IMPACT OF *IN VITRO* CLINDAMYCIN ON THE COMBINATION OF CLINDAMYCIN AND KETOCONAZOLE ON EXOPOLYMER OF *CANDIDA* SPP BIOFILMS OF UROGENITAL ORIGIN

IMPACTO DE LA CLINDAMICINA IN VITRO EN LA COMBINACIÓN CLINDAMICINA-KETOCONAZOL SOBRE EL EXOPOLÍMERO DE BIOPELÍCULAS DE CANDIDA SPP DE ORIGEN UROGENITAL

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ABSTRACT

Introduction: The vaginal mucosa has been widely used for administering antimicrobial agents to treat endogenous infections of the lower genital tract in pregnant and non-pregnant women. *Candida* spp. elaborates biofilms, and its formation is a complex process requiring that fungal cells establish multiple interactions with the medium. Biofilms are surrounded by an exopolymer matrix that can restrict the activity of antibodies, the diffusion of substances, and be associated with antimicrobials, therefore limiting its actions. General antimicrobials and particular anti-mycotic agents can face difficulties to access the cells within the exopolymer matrix. Many formulas used for empirical treatment have improper combinations with limited or null activity on the biofilms. The presence of molecules that cause its inhibition, thus eliminating the exopolymer matrix inducers, or by other mechanism, will allow the specific antimicrobial activity. **Objective:** To show that the activity of clindamycin used in dual formula with ketoconazole works on *Candida albicans* biofilm and on non-*albicans* species of *Candida*. **Methods:** We studied the activity of clindamycin and ketoconazole regarding the adherence and dispersion of biofilms from eight vaginal isolates of *C. albicans* and 7 of non-*albicans Candida*. The isolates were inoculated in three tubes with Sabouraud agar and a glass device to form the biofilm according to a known technique. *Adherence*: Each isolate was incubated for a six-hour period and a combination of clindamycin and ketoconazole from the material of ovules was added and conveniently diluted to one of the tubes of each isolate (62.5/260.4 ug/mL), considering 0 hour. *Dispersion:* The same dilution was added to another tube after 16 hours. The third tube was used as a control without antimicrobials. The reading was carried out with an optical microscope after 24 hours that the clindamycin and ketoconazole combination had been added and colored with crystal violet. They were then evaluated using photographic programs. The activity of clindamycin (62.5 ug/mL) and ketoconazole (260.4 ug/mL) was analyzed alone with a similar technique. We chose vaginal samples from seven patients with vulvovaginal candidiasis and studied them through the cell layer technique. The clindamycin and ketoconazole combination was used for studying the adherence and dispersion. **Results:** *Adherence:* Little influence of clindamycin and ketoconazole was seen in adherence regarding each control. *Dispersion:* The clindamycin and ketoconazole influence was seen in most of the isolates, especially in those of non-*albicans Candida* that showed higher presence of exopolymer matrix.The hyphae were only seen in 1 of 15 isolates of *Candida* spp after the clindamycin and ketoconazole were added at the 16th hour. In biofilms of clinical samples, neither hyphae nor mycotic elements were seen in 5 of 7 compared with the controls. **Conclusion:** According to these results, the use of a clindamycin and ketoconazole combination in biofilms of *Candida* spp results in proper penetration of the antimicrobial agent, which is seen by the biofilm dispersion during 24 hours. Clindamycin does not interfere with the action of ketoconazole, but it would promote its anti-*Candida* activity and would possibly modify surface and EP structures through inhibition of the molecules that facilitate its expression. The *in vivo* model promotes the immunomodulatory activity that *in vitro* models do not. Its combined use in dual formulas would facilitate the antimicrobial activity on *Candida* spp, therefore working as an inhibitor or modifier of the biofilms after dispersion of the exopolymer matrix.

Keywords: clindamycin; ketoconazole; *Candida* spp biofilm; exopolymer; vaginal and cervical infections.

RESUMEN

Introducción: La mucosa vaginal ha sido utilizada largamente para la administración de antimicrobianos destinados al tratamiento de infecciones endógenas del tracto genital inferior (IETGI) en mujeres embarazadas y no embarazadas. *Candida* spp elabora biopelículas (BP) y su formación es un proceso complejo que requiere que las células fúngicas establezcan múltiples interacciones con el medio. Las BP están rodeadas por un exopolímero (EPM) que puede restringir la actividad de anticuerpos, la difusión de sustancias y unirse a los antimicrobianos (AM), limitando su acción. Los antimicrobianos (AM) en general y los antimicóticos en particular (AMC) pueden tener dificultades para llegar a las células dentro del EPS. Muchas de las fórmulas que se emplean para el tratamiento empírico usan combinaciones inapropiadas con limitada o nula actividad sobre las biopelículas (BP). La presencia de moléculas que provoquen su inhibición anulando los inductores del EPM o por otro mecanismo, permitirá la actividad del AM específico. **Objetivo:** demostrar que la actividad de la clindamicina (CLI) en fórmula dual con ketoconazol (KET) actúa sobre BP *Candida albicans* (CA) y especies no *albicans* de *Candida*. (NAC). **Métodos:** estudiamos la actividad de clindamicina-ketoconazol (CK) sobre la adherencia y dispersión de BP de 8 aislamientos vaginales de CA y 7 de CNA. Se inocularon en 3 tubos con caldo Sabouraud y un dispositivo de vidrio para la formación de la BP según técnica ya descrita. *Adherencia:* Se incubaron durante 6 horas y se agregó una combinación de CK proveniente del material de óvulos, diluido convenientemente (62,5/260,4 ug/ml), a uno de los tubos de cada aislamiento tomándose como hora 0. *Dispersión:* esa misma dilución se agregó a otro tubo a las 16 horas. El tercer tubo quedó como testigo sin antimicrobianos. La lectura se efectuó con microscopio óptico a las 24 horas de agregada la combinación CK previa tinción con cristal violeta y se evaluaron con programas fotográficos. Por separado analizamos la actividad de CLI (62,5 ug/ml) y KET (260,4 ug/ml) con técnica similar. Seleccionamos las muestras de 7 pacientes que demostraron candidiasis vulvovaginal (CVV) y las estudiamos con la técnica de capas celulares. Se empleó la combinacion CK para el estudio de la adherencia y dispersión. **Resultados:** *Adherencia* se demostró poca influencia de CK en la adherencia con respecto a cada testigo. *Dispersión:* la influencia de CK se demostró en la mayoría de los aislamientos particularmente en los de CNA que mostraron una mayor presencia de EPM. Las hifas solo se observaron en 1/15 de los aislamientos de *Candida* spp cuando se agregó CK a las 16 horas. En las BP de las muestras clínicas no aparecieron hifas ni otro elemento micótico en 5/7 con respecto a los testigos. **Conclusión:** Según estos resultados el uso de una combinación de CK en BP de *Candida* spp, resulta en una adecuada penetración del AMC demostrada por la dispersión de la BP al cabo de 24 horas. Clindamicina no interfiere con la acción del ketoconazol sino que promovería su actividad anti-candida modificando posiblemente estructuras de superficie y la del EP por inhibición de las moléculas que facilitan la expresión del mismo. *In vivo* promueve la actividad inmunomoduladora que no se puede demostrar con este modelo *in vitro.* Su uso combinado en fórmulas duales facilitaría la actividad del AMC sobre *Candida* spp actuando como inhibidora o modificadora de las BP mediante la dispersión del EPM.

Palavras claves: clindamicina; cetoconazol; biopelícula; exopolímero; infecciones vaginales y cervicales.

INTRODUCTION

The vaginal mucosa has been widely used for administering antimicrobial agents to treat endogenous infections of the lower genital tract in pregnant and non-pregnant women. *Candida* spp. elaborates biofilms (BF), and its formation is a complex process requiring that fungal cells establish multiple interactions with the medium. They participate both in the colonization and in the progression of the disease[1-3]. Biofilms are surrounded by an exopolymer matrix (EP) that can restrict the activity of antibodies, the diffusion of substances and be associated with antimicrobials (AM), limiting its actions, and therefore they are significantly less susceptible to AM agents[4-6]. The access difficulty of general AM and particular anti-mycotic agents (AMC) to cells within the EP is phenotypically different of their corresponding planktonic or suspended cells[7,8]. Many formulas used for the empirical treatmen thave improper combinations with limited or null activity on the BF. The presence of molecules that cause its inhibition, thus eliminating the EP inducers, or by other mechanism, will allow the specific AM activity.

OBJECTIVE

To show that the activity of clindamycin used in combination with ketoconazole (CK) works on *Candida albicans* (CA) BF and on species of non-*albicans Candida* (CNA).

METHODS

We have analyzed the activity of CK regarding the adherence and dispersion of BF from eight vaginal isolates of CA and seven of CNA. The isolates were inoculated in three tubes with Sabouraud agar and a glass device to form the BF according to a known technique[9].

Adherence study: Each isolate was incubated in triplicate for a six-hour period and a combination of CK from the material of ovules was added and conveniently diluted to one of the tubes of each isolate (62.5/260.4 ug/mL), considering 0 hour.

Dispersion study: The same dilution was added to the second tube after 16 hours. The third tube was used as control without AM.

CLI and KET activity: we studied the activity of CLI (62.5 ug/mL) and KET (260.4 ug/mL) separately by using a technique similar to that previously described. The AM was added after the glass device had been incubated at the 6th (adherence) and 16th hours(dispersion).

Study of the CK and BF combination activity of clinical samples: we chose the samples from seven patients showing CVV that were studied by means of the conventional techniques (pH, amine test, microscopy and cultures). We made the BF by using the technique of cell layers[10]. The CK combination was used for the study of adherence and dispersion by applying the same concentrations used for the study regarding BF of CA and CAN. All glass devices with the BF of CA, CAN submitted to the activity of CK, CLI and KET were stained with crystal violet. The devices with cell layers were stained with Gram staining. The reading was carried out with a microscope (1000x) at 24 hours, and they were photographed with a digital camera and analyzed by means of a program for studying the EPM (Optical Imaging Software).

RESULTS

Biofilms of CA and CNA with CK

Adherence: Little influence of CK was seen in adherence regarding each control.

Dispersion: The CK influence was seen in most of the isolates, especially in those of CAN that showed more presence of EPM. The hyphae were only seen in 1 of 15 isolates of *Candida* spp after the CK was added at the 16th hour.

Biofilms of CA and CNA with CLI and KET

The analysis of BF, submitted to CLI activity, only enabled to observe that the EPM had been dispersed, and the BF of CA and CAN were the only ones that remained. In BF with KET addition, the persistence of the EPM was seen, even though there were some cases of certain alteration of the blastospores that indicate imperfect penetration (**Figure 1**).

Biofilms of CVV with CK

In BF of clinical samples, neither hyphae nor mycotic elements were seen in 5 of 7 compared to the controls (**Figures 2** and **3**).

DISCUSSION

The use of antibacterial and anti-mycotic combinations is very popular for local treatment of genital tract infections, whether by using gels, creams, or ovules and suppositories. They are an important option in the prophylaxis and treatment of superficial infections. In addition, they have low incidence of systemic toxicity and lower development of resistance than drugs of parenteral administration. It is known that 65% of human infections are associated with the formation of BF[7]. The genital tract is not different and we have showed, like other authors, that BF are formed in endogenous infections, such as bacterial vaginosis and CVV, and work as a real reservoir, therefore it is difficult to eliminate them and as such, they are usually associated with recurrent infections[7,10,11].

The combinations used do not always comprise the activity on the BF. We have chosen two of those that use different antimicrobials: clindamycin associated with ketoconazole and that including metronidazole, miconazole, gentamicin, neomycin, polymyxin, and centella.

Clindamycin belongs to the group of lincosamides, together with lincomycin. It is a synthetic derivate of lincomycin that was obtained in 1966. Owing to its larger activity, lower absorption through the gastrointestinal pathway and the larger spectrum, the previous one was replaced in the clinical practice.It was first introduced as an anti-staphylococcus. It was

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later seen as a powerful anti-anaerobe. Although the risk of colitis through *Clostridium difficile* has limited its use, this is a useful antibiotic for treating severe infections by anaerobic microorganisms. Lincosamides are composed of an amino acid (methyl proline) and a sugar (pyranose) that are united by an amide. In the clindamycin, the hydroxyl is replaced in the 7th position by a chloride atom.

Even though clindamycin is bacteriostatic, its bactericide action has been seen against some strains of *Staphylococcus*, *Streptococcus*, and *Bacteroides*.

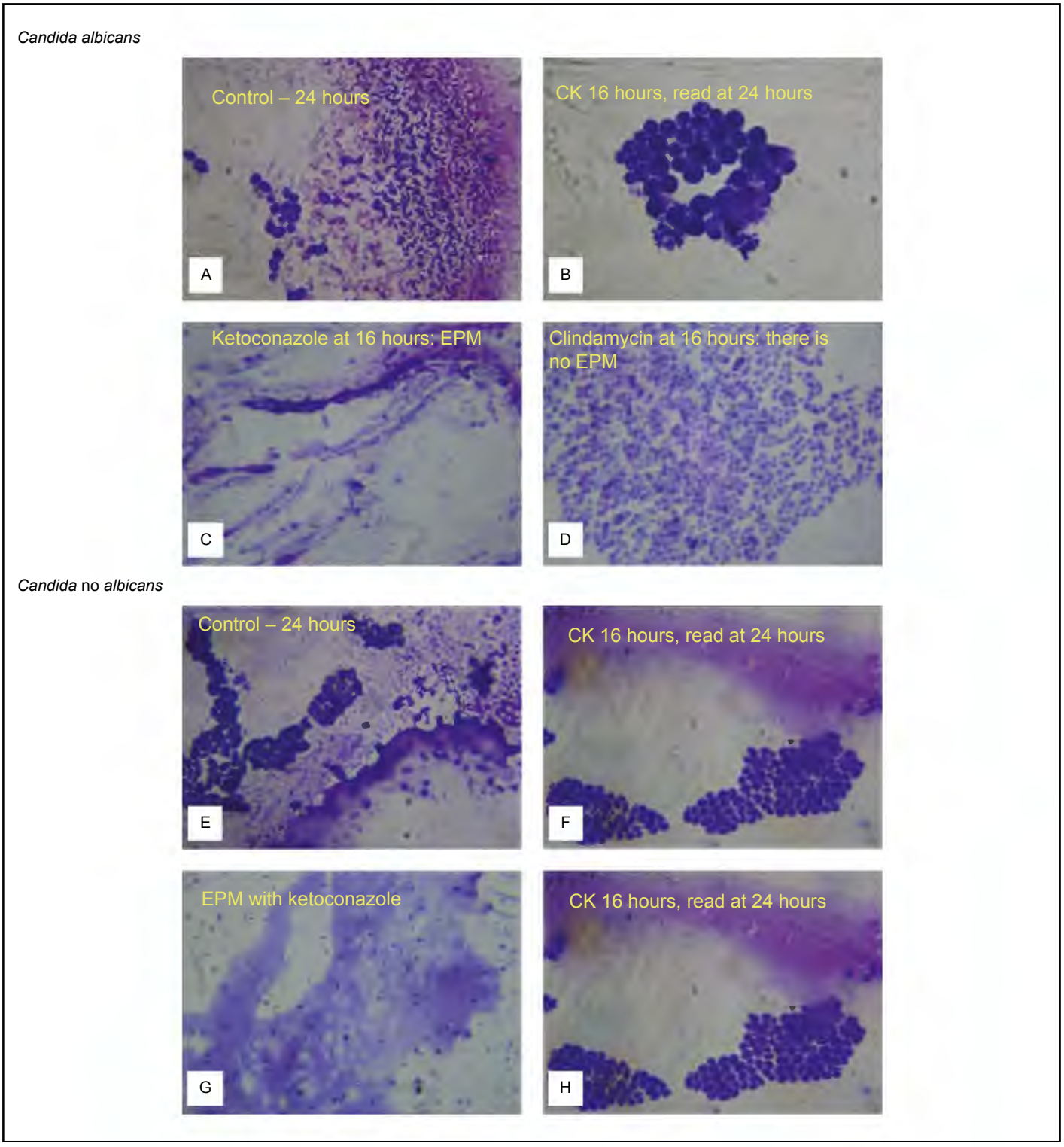

Figure 1 – Biofilms of *Candida albicans* and non *albicans Candida* with the control without antimicrobial exposure, exposure to Clindamycin plus ketoconazole (CK), clindamycin alone and ketoconazole alone.

It also inhibits the bacterial protein synthesis by connecting to the 50S subunit of the bacterial ribosome, thus preventing the beginning of the peptide chain.

The location of connection in the ribosome is the same for macrolide and chloramphenicol, therefore they inhibit their actions by competence and should not be used together because they are antagonistic. When used *in vitro*, they inhibit the production of staphylococci toxins associated with the toxic shock syndrome and prevent the production of BF. By changing the surface molecules, clindamycin facilitates the opsonization, phagocytosis, and intracellular death of bacteria, even in sub-inhibitory concentrations. The consequent alteration of the bacterial wall decreases the adherence capacity of bacteria like of *Staphylococcus aureus* to host cells and facilitates their destruction.

It also has a long-lasting post-antibiotic effect against some susceptible bacteria, possibility owing to the persistence of the drug in the ribosome union place. Its immunomodulatory activity *in vivo* is notorious and it is capable of suppressing the synthesis of toxins in *Streptococcus pyogenes*. Therefore, it is usually associated with the classical treatments using penicillin in severe infections[12].

Metronidazole is a molecule with anti-parasitic and anti-anaerobic activity that lacks immunomodulatory properties. The other antibacterial agents have different effects, but none of them works on BF or penetrate in its inner side.

The azoles that use both preparations have similar pharmacodynamics; however, similarly to all of them, they do not have the ability to penetrate through the EPM that form BF of *Candida* spp. They can work, but they do it slowly until they damage the mycotic elements.

The endogenous infections of the lower genital tract include the CVV, which affect a significant number of women at reproductive age. Topic and systemic medications are used for their treatment. The use of probiotics[13] is also suggested, since they would difficult the formation of hyphae, which is the most virulent form of *Candida* spp.

The pathogenesis of CVV is a multifactorial process and *Candida* spp may interact among themselves and with other microorganisms[14].

This is the reason for antimicrobial combinations. In order to eliminate the participating microorganisms, one has to obtain the penetration of the antimicrobial agents in the infection location and achieve proper concentrations[15]. The BF are usually resistant to many antifungal drugs[16].

Our results show that both the species used in the formation of BF and also those from the patients that we studied with CVV, show an EPM that would make the arrival of anti-mycotic agents difficult. The combined use of CLI and KET improves the activity of KET. The CLI facilitates the dispersion of the EP from the BF, even though it does not adhere. Thus, KET may act more freely on *Candida* cells. This is confirmed with the isolated action of CLI on BF of CA and CAN.

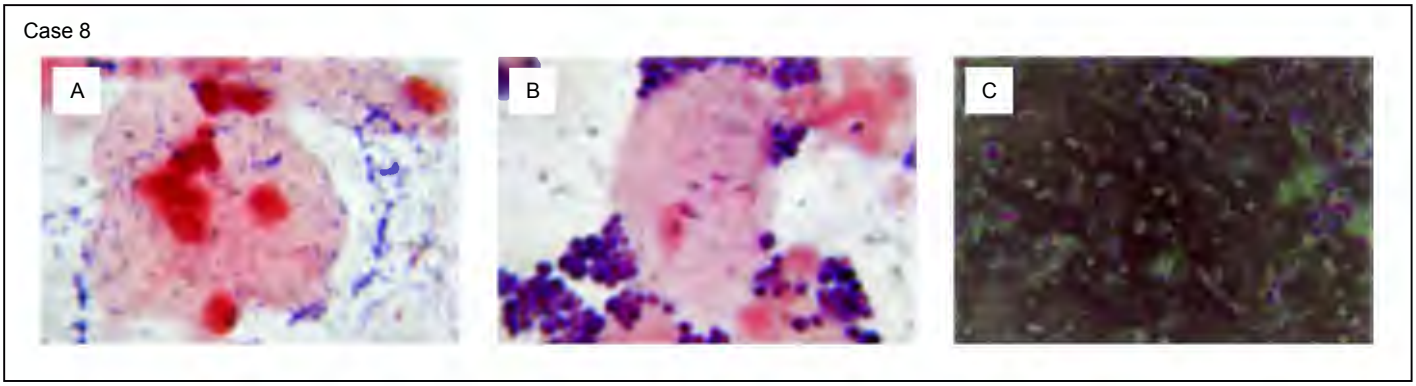

Figure 2 – Case 8 of CVV on cell layers. (A) Original material, presence of scarce mycotic elements and inflammatory response; (B) control cell layer: there is a BF of *Candida albicans*; (C) CK activity on the cell layer of *Candida albicans*: only altered scarce blastospores are seen and there is absence of EPM.

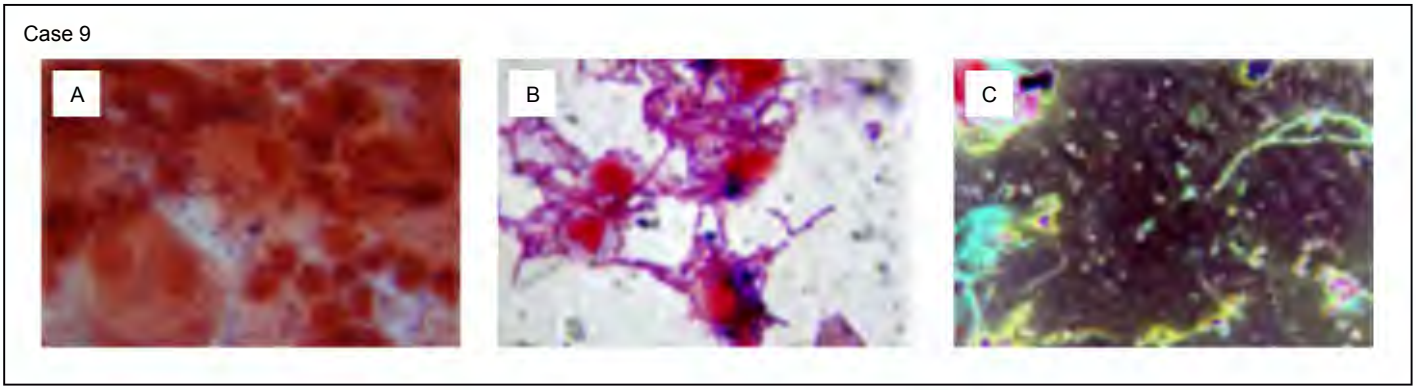

Figure 3 – Case 9 of CVV on cell layers. (A) Original material, presence of scarce mycotic elements and inflammatory response; (B) control cell layer: there is a BF of *Candida albicans*; (C) CK activity on cell layer of *Candida albicans*: only altered scarce blastospores are seen and there is absence of EPM.

It is clear that CLI does not have any action on yeasts, but it facilitates the action of KET and possibly of any other azole. When other antibacterial agents that do not disperse the EPM such as the CLI are used, the efficacy of anti-mycotic agents could be almost null. The activity would be done on the signal molecules, thus interfering with the EP structure that includes blastospores and hyphae. Some activity on the other EP components could be postulated. This would be better manifested *in vivo* since other microorganisms present in the genital tract could contribute to the formation of BF and to the structure of EP[17].

CONCLUSION

According to these results, the use of CK in endogenous vaginal tract infections because of *Candida* spp is more useful than the ketoconazole alone, due to the activity of clindamycin on the EPM architecture that presents the *Candida* spp biofilm. It is usually present in these infections, especially in recurrent or complicated ones.

Conflict of interests

The authors declare no conflicts of interest.

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