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Optimization of Vulvovaginal Candidiasis Induction in *Rattus norvegicus* using Estradiol Benzoate: A Preliminary Study

Dariatul Janah[1], Andi Yasmon[2*], Ibnu Agus Ariyanto[3]

[1]Master of Biomedical Sciences Program, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[3]Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

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Corresponding author:
Andi Yasmon
E-mail address:
Andiyasmon@gmail.com

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ABSTARCT

Introduction. Approximately 75% of women of reproductive age have opportunistic vaginal infection with *Candida albicans* at least once in their lifetime, which is known as vulvovaginal candidiasis (VVC). The increasing global prevalence and emergence of antifungal agents underscore the importance of research into effective prevention and treatment. *In vivo* studies using rats are commonly conducted, as they provide valuable knowledge on reproductive diseases in humans such as VVC. **Methods**. Pseudoestrus was induced in wistar rats by subcutaneous injection of estradiol benzoate at doses of 0.1 and 0.2 mg/rat/week. Cytologic observation using vaginal smear staining is used to detect cornified epithelial cells and leukocytes. VVC was induced by intravaginal administration of *Candida albicans* ATCC10231 (0.5 and 1.0 McFarland) daily for 5 consecutive days. VVC confirmation by microscopic detection of *Candida albicans* pseudohyphae from vaginal swabs, supported by viability testing of vaginal lavage cultures. **Results**. Pseudoestrus induction was optimal at a dose of 0.2 mg/rat/week, as both animals were maintained in this phase for 3 consecutive days. Furthermore, an inoculum of 1.3×10^7 CFU/mL (McFarland standard 1.0) administered for 5 days successfully induced VVC in both animals. Microscopic confirmation revealed intracellular pseudohyphae, supported by the growth of viable *Candida albicans* from vaginal lavage cultures. **Conclusion**. The optimal estradiol benzoate protocol for pseudoestrus induction was 0.2 mg/rat/week, and pseudoestrus was maintained for 3 days without ovariectomy. The optimal VVC rat model was established using intravaginal *Candida albicans* at 1.3×10^7 CFU/mL for 5 days, with viability from lavage cultures of 1.3×10^2 CFU/mL and 0.3×10^2 CFU/mL.

1. Introduction

Vulvovaginal candidiasis (VVC) is an opportunistic fungal infection of the lower female reproductive tract, specifically the vulva and vagina, caused by *Candida* species.[1] More than 90% of cases are attributed to *Candida albicans*, where as the remaining 10% are caused by *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis*.[2,3] VVC is the second most common vaginal infection among women, with approximately 75% of women of reproductive age experiencing at least one episode in their lifetime. In addition, approximately 5–9% of women experience recurrent infections exceeding four episodes per year.[3,4] Characteristic symptoms such as itching, burning sensation, redness, and thick discharge are often overlooked but significantly affect reproductive health and quality of life.[3] Furthermore, in 2022 the World Health Organization (WHO) classified *Candida albicans* as a critical priority pathogen in the Fungal Priority Pathogen List (FPPL).[5] Rather than solely emphasizing the global emergence of antifungal resistance, this classification highlights the urgent need to establish robust, reproducible, and less invasive animal models. Such models are critical for translational research to better understand host-pathogen interactions and to evaluate the efficacy of novel, complementary therapies such as probiotics.[5]

Animal models, particularly female rats (*Rattus norvegicus*) are widely used in *in vivo* biomedical and medical research to study VVC because they provide important insights into human reproduction and host immune responses.[6,7] However, the rat vagina naturally possesses an efficient self-clearing mechanism and is resistant to fungal colonization. Traditionally, rendering rats susceptible to *C. albicans* infection requires exogenous hormonal manipulation, which is most frequently combined with surgical ovariectomy to suppress local immunity and eliminate the variability of endogenous estrogen.[8,9] While ovariectomy guarantees a stable pseudoestrus phase, it severely disrupts basal host physiology by depleting all ovarian hormones and profoundly altering systemic immune responses. Furthermore, ovariectomy has been shown to artificially deplete native *Lactobacillus* populations in

the vaginal microbiota, which drastically deviates from the pathophysiology of intact human females.[10] Therefore, a non-ovariectomized model is highly preferable for translational studies, as it better mimics natural human physiology and provides a more accurate *in vivo* environment for evaluating therapies. The present study addresses this gap by attempting to establish a less invasive and highly reproducible VVC rat model using estradiol benzoate alone, presenting a significant methodological refinement over traditional invasive protocols.

The estrous cycle in rodents shares functional parallels with the human menstrual cycle, consisting of four phases (proestrus, estrus, metestrus, and diestrus) and lasting approximately 4-5 days.[11,12] The estrus phase in rats can be prolonged into a pseudoestrus phase by injecting synthetic estrogen hormones such as estradiol benzoate.[8] As a lipophilic molecule, estradiol can cross the cell membrane via passive diffusion and reach the cytoplasm, where it binds to intracellular estrogen receptors.[13] When combined with sesame oil, which contains lipophilic fatty acids and antioxidants, estradiol benzoate is slowly released, effectively maintaining estrogen levels to enable pseudoestrus induction over several days.[14]

During this phase, vaginal epithelial cells undergo cornification, providing a dense keratinized substrate for *Candida albicans* colonization and pseudohyphal invasion. Crucially, estrogen signaling directly modulates local innate immunity. High estrogen states suppress protective mucosal immunity and place recruited polymorphonuclear neutrophils (PMNs) into a state of functional anergy.[15] Although estrogen administration drives a massive influx of neutrophils to the vaginal lumen, these innate immune cells fail to execute effective phagocytosis. This dysfunctional neutrophil infiltration allows *Candida* virulence factors, such as Secreted Aspartyl Proteases (SAPs), to evade immune clearance and induce severe immunopathology and epithelial damage.[15,16,17]

Despite the success of various earlier VVC models, significant knowledge gaps remain regarding the optimal hormone dosage and *Candida* inoculum concentrations in non-ovariectomized rats. Previous studies often report inconsistencies; suboptimal estrogen doses fail to overcome endogenous hormonal fluctuations, preventing the establishment of the infection, whereas excessively high doses can induce systemic toxicity. Similarly, the use of overwhelmingly high *C. albicans* inoculums in previous studies can mask natural host-pathogen dynamics and lead to exaggerated inflammatory responses.[18] Thus, optimizing the dose and volume of the hormone administered without ovariectomy is critically important.[19] This preliminary study aims to address the limitations of earlier protocols by determining the specific threshold dose of estradiol benzoate (0.1 mg and 0.2 mg) required to reliably induce pseudoestrus without the confounding effects of ovariectomy. By doing so, this study will reduce animal suffering and identify the most effective *Candida albicans* inoculum size necessary to trigger a sustained active infection over a defined time frame, thereby establishing a robust and physiologically relevant baseline for future VVC research.

2. Methods

Study Design

This preliminary study employed a pure experimental *in vivo* design using a rat model of VVC induced during the pseudoestrus phase with estrogen hormone. Hormone injection and intravaginal administration of *Candida albicans* ATCC10231 suspension served as the independent variables, which influenced changes in the estrous cycle of female rats (*Rattus norvegicus*) and VVC induction as the dependent variable. The study was conducted from February to March 2026 in the animal laboratory and the Mycology and Microbiology laboratory of the Faculty of Medicine, Universitas Indonesia (UI).

Candida albicans Isolate

The fungal isolate used was *Candida albicans* ATCC10231 to induce VVC from the Clinical Microbiology Department of FKUI, subcultured on SDA medium was used. *Candida albicans* colonies were resuspended in sterile 0.85% NaCl to achieve standart McFarland turbidity standards of 0.5 and 1.0, corresponding to approximately 10^6–10^7 CFU/mL. The *Candida albicans* ATCC10231 suspension was stored in a refrigerator at 4°C.

Ethical Approval

This *in vivo* experimental study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia–Dr. Cipto Mangunkusumo National Hospital (KEPK FKUI-RSCM) with approval number KET-236/UN2.F1/ETIK/PPM.00.02/2026, protocol number 26-01-0069. The number of experimental animals used in the preliminary study followed the ARRIVE 2.0 guidelines and the 3R principles (*Replacement, Reduction, Refinement*). The minimal animal was determined by the number of animals was determined following the *Reduction* principle. Therefore, only 2 animals per group were used, giving a total of 4 female Wistar rats.

Population and Sample Size

Candida albicans ATCC10231 was used to induce VVC in female Wistar strain rats (*Rattus norvegicus*). The study subjects were female Wistar rats meeting the inclusion criteria of being healthy, non-pregnant, with a body weight of 150–180 grams and aged 8–10 weeks. The exclusion criteria was as follows sick, pregnant, or rats under 8 weeks of age. Animal allocation into experimental groups was performed using simple randomization via manual lottery methode. Allocation concealment was maintained by having an independent technician assign the animals, ensuring the primary

investigators were blinded to the group allocations prior to the intervention. Animals were provided with food and water *ad libitum* and housed at a room temperature of 25–26°C with a 12/12-h light/dark cycle, 34%–35% humidity, and 2 rats per cage. The animal housing facility meets all appropriate standards and strictly adheres to the applicable BPOM (Indonesian Food and Drug Authority) guidelines. In accordance with BPOM guidelines, the testing facility must provide appropriate temperature, humidity, lighting, and noise levels tailored to the animals' physiological needs, maintaining the temperature at 22 ± 3°C, relative humidity at 30–70%, and a 12-h light/12-h dark illumination cycle.[20]

Preliminary Study of Wistar Rats

Female Wistar strain rats (*Rattus norvegicus*) were acclimatized for 7 days, after which the preliminary study was conducted. The study comprised 2 groups with 2 rats per group, giving a total of 4 rats. The preliminary study aimed to optimize all procedures, from pseudoestrus induction to VVC induction through intravaginal administration of *Candida albicans* ATCC10231. The steps of the preliminary study were as follows:

Pseudoestrus Induction in Rats

Pseudoestrus was induced in Wistar rats via subcutaneous (SC) injection of estradiol benzoate at doses of 0.1 and 0.2 mg/rat/week. The hormone was dissolved in sterile sesame oil (filtered through a 0.22 µm membrane) and administered beneath the abdomen in three divided injections (50 µL or 100 µL per injection). This preliminary study aimed to confirm the optimal dose of estradiol benzoate required to properly induce and maintain the pseudoestrus phase.

Daily monitoring of the estrous cycle was conducted each morning by observing the external vaginal morphology and evaluating vaginal cytology. Vaginal swabs were collected using sterile saline-moistened cotton swabs. Smears were immediately prepared on glass slides, fixed in methanol, air-dried, and stained with crystal violet for 3–4 minutes. Following a tap water rinse and air-drying, the slides were mounted with Entellan, coverslipped, and examined under a light microscope. The pseudoestrus phase was confirmed at 10× and 20× objective magnifications by the predominant presence of large, irregularly shaped, anucleate (cornified) epithelial cells, alongside a distinct absence of leukocytes and nucleated epithelial cells.[12,21]

Due to personnel limitations in this preliminary study, microscopic assessments for pseudoestrus identification were performed by the primary investigator without blinding procedures. To minimize bias and ensure objectivity, the estrous cycle phases were strictly evaluated based on the established morphological criteria for rodent vaginal cytology, as described by Begum et al., (2020) and Ajayi et al., (2020). However, the lack of an independent, blinded assessment is acknowledged as a limitation.

Figure 1. Ampule 1 mL containing 5 mg of estradiol benzoate hormone (left) and the estrogen hormone suspension dissolved in sterile sesame oil (right)

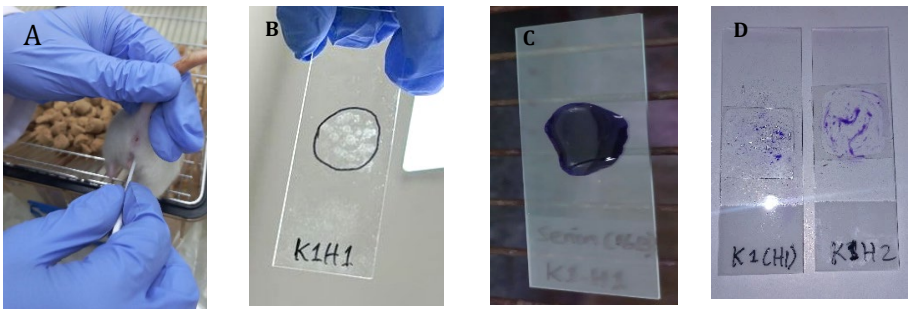

Figure. 2. Cytological examination of the estrous cycle via vaginal swab for smear preparation using simple staining: a) vaginal swab in rats, b) vaginal smear on a glass slide, c) crystal violet staining, d) slide mounted with Entellan and coverslip

Figure. 3. Standart McFarland 0.5 and 1.0 measurements of *C. albicans* ATCC10231 suspension using a Nephelometer

Induction of Vulvovaginal Candidiasis in Rats

Vulvovaginal Candidiasis was induced in female rats by intravaginal administration of a suspension containing *Candida albicans* ATCC10231 cells. The *Candida albicans* isolate was suspended in 100 µL of sterile 0.85% NaCl at two McFarland turbidity standards 0.5 and 1.0 measured using a Nephelometer. A 10 mL stock of 0.85% NaCl was prepared by mixing 9.4 mL of 0.9% NaCl with 0.6 mL of sterile distilled water. The McFarland standard suspensions were quantified by serial dilution (10^{-3} to 10^{-5}), plated in duplicate on SDA medium and incubated at 37°C for 48 hours. The number of *Candida albicans* colonies grown on SDA was converted to CFU/mL using the following formula:[22]

$$N = \frac{\text{Ɛc}}{\text{V.d}}$$

Notation:

N : Inoculum concentration (CFU/mL)

Ɛc : Number of colonies on the Petri dish

V : Inoculated volume (0.1 mL)

D : First dilution factor within the countable range (*Candida albicans* 30–300)

Intravaginal administration of *Candida albicans* ATCC10231 suspension was performed daily for 5 days, vaginal swab and lavage using physiological saline used to identify and confirm VVC. Swab specimens were examined microscopically using Gram staining. The structures of *Candida albicans* included pseudohyphae, intracellular pseudohyphae, budding cells, and spores. Cultures were observed and the number of *Candida albicans* colonies was quantified in colony-forming units per milliliter (CFU/mL).

Microscopic Examination of *Candida albicans*

Gram-stained vaginal swab smears were used for microscopic examination of the fungus to visualize *Candida albicans* structures, including pseudohyphae, yeast cells, and spores.[23] Gram staining was performed by applying crystal violet to cover the entire smear surface during 1 minute, followed by gentle rinsing with running water. Iodine was then applied and left for 1 minute before carefully rinsing again with running water to prevent specimen loss. Decolorization was performed with alcohol for 15–30 s, followed by safranin counterstaining for 30–60 s. The slides were examined under a microscope at 10× and 20× objective magnification, at which yeast cells and pseudohyphae were clearly visible. Although a formal blinding procedure was not implemented, potential observer bias during the microscopic detection of pseudohyphae from vaginal smears was carefully minimized through strict adherence to standardized criteria and cross-verification by a second, independent observer.

***Candida albicans* from Vaginal Lavage**

Fungal culture from vaginal lavage was used for semiquantitative assessment by colony counting of *Candida albicans* in CFU/mL using the total plate count (TPC) method on SDA medium. The entire procedure involved vulvovaginal lavage of the rats with 100 µL of sterile 0.9% NaCl. The lavage fluid was collected in a sterile microtube and immediately cultured on SDA medium. The full procedure involved two rounds of vulvovaginal washing with 100 µL of sterile 0.9% NaCl. The collected fluid was pooled in a sterile microtube and homogenized by vortexing. A volume of 100 µL of fluid was plated on SDA using the spread method with an L-spreader and incubated at 37°C for 48 hours. *Candida albicans* colonies were counted within the range of 30–300 CFU/mL. To ensure the reliability of the quantitative data, the counting and identification of *Candida* colonies on SDA from vaginal lavage were evaluated using objective literature guidelines and cross-verified by the independent observer.

Animal welfare, body weight, and signs of distress for example, lethargy, hunched posture, ruffled fur were monitored daily. Humane endpoints requiring immediate euthanasia were defined as severe distress or >20% body weight loss. Rats were euthanized via an intraperitoneal (IP) overdose of ketamine and xylazine (KX), three times the standard anesthetic dose of 90 mg/kg and 10 mg/kg respectively. [24]

Statistical Analysis

Given the exploratory nature of this preliminary study and the small sample size (n = 2 rats per group), all data were analyzed descriptively. Consequently, no inferential statistical tests were performed.

Table 1. Estrous cycle cytological observations in rats induced with estradiol benzoate

Induction day	K1H1 (0.1 mg)	K1H2 (0.1 mg)	K2H1 (0.2 mg)	K2H2 (0.2 mg)
D0*	Estrus	Diestrus	Diestrus	Diestrus
D1	Pseudoestrus	Pseudoestrus	Metestrus	Metestrus
D2	Pseudoestrus	Pseudoestrus	Pseudoestrus	Pseudoestrus
D3	Proestrus	Pseudoestrus	Pseudoestrus	Pseudoestrus
D4*	Metestrus	Pseudoestrus	Pseudoestrus	Pseudoestrus
D5	Pseudoestrus	Proestrus	Proestrus	Proestrus
D6	Pseudoestrus	Proestrus	Pseudoestrus	Pseudoestrus
D7	Pseudoestrus	Metestrus	Pseudoestrus	Pseudoestrus
D8*	Pseudoestrus	Diestrus	Pseudoestrus	Pseudoestrus

*: Days of estradiol benzoate injection. K1H1: Animal 1 Group 1; K1H2: Animal 2 Group 1

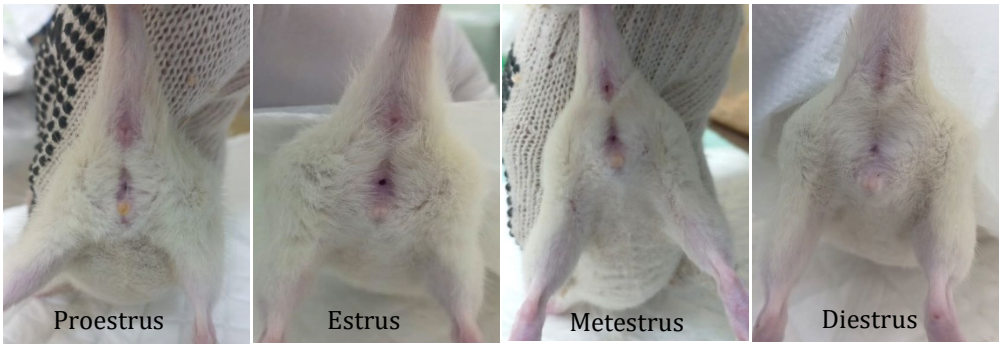

Figure 4. External vaginal morphology of female Wistar rats (*Rattus norvegicus*) observed directly through the 4 phases of the estrous cycle following estradiol benzoate hormone injection

3. Results

Preliminary study of the Rat Model

Pseudoestrus Induction in Rats

The progression of estrous cycle phases following estradiol benzoate induction is illustrated in the Table 1. The 0.2 mg dosage (K2H1 and K2H2) demonstrated a more stable and uniform maintenance of the pseudoestrus phase compared to the 0.1 mg dosage (K1H1 and K1H2), which exhibited frequent phase transitions throughout the observation period.

Estradiol benzoate at 0.1 mg/rat/week (50 µL) in Group 1 successfully induced pseudoestrus 1 day after induction (D1). However, the pseudoestrus phase was not uniform in both rats, indicating inconsistent induction capability (Table 1). In contrast, estradiol benzoate at 0.2 mg/rat/week (100 µL) in Group 2 successfully induced the pseudoestrus phase on the second day after induction (D2) and maintained it consistently for 3 consecutive days, with uniform results in both rats demonstrating stable and consistent pseudoestrus induction (Table 1). Based on these preliminary results, the optimal protocol was a 100 µL suspension volume of estradiol benzoate at 0.2 mg/rat/week for inducing pseudoestrus in female Wistar rats (*Rattus norvegicus*).

Direct morphological observation of rat vaginas throughout the estrous cycle revealed that the vaginal opening was widely open in all rats during the pseudoestrus phase (Figure. 4). The size of the vaginal opening varied and was inconsistent during the proestrus, metestrus, and diestrus phases. Therefore, vaginal morphology alone could not be used as a reliable reference for identifying the phases of the rat estrous cycle. Identification and detection of each estrous cycle phase in this preliminary study were performed through microscopic examination of vaginal epithelial tissue from vaginal smears.

One of the characteristic phases of the female rat estrous cycle is pseudoestrus, which is induced by the injection estrogen hormone. The observed pseudoestrus phase was characterized by a predominance of large, anucleate, cornified epithelial cells with irregular shapes, clearly distinguishing it from other phases. Once the pseudoestrus phase was established, VVC induction was initiated in the rats.

Induction of Vulvovaginal Candidiasis in Rats

A rat model of VVC was established by intravaginal administration of *Candida albicans* ATCC10231 suspension at two standart McFarland turbidity concentrations: 0.5 and 1.0. Intravaginal administration of *Candida albicans* at a standard McFarland 1.0 successfully induced VVC in both rats, whereas the standard McFarland 0.5 induced VVC in only one rat after 5 days.

The occurrence of VVC was confirmed by the microscopic detection of pseudohyphal structures from vaginal swabs and the evaluation of colony morphology, which were accompanied by the direct observation of a thick, viscous, cream-colored vaginal discharge resembling leukorrhea. However, it must be acknowledged that because this confirmation

relied primarily on conventional colony morphology and microscopic examination, these methods alone may not provide definitive species-level identification.

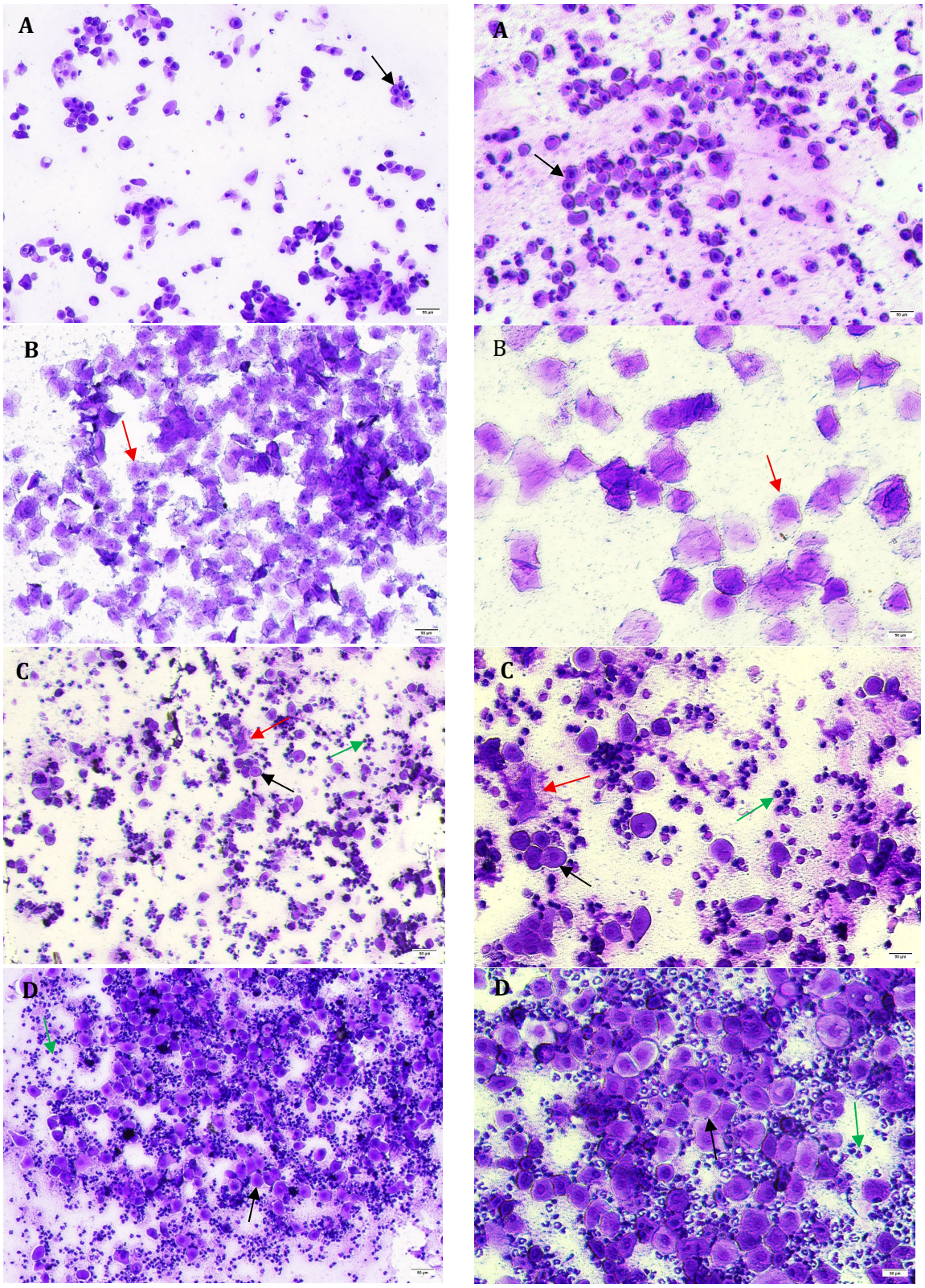

Figure 5. Cytological examination of crystal violet-stained vaginal smears was cytologically examined to determine the estrous cycle phase in rats: A) Proestrus, B) Pseudoestrus, C) Metestrus, D) Diestrus. Black arrows: nucleated epithelial cells; red arrows: anucleated cornified epithelial cells; green arrows: leukocytes. Light microscopy at 10× (left) and (right) 20× objective magnification

Table 2. VVC induction via intravaginal *Candida albicans* ATCC10231

Day of VVC induction	K1H1 (0.5)	K1H2 (0.5)	K2H1(1.0)	K2H2 (1.0)
D0	No *C. albicans* growth on vaginal lavage culture	No *C. albicans* growth on vaginal lavage culture	No *C. albicans* growth on vaginal lavage culture	No *C. albicans* growth on vaginal lavage culture
D1	No signs of discharge	No signs of discharge	Discharge (thick white mucus)	No signs of discharge
D2	No signs of discharge	No signs of discharge	Discharge (thick white mucus)	No signs of discharge
D3	Discharge	Discharge	Discharge (thick white mucus)	No signs of discharge
D4	No signs of discharge	No signs of discharge	Discharge reduced	No signs of discharge
D5	Intracellular *Candida* pseudohyphae detection in epithelial cells	Cytology: *Candida* pseudohyphae	Cytology: intracellular *Candida* pseudohyphae detected in epithelial cells	Cytology: no *Candida* were detected pseudohyphae

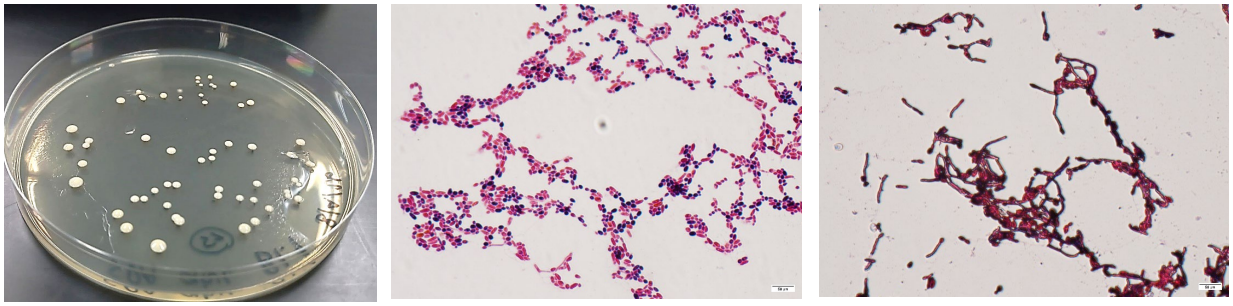

Figure. 6. Morphology of *C. albicans*. Left: *C. albicans* colonies on SDA; center: Gram staining *C. albicans* yeast cells; right: *C. albicans* pseudohyphae from rat vaginal lavage culture (40× objective magnification)

Table 3. Number of *C. albicans* colonies grown on SDA from rat vaginal lavage culture

Day of induction	K1H1 (0.5)	K1H2 (0.5)	K2H1 (1.0)	K2H2 (1.0)
D0	0 CFU/mL	0 CFU/mL	0 CFU/mL	0 CFU/mL
D5	0 CFU/mL	0 CFU/mL	1.3×10^2 CFU/mL	0.3×10^2 CFU/mL

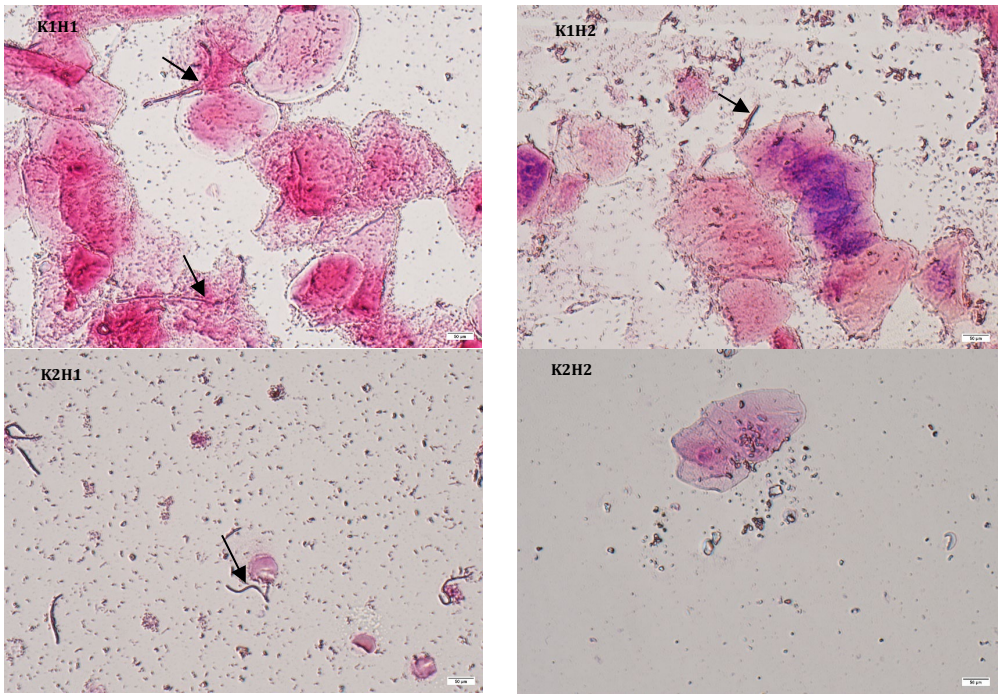

Figure. 7. Gram staining for the identification and confirmation of VVC from rat vaginal swabs. Intracellular *C. albicans* pseudohyphae invading cornified epithelial cells and extracellular pseudohyphae, (light microscopy, 40× objective magnification)

The vaginal swab prior to *C. albicans* induction showed no yeast cells or pseudohyphae. Similarly, vaginal lavage cultures before to VVC induction showed no *C. albicans* colony growth. Microscopic examination of vaginal smears revealed C. albicans pseudohyphae, including structures invading epithelial cells (intracellular pseudohyphae) after 5 days of VVC induction with McFarland 1.0. In addition, *C. albicans* colony growth from vaginal lavage on SDA medium confirmed the growth and viability of the organism within the rat vagina. Characteristic *C. albicans* colonies on SDA were cream-colored and round, with a dry, nonmucoid surface, smooth colony margins, a wrinkled appearance in older colonies, and an acidic odor (Figure. 6). Direct examination of the rat vagina also revealed the presence of thick, white-to-cream-colored mucous exudate from the vagina resembling discharge during VVC induction, further also correlating with the cytological findings.

During the induction of VVC, vaginal lavage cultures on SDA medium confirmed the colony growth of *C. albicans*. Gram staining of these cultures revealed predominantly yeast cell and pseudohyphal as well as morphologic transition structures (Figure. 6).

Candida growth detection after successful pseudoestrus induction and before VVC induction showed no *Candida* colony growth in vaginal lavage cultures on SDA for any of the rats. After 5 days of VVC induction with intravaginal *C. albicans*, vaginal lavage cultures demonstrated typical *C. albicans* colony growth in all animals in the McFarland 1.0 treatment group, with colony counts of 1.3×10^2 CFU/mL and 0.3×10^2 CFU/mL on Day 5 post-induction (Table 3). *Candida* colonies were not detected in the McFarland 0.5 group. VVC identification and confirmation in rats were performed using vaginal smear examination. Gram staining was used to visualize cornified vaginal epithelial cells and C. albicans pseudohyphal structures (Figure. 7). The detection of free pseudohyphae and intracellular pseudohyphae (invading epithelial cells) served as definitive markers of *C. albicans* infection, the primary causative agent of VVC. Intravaginal administration of *C. albicans* at McFarland 1.0 in both rats of group 2 resulted in detection of intracellular pseudohyphal structures in both groups, confirming the establishment of VVC. In the McFarland 0.5 group, only one rat (K1H2) had pseudohyphae. Therefore, a *C. albicans* suspension at McFarland 1.0 with an inoculum of 1.3×10^7 CFU/mL was determined to be the optimal concentration for inducing VVC with a colony of 1.3×10^7 CFU/mL.

4. Discussion

Elevated estrogen levels are a risk factor for VVC in fertile and pregnant women. VVC induction in female rat models is optimally achieved during the pseudoestrus phase through exogenous estrogen administration. Mechanistically, exogenous estrogen induces epithelial keratinization and promotes glycogen accumulation in the vaginal tissues, providing an abundant carbon source for fungal growth. Furthermore, estrogen exposure alters the vaginal microbiota and profoundly suppresses local innate immunity, notably by reducing neutrophil activity and migration into the vaginal lumen. The purpose of estrogen administration is to support immunosuppression and enhance *Candida albicans* virulence by reducing phagocytosis, since neutrophils and other leukocytes are generally absent during pseudoestrus, thereby facilitating colonization and invasion of the vaginal epithelium by *C. albicans*.[7] A preliminary study of pseudoestrus induction using estrogen in wistar rats is highly dependent on the accuracy of the dose and volume of estradiol benzoate administered. As described by Begum et al. (2020), cytologic examination remains the standard procedure for accurately determining each phase of the estrous cycle using simple staining. This method for detecting the estrous cycle phase in rats through cytological observation is simple and rapid.[21] Vaginal smear examination is the most commonly used technique for determining the phase of the estrous cycle because it provides clear cellular imagery, particularly of the pseudoestrus phase, which is characterized by the predominance of large, anucleate cornified epithelial cells with irregular shapes.[12] Although cytologic examination requires some skill in microscopically evaluating vaginal secretion cells and takes additional time, it is accurate and reliable. [12,25] In rats, a thick, mucus-like vaginal discharge was observed only during the diestrus during the estrous cycle. This finding is consistent with previous studies reporting high mucus volumes during diestrus, reflecting the abundance of leukocytes that characterize this phase.[26]

External vaginal structure from rats could not be used to reliably identify the estrous cycle phase. No significant differences were observed between phases, particularly among proestrus, metestrus, and diestrus. In contrast, the vaginal structure during the estrus or pseudoestrus consistently showed a widely open vaginal orifice. This occurs because ovulation and peak sexual receptivity occur during estrus, when the female is ready for copulation.[27] These findings are consistent with the literature indicating that external vaginal morphology cannot accurately differentiate the phases of the rat estrous cycle.[12] Vaginal opening is associated with peak estradiol concentration during ovulation. Therefore, the administration of exogenous estrogens, such as estradiol benzoate, aims to maintain high estrogen concentrations to sustain the pseudoestrus phase.[12] Estradiol benzoate at 0.1 mg/50µL rapidly triggered pseudoestrus but failed to maintain it consistently and uniformly. This inconsistency indicates that the minimum hormonal threshold required to sustain the pseudoestrus phase was uniformly achieved. These

results are consistent with those of previous studies showing that estradiol benzoate at 0.1 mg/50µL induced pseudoestrus in only 50% of rats.[8] In contrast, the 0.2 mg/100µL concentration and volume demonstrated a more stable and consistent effect. Pseudoestrus was induced on the second day post-induction and maintained consistently for 3 consecutive days, with uniform results across all animals. Therefore, 100 µL of estradiol benzoate at 0.2 mg/rat/week was established as the optimal protocol, providing adequate and stable hormonal exposure. These results are consistent with those of Carrara et al. (2010), who reported that estradiol benzoate at 0.2 mg/rat/week could induce pseudoestrus in 100% of Wistar rats without ovariectomy, thereby reducing animal suffering.[8] This optimized pseudoestrus induction protocol serves as a reliable model for minimizing biological undefined variability, particularly in biomedical research using Wistar rats, including fungal infections such as VVC.

The successful establishment of a VVC model in this study began with the validation of vaginal microflora sterility before induction. Negative vaginal swab results and the absence of colony before the intervention confirmed that the Wistar rats were free from natural *C. albicans* colonization. This confirms that the infection evidenced by intracellular pseudohyphal structures and clinical manifestations such as cream-colored thick vaginal exudate arose as a direct result of intravaginal *C. albicans* infection. McFarland standard 0.5 (6.8×10^6 CFU/mL) produced an inconsistent infection rate, inducing VVC in only one of two rats. In contrast, McFarland standard 1.0 (1.3×10^7 CFU/mL) administered over 5 consecutive days demonstrated a consistent both animals infection rate. Inducing VVC in all animals in the treatment group. These preliminary findings indicate that approximately 1.3×10^7 CFU/mL represents the minimum optimal infective dose required to overcome the local vaginal immune defenses of rats in the pseudoestrus state, thereby causing VVC.

Pathogenicity and viability of *C. albicans* in rat vagina. Definitive VVC confirmation was achieved cytological and microbiological methode on day 5 after induction. Microscopic examination of vaginal smears using simple and Gram staining revealed cornified vaginal epithelial cells and intracellular *C. albicans* pseudohyphae invading vaginal epithelial cells. The pseudohyphal structure represents the primary virulence mechanism of *C. albicans*, the ability to undergo morphological transition (polymorphism) from yeast cells to a mold form with pseudohyphae, which triggers vaginal tissue damage and inflammation in VVC.[28] Viability was confirmed through the successful reisolation of colonies from vaginal lavage. Before VVC induction, vaginal lavage cultures showed no *Candida* colony growth. After 5 days of VVC induction, vaginal lavage cultures demonstrated typical *C. albicans* colony growth in all animals in the McFarland 1.0 treatment group, with colony counts of 1.3×10^2 CFU/mL and 0.3×10^2 CFU/mL on Day 5. No *Candida* was detected in the McFarland standard 0.5 group. The characteristic macroscopic appearance of *Candida* included round, cream-colored colonies with a dry, nonmucoid surface and wrinkled appearance in older colonies. *Candida* growth from vaginal lavage demonstrated that fungal cells not only colonized the vagina but also actively replicated within it.

C. albicans suspension at McFarland standard 1.0 (1.3×10^7 CFU/mL) maintained for 5 days constituted a promising protocol for establishing a stable VVC animal model. The detected intracellular pseudohyphal structures served as strong evidence of active infection, validating this model for use in subsequent antifungal therapy or probiotic candidate efficacy testing. This study required only 1.3×10^7 CFU/mL of *C. albicans* over a 5-day period to successfully induce VVC, whereas previous studies, such as Carrara et al. (2010), induced VVC using a considerably higher inoculum concentration (5×10^8 CFU/mL) over a longer duration of 7 days.[8]

Several potential explanations can account for these methodological differences. First, strain variation likely plays a critical role; the specific *C. albicans* strain utilized in this current study may possess higher intrinsic virulence, dimorphic capacity, or superior adhesion capabilities to the vaginal epithelium compared to the isolate used by Carrara et al. Second, differences in hormonal exposure dynamics specifically the dosage, frequency, and tissue saturation of the estradiol benzoate administered can create varying degrees of local immunosuppression and glycogen availability, thereby accelerating or delaying how rapidly the infection establishes. Finally, differences in innate animal susceptibility, which can be influenced by the specific sub-line genetics of the Wistar rats, age, stress levels, or variations in their baseline vaginal microbiota, could significantly alter the threshold required for successful *Candida* colonization.

Considering the preliminary nature of this study and the fact that only four animals were included, indicating that Wistar rats may represent a promising model for VVC. This promising Wistar rat model is expected to provide a foundation for designing studies with larger treatment groups such as investigating the potential of active compounds, novel drugs, or probiotics for vaginal *Candida* infection prophylaxis. Therefore, this preliminary study achieved VVC induction in a shorter time frame and at a lower *C. albicans* concentration. This optimized Wistar rat model for VVC is expected to provide a foundation for designing studies with larger treatment groups such as investigating the potential of active compounds, novel drugs, or probiotics for vaginal *Candida* infection prophylaxis.

Future recommendations to validate and refine this VVC model, future research should increase the

sample size to enhance statistical power and incorporate an ovariectomized control group for comparative performance analysis. Further studies must integrate robust diagnostic parameters, including histopathological examination and the quantification of local immune biomarkers, to clarify host-pathogen dynamics. Additionally, adopting molecular identification techniques such as PCR is essential for definitive *C. albicans* confirmation. Addressing these improvements will enable the reliable application of this model for large-scale therapeutic testing of novel drugs and probiotic candidates.

5. Conclusion

Wistar rats (*Rattus norvegicus*) are an excellent animal model for vulvovaginal candidiasis (VVC) following pseudoestrus induction with estradiol benzoate hormone. VVC was microscopically confirmed by detecting intracellular pseudohyphae in vaginal smears, supported by the growth of viable *Candida albicans* colonies from vaginal lavage. Overall, the findings suggest that estradiol benzoate at 0.2 mg/rat/week combined with intravaginal inoculation of 1.3×10^7 CFU/mL of *Candida albicans* may provide a reproducible non-ovariectomized model of vulvovaginal candidiasis. Furthermore, this protocol avoids ovariectomy and may potentially reduce invasiveness.

6. Author Contribution

D.J., A.Y., and I.A.A conceptualized the concept. Conceptualized the research idea, developed the concept and outline, collected data and perfomed analysis, drafted and revised the manuscript, and approved the final version. A.Y. and I.A.A provided supervision (supporting), guidance on idea development, conceptualization, and manuscript writing.

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