Int J Clin Exp Med 2017;10(4):6396-6404
www.ijcem.com /ISSN:1940-5901/IJCEM0049406

Original Article

Ginseng stem-leave saponins (GSLS) could enhance the activity of fluconazole (FLC) against genital candidiasis

Xiangting Lu[1*], Xin Huang[1*], Miao Jiang[1], Arun Karki[1], Yiyang Wen[1], Hui Shen[2], Simin Chen[3], Maomao An[3]

[1]*Department of Dermatology, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai, PR China;* [2]*Department of Laboratory Diagnosis, Changhai Hospital, Second Military Medical University, Shanghai, PR China;* [3]*Department of Pharmacology, Tongji University School of Medicine, Shanghai, PR China.* [*]*Equal contributors.*

Received January 21, 2017; Accepted February 16, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Background: Limitations of antifungal agents used in the treatment of genital candidiasis, as the emergence of resistant strains, more efforts are needed to find new antifungal agents. Methods: we performed in vitro drug susceptibility testing for GSLS against 31 *Candida* spp. clinical isolates following Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines. Flow cytometer (FCM) and transmission elcetron microscopy (TEM) were used to assess the alterations induced by GSLS in *C. parapsilosis* cells. Experiments in mice were performed to determine the antifungal activity of GSLS against genital candidiasis. Results: The 24 h of minimum inhibitory concentration (MIC_{50}) of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata* were < 0.49 μg/ml, 0.98 μg/ml, < 0.49 μg/ml, 1.95 μg/ml, respectively. A series of events related to alterations were observed in GSLS-treated *C. parapsilosis*, including the proportion of G_0/G_1 phase cells increased, the proportion of S-G_2-M phase cells decreased, proliferation index (PI) decreased, extensive chromatin condensation, nuclear fragmentation, and abundant cell sap released. Additionally, at day 3 post-medcation, the clone forming unit (CFU) counts of group C (treated with FLC) and D (treated with FLC + GSLS) were 0.16 ± 0.24, 0.06 ± 0.13, respectively. At day 7 post-medcation, the CFU counts of group C and D were both 0.00 ± 0.00. Conclusions: GSLS possesses activity against genital *Candida* spp. clinical isolates in vitro and in vivo. GSLS could be a potential candidate to fight against genital candidiasis by inhibiting the synthesis of DNA, cellular division and the proliferation activity of *Candida*.

Keywords: Ginseng stem-leave saponins, fluconazole, genital candidiasis, mechanism

Introduction

Genital candidiasis mainly includes the female vulva and vaginal *Candida*, *Candida* balanitis, urethritis in male. Vulvovaginal candidiasis (VVC) is a common opportunistic fungal infection in women at reproductive age, which caused by *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* etc, accounting for 95% in genital candidiasis, the main pathogen was *C. albicans*, about 70%~90% [1-3]. According to reports [4, 5] in the world about 70%~75% of all women of reproductive age had at least one case of VVC during their lifetime, 40%~50% of the patients had recurrent experience, while 5%~8% women developed recurrent Vulvovaginal Candidiasis (RVVC). Sexual transmission was considered the main reason for the frequent recurrence of genital candidiasis. *C. albicans* and non-*albicans* species tended to exhibit decreased susceptibility to azoles and even resistance was another difficulty for the treatment of VVC [2, 6]. It has the characteristics of obvious symptoms, sexual partners with the same patient, easy to relapse etc, which have exerted a serious influence on the physical and mental health and quality of life of patients and their families [7].

Recently, the non-*albicans* species morbidity has increased noticeably especially in postmenopausal women [8]. It has been reported that VVC induced by *C. albicans* were prone to drug resistance due to overusing and misusing of antifungal agents, and non-*albicans* species such as *C. krusei*, were resistant to FLC [2, 9]. Unsatisfied results are often achieved when using conventional treatment for VVC. Therefore, more anti-genital candidiasis agents with low toxicity and high efficiency are urgently

needed. Traditional Chinese Medicines (TCM) have a long history in the treatment of fungal infections, and have accumulated rich experience. So the selected drugs with anti-*Candida* activity from TCM, and combined with western medicines, which not only increase therapeutic efficacy, but also reduce toxicity, side effects and prevent the emergence of drug resistance [10, 11].

In this study, we demonstrated that GSLS had the activity against genital *Candida* spp. clinical isolates in vitro susceptibility testing. GSLS could induce the alteration of the cell cycle of *C. parapsilosis* and change the ultrastructural of *C. parapsilosis* by FCM assay and TEM. Animal experiments further identified that GSLS could enhance the activity of FLC against genital candidiasis.

Materials and methods

Reagents

FLC powder was provided by Shanghai three dimensional Pharmaceutical Co., Ltd (purity 99.9%, Shanghai, China). GSLS was purchased from National Institute for the Control of Pharmaceutical and Biological Products. MOPS was purchased from Sigma company. DMSO was obtained from American Amersco Corporation.

Animals

ICR strain of female mice (8-9 weeks) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All procedures performed on mice were approved by the Animal Care and Use Committee of Shanghai Tongji Hospital, China.

Fungi and identification of fungal species

The 31 strains were isolated from patients with genital *Candida* infection in the Department of Dermatology of Tongji Hospital Affiliated to Tongji University. Isolates were obtained from the male and female external genital organs and other parts of the female vagina. *C. krusei* (ATCC6258) and *C. parapsilosis* (ATCC22019) were selected as quality control strains, which were obtained from Centre of fungal preservation of the Changzheng Hospital (Second Military Medical University, Shanghai). The clinical isolates were identified with CHROMagar and API-20CAUX.

In vitro susceptibility testing

GSLS susceptibility testing was performed following Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines [12]. GSLS was diluted twofold so as to make a final concentration ranging from 0.49~250 μg/ml. Prepared the inoculum by using sterile 0.85% saline. Adjusted the cell density to a 0.5 McFarland turbidity standard with spectrophotometric methods. Using filtered Rosewell Park Memorial Institute (RPMI) 1640 broth to diluted suspensions 1000-fold to a concentration of 1~5 × 10^6 CFU/ml. Plated 100 μl of the suspensions into each well containing 100 μl of GSLS. Incubated trays at 35°C for 24 h and 48 h. MIC values were determined as the GSLS concentration at which 50% fungal growth was inhibited (MIC_{50}) [13].

Determination of the cell cycle and PI of C. parapsilosis by FMC

C. parapsilosis (ATCC22019) suspensions were treated with different doses of GSLS and were washed 3 times. Mixed the precipitates and resuspended them with PBS. After 3 times freeze-thaw, washed the suspensions twice with PBS and adjusted to a concentration of 1~5 × 10^6 CFU/ml. Subsequently, added 1 ml propidium iodide, held the suspensions at 4°C for 12 h and 24 h, then washed away propidium iodide, filtered the samples with 300-well nylon net [14, 15]. Finally, the alteration of cell cycle and PI of *C. parapsilosis* were detected by flow cytometry (BD, FACS) [16, 17]. PI formula is as follows:

$$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%.$$

The ultrastructure of C. parapsilosis under TEM

C. parapsilosis (ATCC22019) were incubated with GSLS or normal saline (NS) for 12 h and 24 h at 35°C. Collected the samples in due time and centrifugated at 3000 rpm for 10 min. Discarded the supernatant and washed the precipitate 3 times with PBS. Then samples were pre-fixed with 2% glutaraldehyde, fixed with 1% osmium tetroxide and underwent a gradient of dehydration with ethanol and acetone. Subsequently, embedded samples with resin and double-stained. Ultimately, observed ultrathin sections under TEM (Hitachi H-7000, Japan) [15].

Table 1. Effects of GSLS against genital *Candida* spp. clinical isolates (μg/ml)

Species (Number)	MIC_{50}	
	24 h[a]	48 h
C. albicans (16)	< 0.49	0.98
C. tropicalis (4)	0.98	31.25
C. krusei (5)	< 0.49	1.95
C. glabrata (6)	1.95	15.63

[a]h, MIC_{50} s of GSLS against genital *Candida* spp. clinical isolates were determined at 24 and 48 h of incubation.

In vivo animal study

Murine vulvovaginal candidiasis model: ICR strain of female mice (8-9 weeks) were injected subcutaneously with estradiol benzoate (0.05 mg/day) for 6 days before inoculation with *C. albicans* standard strain (ATCC64548) until the end of the experiment. Mice were inoculated in the vaginas with 20 μl of a suspension containing indicated live *C. albicans* (5×10^4/CFU), then inverted mice for 1~2 min. After 3 days and 7 days of infection, 40 μl sterile PBS was injected into the vaginas for 4~5 times, respectively. Lavage fluid was dissolved in 1 ml sterile PBS, 200 μl suspension was inoculated in the Sabouraud culture dish containing Gentamicin at 35°C for 48 h. Then CFU count was performed. When CFU ≥ 10×10^3 CFU/ml, indicating that the model building was successful. Degree of edema and characters of secretions of the vulva in model mice were observed. Taking the vaginal lavage fluid 20 μl, 1000 rpm centrifugal for 15 s, used for sediment image microscopy. Mice were monitored daily and were killed after 3 days or 7 days of infection, The vaginal tissues were removed, and then homogenized in 0.5 ml PBS for fungal burdens measurement or fixed in 10% neutral formalin for Hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) staining [18, 19].

Treatment of mouse model with Candida vaginitis

Successfully induced mice with vulvovaginal candidiasis, female ICR mice were randomly allocated to four different groups. Group A, oral NS 0.2 ml/day, vaginal lavage with NS 10 μl/day; group B, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 μl/day; group C, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 μl/day containing FLC 13 mg/kg; group D, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 μl/day containing GSLS 50 mg/kg. After treatment for 3 days and 7 days, CFU count, H&E and PAS staining were performed.

Statistics analysis

Data were expressed as Mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism V6.02 (GraphPad Software, Inc) and IBM SPSS Statistics Version21 (IBM Corp., USA). One-way ANOVA with Dunnett test was employed to analyze the differences between sets of data. Statistical significance was set at a *p*-value in the figures as: *, $P < 0.05$; **, $P < 0.01$.

Results

In vitro susceptibility testing

The results of GSLS in vitro susceptibility testing for 24 h and 48 h showed that GSLS has activity against genital *Candida* spp. clinical isolates (**Table 1**).

Influence of GSLS on the cell cycle and PI of C. parapsilosis

The result of this study indicated that the GSLS group compared with the negative control, generally the proportion of G_0/G_1 phase increased, the proportion of *C. parapsilosis* S-G_2-M phase decreased, PI decreased (**Figure 1**). The treatment of 125 μg/ml and 250 μg/ml GSLS had significant inhibitory effect on *C. parapsilosis* for 12 h, S phase was markedly inhibited compared with the negative control ($P < 0.01$ and $P < 0.01$, **Figure 1A**, **1C**). After 24 h, the inhibitory effect of GSLS at 62.5 μg/ml on S phase of *C. parapsilosis* was more obvious ($P < 0.01$, **Figure 1B**, **1D**) and the inhibitory effect of GSLS at 125 μg/ml and 250 μg/ml on S phase of *C. parapsilosis* were also significant ($P < 0.01$ and $P < 0.05$, respectively; **Figure 1B**, **1D**). The proportion of S phase in GSLS groups were significantly lower than that of negative control for 12 h and 24 h (**Figure 1A-D**). Additionally, PI of *C. parapsilosis* decreased with the increase of GSLS concentration (**Figure 1E**). The treatment of 125 μg/ml and 250 μg/ml GSLS for 12 h and 24 h, PI values were significantly lower compared to that of the negative control, respec-

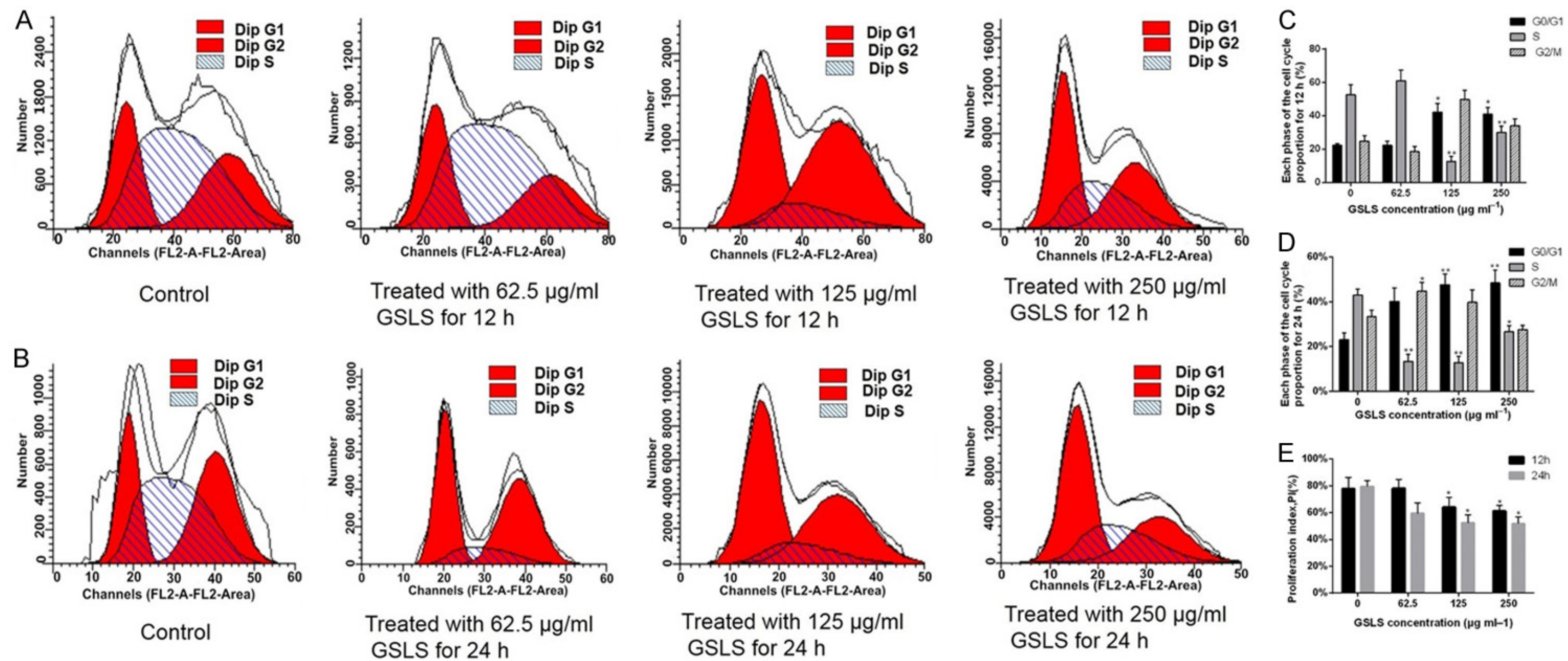

Figure 1. Influences of GSLS on the cell cycle of *C. parapsilosis*. A. Effect of different GSLS concentrations on the cell cycle of *C. parapsilosis* for 12 h; B. Effect of different GSLS concentrations on the cell cycle of *C. parapsilosis* for 24 h; C. Quantitative analysis of the influence of GSLS on the cell cycle of *C. parapsilosis* for 12 h ($\bar{x}$ ± s); D. Quantitative analysis of the influence of GSLS on the cell cycle of *C. parapsilosis* for 24 h ($\bar{x}$ ± s); *C. parapsilosis* fungi suspensions were treated with different doses of GSLS and incubated at 35°C for the corresponding period. The changes of cell cycle of *C. parapsilosis* were detected by FCM. E. PI value of *C. parapsilosis* after exposure to 62.5, 125 and 250 μg/ml GSLS for 12 h and 24 h, respectively, the group with no treatment of agent was set as control. Compared to the control, *, $P < 0.05$; **, $P < 0.01$.

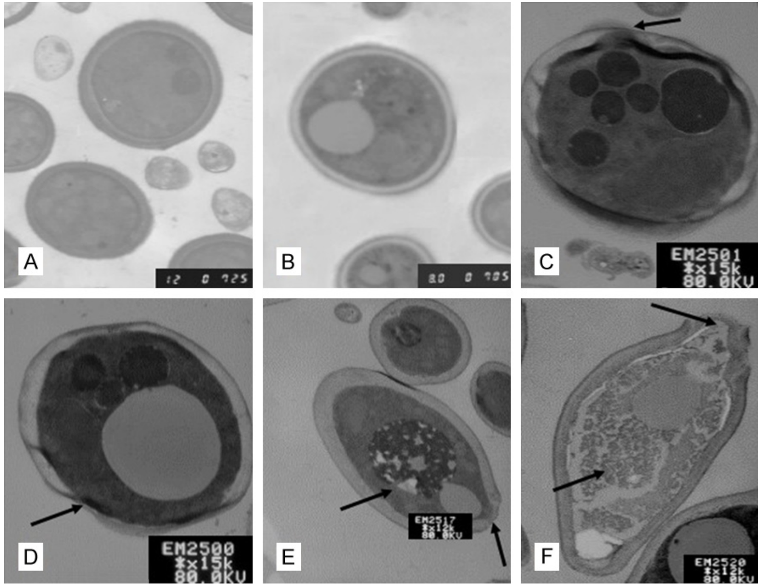

Figure 2. Influences of GSLS on the ultrastructure of *C. parapsilosis* were observed by TEM. (A) Treated with NS (× 20000, 12 h); (B) Treated with NS (× 20,000, 24 h); (C) Treated with 62.5 μg/ml GSLS (× 20000, 12 h); (D) Treated with 125 μg/ml GSLS (× 20000, 12 h); Arrows indicate a slight shrinkage in the wall and membrane of *C. parapsilosis* in (C and D); (E) Treated with 62.5 μg/ml GSLS (× 20,000, 24 h). Arrows indicate the damaged wall, non-homogenous cytoplasm, bubble formation and patchy areas with low electron density; (F) Treated with 125 μg/ml GSLS (× 20,000, 24 h). Arrows indicate extensive chromatin condensation, nuclear fragments, dissolved organelles and broken cytoplasm.

tively ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, respectively; **Figure 1E**). These suggested GSLS could inhibit the synthesis of *C. parapsilosis* DNA, suppress cellular division, regulate cell cycle and inhibit the proliferation activity of *C. parapsilosis.*

Effects of GSLS on the ultrastructure of C. parapsilosis

In control group, incubation with NS for 12 h, *C. parapsilosis* was round or oval with intact wall and membrane, an intact nuclear membrane, nucleus and nucleoli could be clearly observed, (**Figure 2A**). After 24 h incubation, no obvious changes were found in the ultrastructure of *C. parapsilosis* (**Figure 2B**). Treated with 62.5 μg/ml and 125 μg/ml GSLS for 12 h (**Figure 2C**, **2D**), a slight shrinkage in the wall and membrane of *C. parapsilosis* was observed, intracellular lipid droplets increased significantly, vacuoles increased, and its nuclear was intact, clear but irregular. Up to 24 h incubation with GSLS at 62.5 μg/ml, the wall was damaged, the cytoplasm was non-homogenous, bubble was formation and patchy areas with low electron density were exhibited in *C. parapsilosis* (**Figure 2E**). The cells exposed to 125 μg/ml GSLS for 24 h showed the wall border of *C. parapsilosis* was blurred and dissolved, extensive chromatin condensation, nuclear fragments, dissolved organelles and broken cytoplasm (**Figure 2F**).

Murine vulvovaginal candidiasis model

After inoculation of *C. albicans* in mice for 3 days, some hyphae and spores could be seen by vaginal lavage fluid smear fungal microscopic examination (**Figure 3A**). At day 7 post-infection, massive hyphae and spores were discovered (**Figure 3B**). Obvious swelling of the vulva, milk white paste secretions increased significantly. Moreover, with prolonged infections, HE staining revealed that inflammatory influx of vaginal tissues were aggravated in mice (**Figure 4A**). PAS staining also identified a large number of hyphae and spores (**Figure 4C**).

Effects of GSLS combined with FLC in the treatment of Candida vaginitis in mice

After treatment for 3 days, vaginal lavage fluid smear microscopic examination in group A is basically the same as before administration; group B, C (**Figure 3C**) and D showed a small amount of fungal pseudohyphae, blastospores and a large number of vaginal epithelial cells. At day 7 post-medcation, more pseudohyphae and blastospores, a large number of vaginal epithelial cells and milk white paste secretions can be observed in group A; group B, C and D indicated that a small number of vaginal epithelial cells, no fungal pseudohyphae and blastospores were found. HE staining suggested a small number of inflammation cells infiltration in the dermis of vaginas. No inflammatory influx of the superficial layer of the vaginal wall was observed (**Figure 4B**). PAS staining showed no hyphae and spores (**Figure 4D**). In addition, the results of vaginal CFU count in mice confirmed the effect of GSLS combined with FLC could

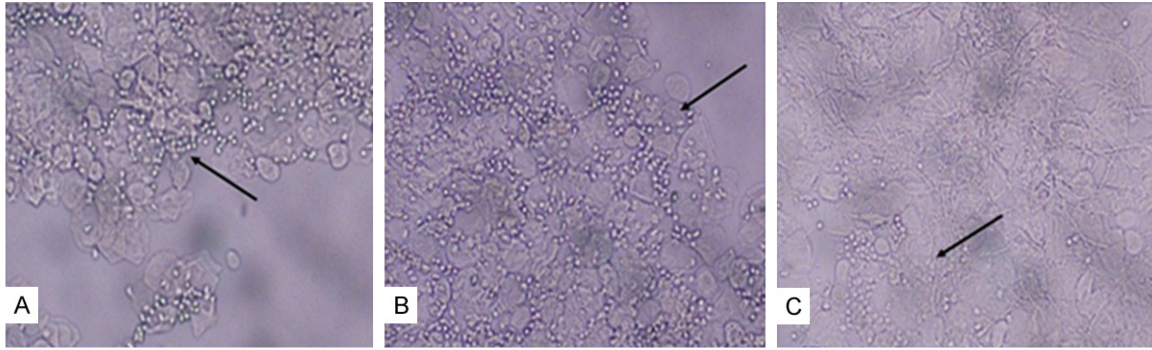

Figure 3. Mice vaginal lavage smear in group C (A. Mice was inoculated with *C. albicans* for 3 days; B. Mice was inoculated with *C. albicans* for 7 days; C. Mice medication for 3 days). Arrows indicate hyphae and spores.

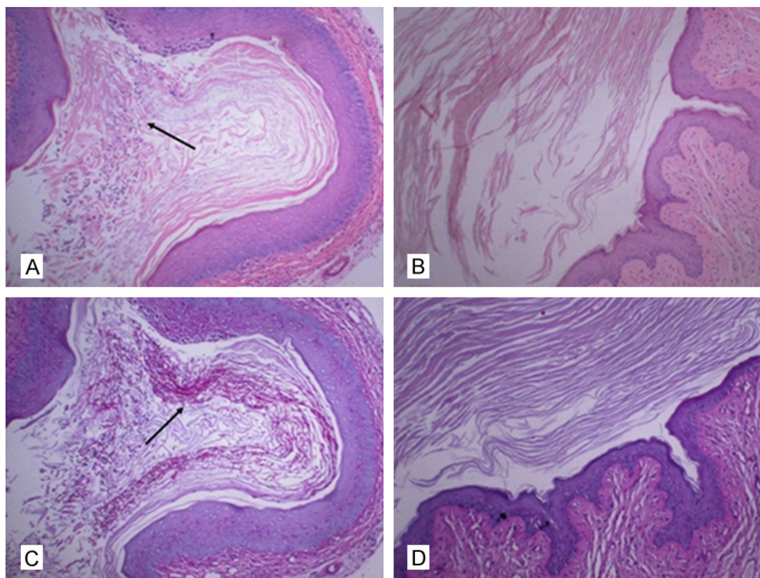

Figure 4. The representative pathological changes of vaginal tissue section of group C. (A) Group C was inoculated with *C. albicans* for 7 days. Arrow indicate inflammatory cells influx; (B) Group C received medication for 7 days. (HE staining in A and B, Magnification = 100 ×); (C) Group C was inoculated with *C. albicans* for 7 days. Arrow indicate *C. albicans* filaments in the tissues; (D) Group C received medication for 7 days. (PAS staining in C and D, Magnification = 100 ×).

against *Candida* vaginitis. No significant difference in the levels of fungal burden of vaginas was observed between group C (FLC + FLC) and group D (FLC + GSLS) ($P > 0.05$, **Figure 5**). Fungal burdens in the vaginas of group D significantly lower as compared to group A (NS + NS) and B (FLC + NS) ($P < 0.01$ and $P < 0.05$, respectively; **Figure 5**).

Discussion

Genital candidiasis is one of the most common diseases of reproductive tract infection [20]. Recently, researches have indicated that the proportion of *C. glabrata* increased continuously in VVC [21, 22]. Richer *et al.* [23] studies also found that the infection rate of non-*albicans* species in the culture of vaginal secretion of RVVC patients was as high as 42%.

In vitro susceptibility test demonstrated that GSLS has antifungal activity against genital *Candida* including *C. albicans* and non-*albicans Candida* species. It has been reported that *C. krusei* isolates were resistant to FLC and the resistant rate was 100% [2]. However, the 24- and 48-hours MIC_{50} of GSLS against *C. krusei were* < 0.49 μg/ml and 1.95 μg/ml, respectively in our study. It means that GSLS could be a potential candidate to fight against *C. krusei*-related infections. Subsequently, we found that GSLS could inhibit the synthesis of *Candida* DNA, suppress cellular division, regulate cell cycle and inhibit the proliferation activity of *Candida* by FCM. Observed from TEM, GSLS could destroy the ultrastructural of *Candida*. We speculated that the antifungal mechanism of GSLS was similar to that of most antifungal drugs, which acted on the cell membrane or cell wall of fungi. Destroyed selective permeability of cell membrane, decreased intracellular osmotic pressure, resulting in the cell content leakage.

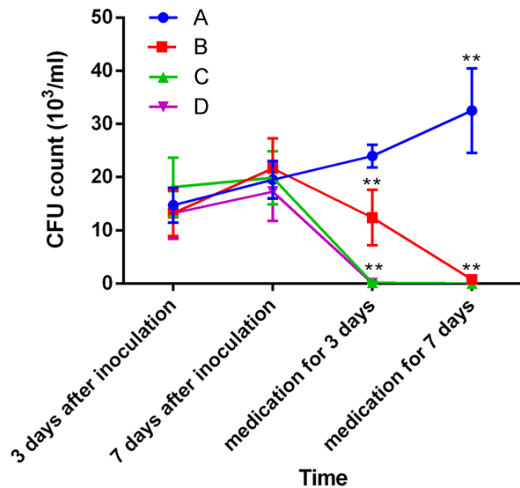

Figure 5. CFU count of mice vaginal lavage fluid at different times. A. Oral NS 0.2 ml/day + vaginal lavage with NS 10 μl/day (n = 8 per group), compared with group D, **, $P < 0.01$; B. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μl/day (n = 8 per group), compared with group D, *, $P < 0.05$; C. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μl/day containing FLC 13 mg/kg (n = 8 per group); D. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μl/day containing GSLS 50 mg/kg (n = 8 per group). CFU count were detected on the vaginal smear at the 3rd day and the 7th day after inoculation with *C. albicans* and after treatment for 3 days and 7 days, respectively.

Disturbed their normal metabolism. The cell death was promoted, and the purpose of inhibiting fungi was achieved. However, we found out that GSLS had the function of inhibiting DNA synthesis and cell division by FCM. Therefore, it can not be ruled out GSLS can directly destroy the nuclear DNA, and then affect the structure and function of the cell organelles, disrupting its normal metabolism, resulting in cell death. So, it is necessary to further study the effect of GSLS on the target site of *Candida.*

The morphological and physiological changes were remarkable after GSLS treatment in the present study, including the alteration of the cell cycle of *C. parapsilosis*, the proportion of G_0/G_1 phase cells increased, the proportion of S-G_2-M phase cells decreased, PI decreased, extensive chromatin condensation, nuclear fragmentation, and abundant cell sap released. These changes suggested that GSLS could inhibit the synthesis of DNA of *C. parapsilosis*. Recently, Yang [15] *et al*. studies exhibited that baicalin could inhibit the activities of Ca^{2+}-Mg^{2+} ATPase and SDH, increase cytosolic Ca^{2+} content, and hasten apoptosis rate. Moreover, study performed by Wang [24] *et al*. indicated that baicalin may induce cell death via apoptosis to inhibit the development of *C. albicans* biofilms. Compared with these studies, our work mainly focused on the alteration of the cell cycle of *C. parapsilosis*, and used varied experimental methods and parameters to survey antifungal activity of GSLS. In particular, we carried out animal experiment of GSLS combined with FLC in the treatment of *C. albicans* vaginitis in mice. From the results of these works, it was evident to observe that GSLS could enhance the activity of FLC against genital candidiasis in mice.

Actually, previous studies have reported that some TCM do not have strong antifungal activities (e.g. baicalein [25], gallic acid [26], matrine [27]), but usually owned favourable in vivo antifungal functions (e.g. sodium houttuyfonate [28], Panax Notoginseng Saponins and GSLS [11]). Our findings are consistent with previous studies performed by Keyal Uma [11] *et al*. that FLC used in combination with GSLS is effective against resistant strains of *C. albicans* in vitro. The antifungal activity presented here was relatively strong. Although the combination therapy has become a common clinical practice, as it could significantly improve the antifungal efficacy of each drug compared to each drug used alone and reduce adverse side effects, before widely used in the clinic, we still need for further screening of such combinations [11, 29].

In conclusion, this study demonstrated the activity of GSLS combination with FLC in vivo against genital *Candida*, and the inhibition synthesis of DNA of *Candida*. Therefore, GSLS might be a drug of choice in the treatment of genital Candidiasis infections.

Acknowledgements

This work was supported by the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality (No. 17ZR-1426300); the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality (No. 13ZR1437900); the National Natural Science Foundation of China (No. 81671989); the National Natural Science Foundation of China (No. 81601745).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xin Huang, Department of Dermatology, Shanghai Tongji Hospital, Tongji University School of Medicine, Xin-Cun Road 389, Shanghai 200065, China. Tel: +86-21-66111459; Fax: +86-21-66111329; E-mail: alida_huang@163.com

References

[1] Pericolini E, Gabrielli E, Ballet N, Sabbatini S, Roselletti E, Cayzeele Decherf A, Pelerin F, Luciano E, Perito S, Justen P and Vecchiarelli A. Therapeutic activity of a saccharomyces cerevisiae-based probiotic and inactivated whole yeast on vaginal candidiasis. Virulence 2017; 8: 74-90.

[2] Zhang JY, Liu JH, Liu FD, Xia YH, Wang J, Liu X, Zhang ZQ, Zhu N, Yan Y, Ying Y and Huang XT. Vulvovaginal candidiasis: species distribution, fluconazole resistance and drug efflux pump gene overexpression. Mycoses 2014; 57: 584-591.

[3] Zisova LG, Chokoeva AA, Amaliev GI, Petleshkova PV, Miteva-Katrandzhieva TM, Krasteva MB, Uchikova EH, Kouzmanov AH and Ivanova ZV. Vulvovaginal candidiasis in pregnant women and its importance for candida colonization of newborns. Folia Med (Plovdiv) 2016; 58: 108-114.

[4] Beikert FC, Le MT, Koeninger A, Technau K and Clad A. Recurrent vulvovaginal candidosis: focus on the vulva. Mycoses 2011; 54: e807-810.

[5] Dias LB, Szeszs MW, Filho JM and Hahn RC. Vulvovaginal candidiasis in Mato Grosso, Brazil: pregnancy status, causative species and drugs tests. Braz J Microbiol 2011; 42: 1300-1307.

[6] Guo H, Guan XH, Wang HX, Gao XH, Chen HD and Li JH. Genital candidiasis presenting as pseudomembrane of the urethral meatus. J Dtsch Dermatol Ges 2016; 14: 612-613.

[7] Gamarra S, Morano S, Dudiuk C, Mancilla E, Nardin ME, de Los Angeles Méndez E and Garcia-Effron G. Epidemiology and antifungal susceptibilities of yeasts causing vulvovaginitis in a teaching hospital. Mycopathologia 2014; 178: 251-258.

[8] Hamad M, Kazandji N, Awadallah S and Allam H. Prevalence and epidemiological characteristics of vaginal candidiasis in the UAE. Mycoses 2014; 57: 184-190.

[9] Youngsaye W, Hartland CL, Morgan BJ, Ting A, Nag PP, Vincent B, Mosher CA, Bittker JA, Dandapani S, Palmer M, Whitesell L, Lindquist S, Schreiber SL and Munoz B. ML212: a small-molecule probe for investigating fluconazole resistance mechanisms in candida albicans. Beilstein J Org Chem 2013; 9: 1501-1507.

[10] Chen X, Ren B, Chen M, Liu MX, Ren W, Wang QX, Zhang LX and Yan GY. ASDCD: antifungal synergistic drug combination database. PLoS One 2014; 9: e86499.

[11] Keyal U, Huang X and Bhatta AK. Drug interaction of traditional Chinese medicines with fluconazole against fluconazole resistant strains of candida albicans. Int J Clin Exp Med 2016; 9: 22357-22362.

[12] Rahmoun NM. Clinical Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts, Approved Standard-Third Edition M27-A3 Wayne P.A, 2008. 2013.

[13] Espinel-Ingroff A, Canton E, Peman J, Rinaldi MG and Fothergill AW. Comparison of 24-hour and 48-hour voriconazole MICs as determined by the clinical and laboratory standards institute broth microdilution method (M27-A3 document) in three laboratories: results obtained with 2,162 clinical isolates of candida spp. and other yeasts. J Clin Microbiol 2009; 47: 2766-2771.

[14] Qi X, Zhou CP, Xu W, Cao Y, Ling H, Ning CW, Ming LC, Xu R, Lamrani M and Mu Y. Novel short antibacterial and antifungal peptides with low cytotoxicity: efficacy and action mechanisms. Biochem Biophys Res Commun 2010; 398: 594-600.

[15] Yang S, Fu Y, Wu X, Zhou Z, Xu J, Zeng X, Kuang N and Zeng Y. Baicalin prevents candida albicans infections via increasing its apoptosis rate. Biochem Biophys Res Commun 2014; 451: 36-41.

[16] Yazdanparast SA, Khodavaisy S, Fakhim H and Shokohi T. Molecular characterization of highly susceptible candida africana from vulvovaginal candidiasis. Mycopathologia 2015; 180: 317-323.

[17] Shirkhani S, Sepahvand A, Mirzaee M and Anbari K. Phospholipase and proteinase activities of candida spp. isolates from vulvovaginitis in Iran. J Mycol Med 2016; 26: 255-260.

[18] Leng Q, Woodle MC, Liu Y and Mixson AJ. Silver adducts of four-branched histidine rich peptides exhibit synergistic antifungal activity. Biochem Biophys Res Commun 2016; 477: 957-962.

[19] Zhang SQ, Zou Z, Shen H, Shen SS, Miao Q, Huang X, Liu W, Li LP, Chen SM, Yan L, Zhang JD, Zhao JJ, Xu GT, An MM and Jiang YY. Mnn10 maintains pathogenicity in candida albicans by extending alpha-1, 6-mannose backbone to evade host Dectin-1 mediated antifungal immunity. PLoS Pathog 2016; 12: e1005617.

[20] Leusink P, Kaptheijns A, Laan E, Van BK and Lagro-Janssen A. Comorbidities among women with vulvovaginal complaints in family practice. J Sex Med 2016; 13: 220-225.
[21] Leeyaphan C, Bunyaratavej S, Foongladda S, Rujitharanawong C, Maneeprasopchoke P, Surawan T, Muanprasat C and Matthapan L. Epidemiology, clinical characteristics, sites of infection and treatment outcomes of mucocutaneous candidiasis caused by non-albicans species of candida at a dermatologic clinic. J Med Assoc Thai 2016; 99: 406-411.
[22] Nash EE, Peters BM, Lilly EA, Noverr MC and Fidel PL Jr. A murine model of candida glabrata vaginitis shows no evidence of an inflammatory immunopathogenic response. PLoS One 2016; 11: e0147969.
[23] Richter SS, Galask RP, Messer SA, Hollis RJ, Diekema DJ and Pfaller MA. Antifungal susceptibilities of candida species causing vulvovaginitis and epidemiology of recurrent cases. J Clin Microbiol 2005; 43: 2155-2162.
[24] Wang T, Shi G, Jing S, Wu D, Yan Y, Zhang M, Cui Y and Wang C. Invitro antifungal activity of baicalin against candida albicans biofilms via apoptotic induction. Microb Pathog 2015; 87: 21-29.
[25] Serpa R, França EJ, Furlanetomaia L, Andrade CG, Diniz A and Furlaneto MC. In vitro antifungal activity of the flavonoid baicalein against candida species. J Med Microbiol 2012; 61: 1704-1708.
[26] Alves CT, Ferreira IC, Barros L, Silva S, Azeredo J and Henriques M. Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against candida species. Future Microbiol 2014; 9: 139-146.
[27] Shao J, Wang T, Yan Y, Shi G, Cheng H, Wu D and Wang C. Matrine reduces yeast-to-hypha transition and resistance of a fluconazole-resistant strain of candida albicans. J Appl Microbiol 2014; 117: 618-626.
[28] Huang W, Duan Q, Fang L, Jing S, Cheng H and Wu D. Sodium houttuyfonate and EDTA-Na 2 in combination effectively inhibits pseudomonas aeruginosa, staphylococcus aureus and candida albicans in vitro and in vivo. Bioorg Med Chem Lett 2015; 25: 142-147.
[29] Bink A, Pellens K, Cammue BPA and Thevissen K. Anti-biofilm strategies: how to eradicate candida biofilms? Open Mycology Journal 2011; 511: 29-38.