VC have been widely applied to study the contributing factors, pathogenesis and treatment of the infection [3, 6]. In these models, the induction of pseudoestrus by subcutaneous injection of estrogen is pivotal for the establishment of persistent infection. The effective dose of estrogen for inducing pseudoestrus and persistent infection is ≥0.2 mg/week in rats and 0.01–0.5

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mg/week in mice [3–6, 8].

For qualitative measurement of the infective level in experimental VC, vaginal lavage culture and wet mount preparation are usually applied to determine the number of colony forming units (CFUs) and hyphal presence, respectively [3, 6]. The vaginal neutrophil count is simple and reliable for assessment of inflammation because other clinical symptoms (i.e., itching, swelling) are difficult to evaluate in this model [14]. Black *et al.* introduced the histological scoring system including fungal level, inflammation level and ultrastructural change for evaluating the infective severity in mouse VC [2]. Recently, an *in vivo* bioluminescence imaging technique has been developed to monitor the spatiotemporal behavior of cutaneous, subcutaneous, and vaginal *C. albicans* infections in live animals and to assess vaginal fungal burden [13]. However, the sensitivity of direct microscopic examination for the diagnosis of VC is 61% [10]. Hyphae could be miscounted, as they grow as a single colony on the agar plate and might not reflect the accurate fungal burden [14]. Repeated irrigation could impact the vaginal fungal titers [6]. Therefore, this study aimed to compare the feasibility of histological scoring and colony count in the assessment of infective severity in mouse VC induced by two different doses of estrogen.

Materials and Methods

Preparation of inoculum

The strain of *C. albicans*(CGMCC 2.2411) was purchased from the Institute of Microbiology, Chinese Academy of Sciences. The solid yeast was grown in liquid medium containing 1% peptone (Oxoid Ltd., Hampshire, England) and 0.1% glucose at 25°C for 16 to 18 h in a shaking water bath, and then cultured on Sabouraud dextrose agar (Oxoid Ltd., Hampshir, England) at 30°C for 48 h. Colonies were harvested using a platinum loop and suspended in sterile phosphate-buffered saline (PBS). The conidial suspension was counted by a hemacytometer and adjusted to the final inoculum of 5×10^6 conidia/ml.

Animals

Specific pathogen-free female BALB/c mice (age, 8–10 weeks; mean weight, 24 ± 4 g) were obtained from the Laboratory Animal Center of Southern Medical University. The mice were kept at a temperature of 22°C with a 12-h light/dark cycle, and provided food and water *ad libitum*. This research was approved by the Laboratory Animal Ethical Committee of Guangdong Medical College.

Table 1. Animal grouping and subcutaneous injection dose of estrogen

Groups	Estrogen*		
	0	0.05 mg	0.1 mg
Non-infection	E0n	E1n	E2n
Infection	E0i	E1i	E2i

*Estrogen treatment given on alternate days.

Construction of a murine model

A total of 180 mice were randomly divided into 6 groups, each containing 30 animals (Table 1). The E0 group was injected subcutaneously with 0.1 ml soybean oil (Shenzhen Nanshun Grease Co., Ltd.., Shenzhen, China), and the E1 and E2 groups were injected subcutaneously with 0.05 mg and 0.1 mg of estradiol benzoate (Shanghai Tongyong Pharmaceutical Co., Ltd.., Shanghai, China) in soybean oil, respectively; hormone treatment started 72 h prior to inoculation and continued every other day until completion of the study. The infection and non-infection groups were inoculated intravaginally with 5×10^4 conidia in 10 μl PBS and 10 μl PBS, respectively. General and vulvovaginal manifestations of all mice were monitored daily.

Direct microscopic examination

Four mice of each group were randomly selected at 3, 7 and 14 days post inoculation (dpi). Vaginal smears were prepared with 10% potassium hydroxide and observed by light microscopy.

Colony count

Three mice of each group were randomly selected and received vaginal lavage at three time points. With the help of a pipette, 0.1 ml PBS was introduced and aspirated in the vaginal cavity 10 times. The 20 μl lavage fluid was admixed with 480 μl PBS, and then 50 μl was plated on Sabouraud dextrose agar containing chloramphenicol (0.05 mg/ml). The CFUs were calculated for 48 h at 30°C. Each sample was cultured twice.

Histopathologic examination

Three mice of each group at the three time points were sacrificed by cervical dislocation. The isolated vaginae

were fixed in 10% neutral formalin solution, and embedded in paraffin. A 4 μm section was cut longitudinally for hematoxylin and eosin and periodic acid-Schiff staining. All slides were observed on a BX51 system microscope (Olympus, Tokyo, Japan), digitized with a DP71 digital camera (Olympus) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Extent of infection and inflammation

Five visual fields of quintile sections were examined at ×400 magnification. The extent of infection and inflammation was evaluated by a modified method based on the report of Black *et al.* [2], and the average scores of five fields were recorded in each section.

Infection degree was scored as (1) absent (score of 0), meaning no yeast elements; (2) mild (score of 1), meaning 1–30 yeast elements in the vaginal lumen and cornified epithelial layer, or ≥5 in the upper 1/3 layer of the vaginal mucosa; (3) moderate (score of 2), meaning 31–60 yeast elements in the vaginal lumen and cornified epithelial layer or ≥5 in the upper 2/3 layer of the vaginal mucosa; and (4) severe (score of 3), meaning >60 yeast elements in the vaginal lumen and cornified epithelial layer or ≥5 in the whole layer of the vaginal mucosa.

Inflammation extent was scored as (1) absent (score of 0), meaning no neutrophils; (2) mild (score of 1), meaning 1–10 neutrophils or 1–3 microabscesses in the epithelial layer and a few neutrophils in the submucosa; (3) moderate (score of 2), meaning 11–20 neutrophils or 4–6 microabscesses in the epithelial layer and some neutrophils in the submucosa; and (4) severe (score of 3), meaning >20 neutrophils, >6 microabscesses or large abscess formation in the epithelial layer and numerous neutrophils in the submucosa.

Statistical analysis

The data were expressed as means ± SEM, and analyzed using ANOVA with a least significant difference post hoc test or Welch's test with Dunnett's T3 post hoc test.

Results

General and vulvovaginal manifestation

Infected mice showed reduced activity and dysphoria at 3 dpi, especially one day following each injection of estrogen. Uninfected mice had mild general symptoms one day after estrogen injection. The vulva appeared red and swollen at 3 dpi, and this was exacerbated by a whitish cheesy discharge after 7 dpi in the E1i and E2i groups. However, vulvovaginal manifestations were absent in the E0i and non-infection groups.

Table 2. Effect of estrogen dose on vaginal fungal burden

Groups	CFU (10^4/ml)		
	3 d	7 d	14 d
E0i	0.73*	0	0
E1i	6.81 ± 0.06	1.57 ± 0.03†	0.13 ± 0.01†‡
E2i	9.81 ± 0.06^{a}	3.43 ± 0.11^{a†}	0.32 ± 0.01^{a†‡}

Data are presented as means ± SEM. *Statistical analysis was not performed because only one animal was positive. ^{a} P<0.05 when compared with the E1i group at the same time points. $^{\dagger}P$<0.05 when compared with day 3. $^{\ddagger}P$<0.05 when compared with day 7.

Direct microscopic examination

There were undetectable fungal elements in non-infection groups at all time points. Fungal elements were present at 3 and 7 dpi but absent at 14 dpi in the E1i and E2i groups. One case was positive only at 3 dpi in the E0i group.

Vaginal fungal burden

No colonization of *C. albicans* was detectable in non-infection groups at all time points. The CFUs in the E1i and E2i groups peaked at 3 dpi, decreased remarkably at 7 dpi and reached a nadir at 14 dpi (Table 2). Fungal burden was higher in the E2i group than in the E1i group at the three time points (P<0.05). In contrast, a fungal titer was only detected at 3 dpi in the E0i group.

Extent of infection and inflammation

In non-infection groups, there were intact mucosa and no detectable yeasts during the observation period, but mild telangiectasia and a few inflammatory infiltrates were present in the lamina propria. In the E0i group, there were a small amount of yeasts only at 3 dpi and a small amount of inflammatory cells at the three time points. In the E1i and E2i groups (Fig. 1), there were a relatively intact mucosa and adherence of numerous yeasts at 3 dpi; an inflammatory response and hyphal penetration in the superficial mucosa at 7 dpi; and neutrophil invasion and microabscesses in the mucosa and mixed inflammatory infiltrates in the lamina propria at 14 dpi, but fungal elements were diminished.

Fungal infection started at 3 dpi, peaked at 7 dpi and

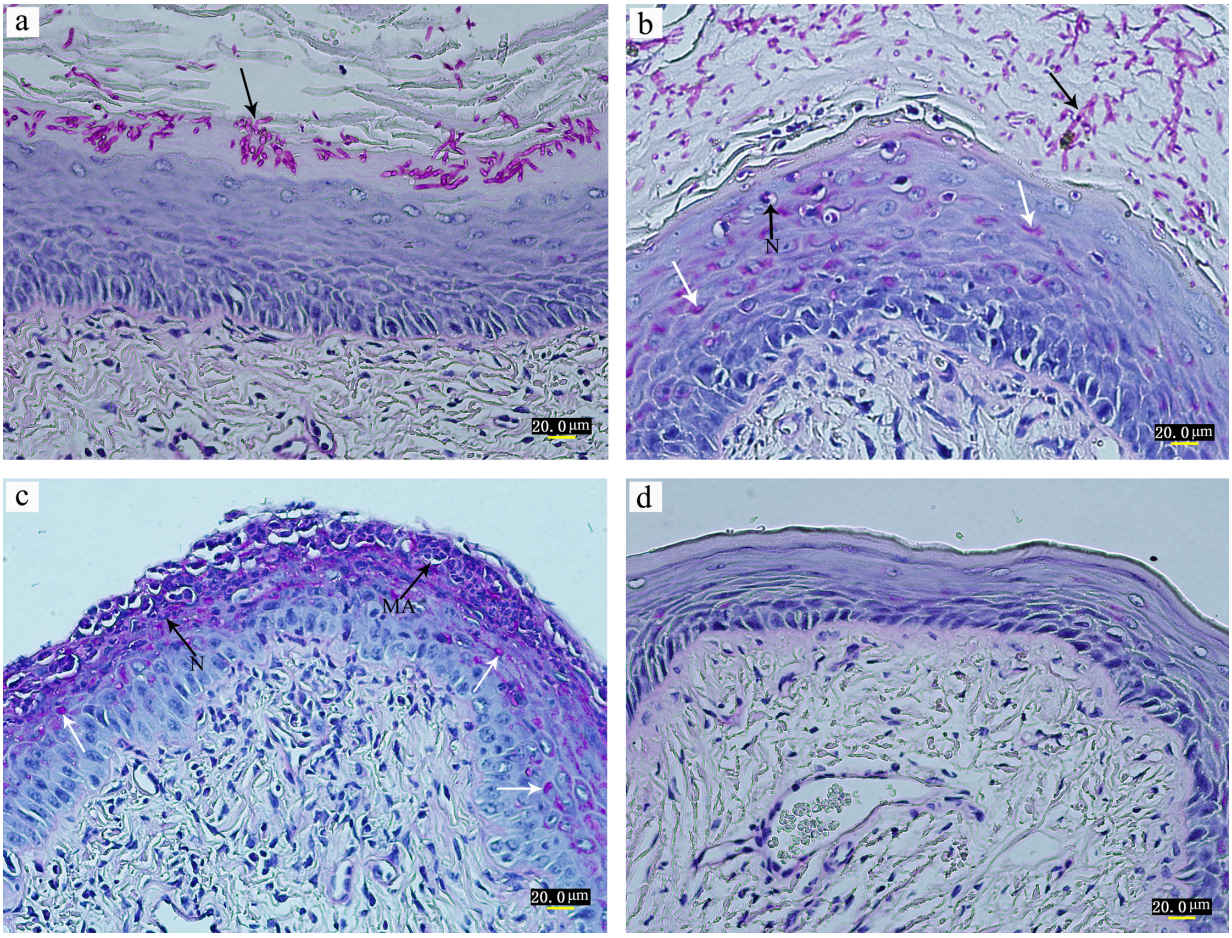

Fig. 1. Infection and inflammation extent in vaginal histopathology (PAS stain, 400×). (a) Numerous pseudohyphae and blastoconidia (black arrow) adhered to the cornified epithelium in the E2i group at 3 dpi (infection degree, score=3; inflammation extent, score=1). (b) Pseudohyphae and blastoconidia (black arrow) adhered to the cornified epithelium, and endocytosed hyphae (white arrows) and neutrophil (N) infiltration in the mucosa in the E2i group at 7 dpi (infection degree, score=3; inflammation extent, score=2). (c) Subcorneal microabscesses (MA), neutrophil (N) infiltration and endocytosed hyphae (white arrows) in the mucosa in the E2i group at 14 dpi (infection degree, score=3; inflammation extent, score=3). (d) Intact mucosa without neutrophil infiltration and hyphal invasion in the E2n group at 7 dpi (infection degree, score=0; inflammation extent, score=0).

decreased at 14 dpi in the E1i and E2i groups (P<0.05), and it was higher in the E1i and E2i groups compared with the E0i group (P<0.05) (Table 3). Inflammation response started at 3 dpi, increased at 7 dpi and peaked at 14 dpi in infection groups (P<0.05), and it was higher in infection groups than that in non-infection groups (P<0.05) (Table 4).

Discussion

The estrous cycle of rodents is similar to that of humans, but lasts only 4–6 days [3]. The capacity of estrogen to induce VC is definite, and the possible mechanisms are mainly as follows: (1) it converts the vaginal columnar epithelium into a thick stratified squamous epithelium and adds to glycogen content and growth substrates [5, 14]; (2) it facilitates *C. albicans* adhesion, growth, colonization and germ tube formation [8, 15]; and (3) it suppresses cellular immunity and delayed-type hypersensitivity [8]. The present study showed that estrogen treatment increased the vaginal fungal burden and extent of infection and inflammation compared with the control group, and 0.3 mg/week estrogen generally induced more severe infection and inflammation than 0.15 mg/week estrogen did. However, only one mouse presented with positive mycological results at 3 dpi in the

Table 3. Effect of estrogen dose on infection degree

Groups	Infection degree (scores of 0–3)		
	3 d	7 d	14 d
E0i	0.33 ± 0.16	0.39 ± 0.13	0.24 ± 0.11
E1i	1.80 ± 0.15[a]	2.56 ± 0.21[a†]	2.28 ± 0.12[a†‡]
E2i	1.92 ± 0.14[a]	2.77 ± 0.17[ab†]	2.45 ± 0.23 [a†‡]

Data are presented as means ± SEM. [a] $P<0.05$ when compared with the E0i group at the same time points. [b] $P<0.05$ when comparing the E1i and E2i groups at the same time points. [†] $P<0.05$ when compared with day 3. [‡] $P<0.05$ when compared with day 7.

Table 4. Effect of estrogen dose on inflammation extent

Groups	Inflammation extent (scores of 0–3)		
	3 d	7 d	14 d
E0n	0.11 ± 0.04	0.24 ± 0.06	0.18 ± 0.07
E0i	0.26 ± 0.06	0.47 ± 0.03[a†]	0.53 ± 0.05[a†]
E1n	0.38 ± 0.09	0.47 ± 0.06	0.52 ± 0.07[†]
E1i	1.61 ± 0.12[ab]	2.41 ± 0.21[ab†]	2.72 ± 0.28[ab†‡]
E2n	0.44 ± 0.06	0.51 ± 0.07	0.75 ± 0.13[†‡]
E2i	1.94 ± 0.17[abc]	2.56 ± 0.27[abc†]	2.81 ± 0.23[ab†‡]

Data are presented as means ± SEM. [a]$P<0.05$ when comparing the infection and non-infection groups at the same time points. [b]$P<0.05$ when compared with the E0i group at the same time points. [c]$P<0.05$ when comparing the E1i and E2i groups at the same time points. [†]$P<0.05$ when compared with day 3. [‡]$P<0.05$ when compared with day 7.

mice without estrogen treatment. These results testify further that estrogen is a prerequisite for the construction of mouse VC models.

Quantification of CFUs from vaginal lavage is the standard technique for assessment of vaginal fungal burden [3, 5]. *Candida* blastoconidia and hyphae normally did not penetrate beyond the superficial layer of the vaginal epithelium, and residual fungal elements were seldom found in the sections of post-lavage vaginal tissues [14]. Nevertheless, the hyphae aggressively penetrated into the mucosal epithelium and were more densely packed in the cornified epithelium at 7 dpi [2]. We also found that hyphal penetration occurred in the superficial mucosa at 7 dpi. In the meantime, the fungal elements of inoculum were inevitably present in the early vaginal lavage. Additionally, we observed that CFU count decreased and infection degree increased gradually with time. Vaginal wash can dislodge only superficial yeasts in the lumen, but not the deeper hyphae. It is uncertain how many colonies may originate from the hyphal fragments [2]. Therefore, the CFU count could not reflect the accurate fungal burden and might be an unreliable indicator of infective severity.

The vaginal neutrophil count is a simple and reliable indication of inflammation in the mouse VC model, and hyphal scoring is also useful for the assessment of infection [14]. Although the histological scoring system provided by Black *et al.* is a valuable index for evaluating the infective severity in mouse VC [2], a few drawbacks are present in the practice. First, the yeast elements in the vaginal lumen and cornified epithelial layer cannot be fully determined, and hyphal penetration and induced endocytosis may attract insufficient attention in the grading scale of infection degree. The induced endocytosis and active penetration are involved in the adhesion and epithelial invasion of *C. albicans*, and may ultimately lead to cell damage [11]. Meanwhile, the breakpoint of 50 yeast elements for stratifying into mild and florid infection seems to be not meticulous. Therefore, the infection degree was revised to 4 levels, in which the count of yeast elements in the vaginal lumen and cornified epithelial layer or in the vaginal mucosa were all considered. Second, although the inflammation level included 4 categories, moderate and severe inflammation was not quantitative. Thus, we tried to utilize the breakpoint of 11–20 neutrophils or 4–6 microabscesses in the epithelial layer as a grading index. Third, the so-called ultrastructural change could be arbitrary because the vascular shape might be often affected by the cut surface; in the present study, submucosal necrosis was always absent, so the ultrastructural change was omitted. The modified histological scoring system comprises the infection degree and inflammation extent, and each has 4 scores. In the present experiment, the CFU count approached the maximum at 3 dpi and decreased remarkably after 7 days. Infection score increased gradually during the first 7 days and decreased at 14 dpi, while inflammation extent was progressively exacerbated over the course of 14 days. Vulval symptoms were generally correlated with the inflammation classification in the infected mice. Although endocytosed hyphae might sometimes be difficult to count at 7 dpi, the modified histological scoring system could be more suitable for evaluation of infectivity and dynamic change in experimental VC.

The infection score and fungal burden decreased gradually without antifungal treatment during the experimental period even if estrogen was continuously administered. The innate immunity is now considered to be implicated in both resistance and susceptibility to VC

or recurrent VC. Vaginal epithelial cells represent the predominant resident innate immune cell in the vagina, and can inhibit the growth of *C. albicans in vitro* and modulate the inflammatory response to microbicides [1, 7]. Vaginal epithelial cells may retard or arrest growth rather than kill *C. albicans* through a carbohydrate moiety in a noninflammatory manner [12]. Consequently, *C. albicans* can be naturally eliminated in the mouse VC with time.

In conclusion, the modified histological scoring system might be more feasible than the colony count for evaluation of infectivity and dynamic change in experimental VC. Vaginal lavage can be done more than once in the same mouse, but histological scoring requires euthanization of the mouse. Colony counting after vaginal lavage may be a first step in detection of VC, and if no colonies are observed, then one can perform histological scoring, the latter being able to also detect the severity of the infection.

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