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Comparison of antifungal effect of nanosilver particles alone and in combination with current drugs on candida species isolated from women with recurrent vulvovaginal candidiasis

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ABSTRACT

Vulvovaginal candidiasis may appear as an acute, chronic or recurrent genital infection that is caused due to overgrowth of *Candida* species, especially *Candida albicans*. Long-term, repetitive administration of common antifungal drugs causes resistance of candida species to these drugs. Therefore in order to achieve an optimum therapy we decided to examine the effects of common drugs in combination with nanosilver particles on the *Candida* species isolated from recurrent vaginal Candidiasis. This experimental study was performed on 30 *Candida* species isolated from women suffering from recurrent candidiasis. Direct a microscopic examination, culture and complementary tests such as culture on *Candida* Chrome Agar, formation of Germ tube, temperature test and sugar assimilation API 20c AUX were used to identify isolated *Candida* species. Minimum inhibitory concentration (MIC) of each common drug, nanosilver particles alone and their combination with nanosilver particles were performed with microdilution method according to NCCLS guideline and the results were analyzed with logistic regression, Chi-square and Mann – Whitney U test. In this study *Candida albicans*, *C. glabrata*, *C. Krusei*, *C. tropicalis*, *C. parapsilosis* and *C. famata* were isolated and identified, respectively, according to their abundance. The MIC_s of common drugs ranged from 4 to 128 µg/ml. The antifungal activities of these drugs in combination with nanosilver particles were more than when they were each used alone. Appropriate diagnosis of infection in laboratory, using in vitro susceptibility tests and administration of topical formulations of nanosilver particles in combination with current drugs can be useful for treating vaginal candidiasis and preventing the disease recurrence.

Keywords: Vulvovaginal candidiasis, Nanosilver, Current drugs, combination.

INTRODUCTION

Vulvovaginal Candidiasis (VVC) is a mycotic infection of women's genital tract mucosa which occurs due to the overgrowth of candida's various species, particularly *C. albicans*, at the fertilization age, usually between 25 and 35 years [6, 8].

It is estimated that 75% of all women experience an episode of the disease in their life time and 40-50% of them will develop recurrent infections and approximately 5% of women are diagnosed as chronic infections [13, 15]. Variation in the incidence of vulvovaginal candidiasis has been reported in communities of Iran. The disease has been reported with different prevalence in different areas of Iran, e.g. 43% in Mashhad, 19.8% in Kerman, 22.3% in Kashan and 26.7% in Sari [1, 7]. *Candida albicans* is responsible for 80 to 90% of episodes of vulvovaginal candidiasis, followed by *Candida glabrata* which causes 5-15% of infections [8].

There are a few types of drugs commonly administered for treatment of vulvovaginal candidiasis including nystatin and azolic drugs—clotrimazole, miconazole and fluconazole—that are administered in the form of vaginal cream and tablets [18]. In case of treatment failure with clotrimazole as the first-line therapy for the disease, fluconazole may be orally administered, as a substitute, in a single dose [18].

Limited number of the drugs available for the treatment, high frequency of infection, and multiplicity of candidal agents causing the infection are considered as factors which necessitate repetitive and long-term use of such drugs. This condition paves the way for resistance and, probably, recurrence of the infection that have recently been regarded as the main therapeutic problem [15, 18].

Biological properties of nanosilver particles go back to their both physical and chemical features, which enable them to get access to tissues, cells, and biological molecules in the human body [2, 3]. In a study, antibacterial activity of nanosilver particles against dermatophytes and candida species has been reported [14].

The main objectives of combining two or more antibiotics are to delay the Anti microbial resistance, establish a desirable synergism of therapeutic effect, and reduce the side effects. Congruous with this attitude were conducted a number of studies, say, evaluating the antifungal activity of fluconazole alone and in combination with Nanosilver and its effect on candida albicans, which indicated their synergistic effect on each other [9, 10].

Taking the above mentioned issues into account, this study was designed to investigate antifungal activity of typical antifungal drugs in combination with nanosilver particles on isolated candida species from patients suffering recurrent vulvovaginal candidiasis.

MATERIALS AND METHODS

This experimental study was performed through the following steps:

1. Supply of materials: *Candida* species were obtained from patients suspected with recurrent vulvovaginal candidiasis who had referred to gynecological clinic of Lolagar Hospital.

Nanosilver colloidal solution with the concentration of 4000 ppm (33nm) was purchased from the Nanonab Pars company. Fluconazole, clotrimazole and nystatin were purchased from Sigma Company, and Ketoconazole was obtained from Behvarz pharmacy laboratory as dedication.

2. Preparation of samples: The specimens were collected by gynecologists and obstetricians by entering a sterile cotton swab inside vagina so as to take vaginal discharge. The swabs were then transferred to a tube containing 1 ml sterile normal saline and were immediately transported to the laboratory.

3. Specimen processing and identification of species: To confirm the initial clinical diagnosis, both direct microscopic examination for budding yeast cells and culture examination were performed. Along with the latter examination colony counting was made and considered positive if it exceeded 1000 in volume unit.

Investigation of color changes in the culture made on CHROM agar *Candida* plates, germ tube formation test, clamidoconidi formation on corn meal Agar medium, heat tolerance test, and assimilation of carbohydrate supplying API20c AUX kit were utilized to definitely identify the species.

4. Assay for drugs susceptibility: The assay was fulfilled through the following processes:

4-a) Preparation of culture media: RPMI powder obtained from Sigma Company was dissolved in water and sodium bicarbonate (2 g/l) and added to the medium. Then the medium was filtered and distribute and transferred to tubes and stored at 4°C. While using the medium, 1 ml glutamine was added to 100 ml medium [12, 16, 19].

4-b) Preparation of suspension of Candida: Stock inoculum suspensions of the candida species were obtained from 24-h-old cultures on Sabouraud Dextrose Agar (SDA) at 30°C, the candida species was diluted by 1 ml normal saline to provide inoculum suspensions. The turbidity of the candida suspensions was adjusted by the spectrophotometry to create 90% transmission at 530 nm, representing 1×10^6 CFU/ml. It was diluted 1000 times to provide a suspension of 1×10^3 CFU/ml [12, 16, 19].

4-c) Preparation of medicomments serial dilutions: Nanosilver with the features mentioned above was diluted using sterile distilled water to achieve 128 µg/ml concentration; serial dilutions were then prepared from 64 to 0.5 µg/ml applying the RPMI medium above. On the other hand, 0.0128 gr of each antifungal drug powder was dissolved in a 10 ml solvent (distilled water or dimethyl sulfoxide, depending on the type of the drug) and was then left for 30 min at the laboratory conditions to dissolve completely, to obtain the concentration of 1280 µg/ml. Serial dilutions were prepared from 128 to 1 µg/ml by using RPMI medium for determination of MIC values [12, 16, 19].

4-d) Determiation of MIC: Drug susceptibility testing was performed in sterile, U-bottomed 96-well microtiter plates. 50 µl of common antifungal and 50 µl of nanosilver solutions were added to 8 columns and 8 rows of microtiter plate, at series of concentrations, respectively. Also 100 µl of common antifungal and nanosilver solutions were added to microtiter plates, at series of concentrations, separately. Finally, 100 µl of yeast suspension was added to all microtiter plates and were placed on shaker for 3 to 5 min and were incubated at 37°C for 24, 48 and 72 hours. This process was repeated 3 times for each sample and the averages of 3 independent experiments were considered as the MIC value. The fractional inhibitory concentration (FIC) indexes were calculated as a summation of the MIC for antifungal drug in the combination/MIC for antifungal drug alone and the MIC for nanosilver in the combination/MIC for nanosilver alone. The effects of the drugs and nanosilver were interpreted to be indicative of synergy, partial synergy, additive, indifferent, or antagonistic when the FIC indexes were ≤ 0.5 , 0.5 to 1, 1, 1 to 4 or $4 \leq$, respectively. The data were analyzed using man-whitni U test, chi-squire test and logistic regression [12, 16, 19].

RESULTS AND DISCUSSION

Totally 30 cases of recurrent vaginal candidiasis were found to be caused by the following etiologic agents including *C. albicans* (63.3%), *glabrata* (13.3%), *krusei* (10%), *tropicalis* (6.6%), *parapsilosis* (3.3%), and *famata* (3.3%). Subsequent to statistical analysis of the relevant data base, findings about the activity of medicaments against these candida species are summarized in the tables (1-3):

Table 1: MICs mean of any drugs on based µg/ml after 72 hours

drug	samples	average	The minimum MIC	The maximum MIC
Fluconazole	30	87.2000	4.00	128.00
Ketoconazole	30	75.5333	1.00	128.00
Clotrimazole	30	105.60	32.00	128.00
Nystatin	30	49.6000	16.00	128.00
Nanosilver	30	37.3333	16.00	64.00

Chi-square test was used to evaluate relationship between the type of drug combination and synergistic effects which this test is significant ($P < 0.0001$). (table 2)

Table 2: The effects of Current drugs in combination with Nanosilver on Candida species

FICI (72h)	drugs				total
	nanosilver and fluconazole	nanosilver and nystatin	nanosilver and ketoconazole	nanosilver and clotrimazole	
FICI ≤ 0.5	15 50%	11 36.7%	19 63.3%	4 13.3%	49 40.8%
0.5 < FICI < 1	8 26.7%	13 43.3%	9 30.0%	9 30.0%	39 32.5%
FICI = 1	0 0%	3 10.0%	0 0%	1 3.3%	4 3.3%
1 < FICI < 4	7 23.3%	3 10.0%	2 6.7%	16 53.3%	28 23.3%
total	30 100.0%	30 100.0%	30 100.0%	30 100.0%	120 100.0%

Figure 1: comparison of MIC mean of each drug on Candida species at different times

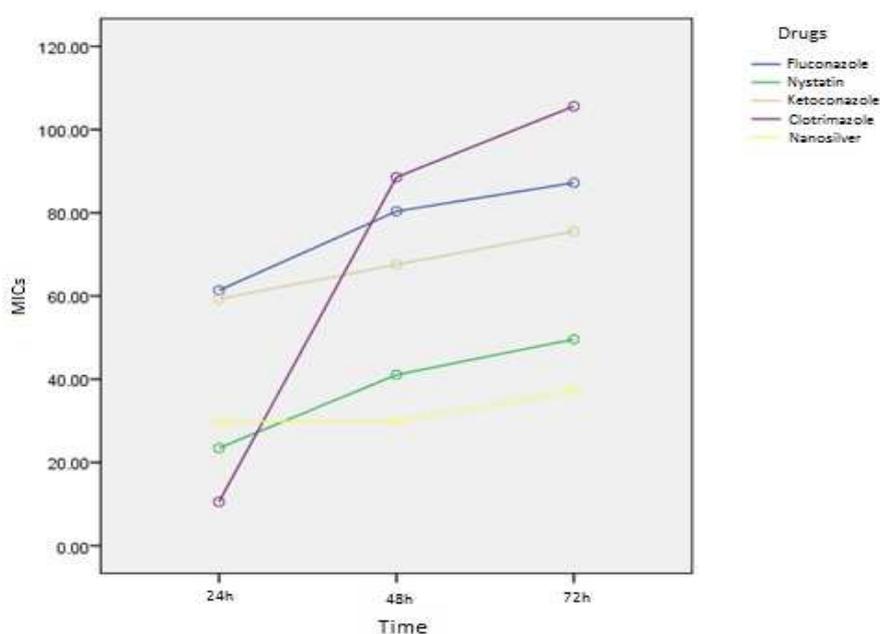


Figure 2: comparison of FIC mean of each drug in combination with nanosilver on Candida species at different times

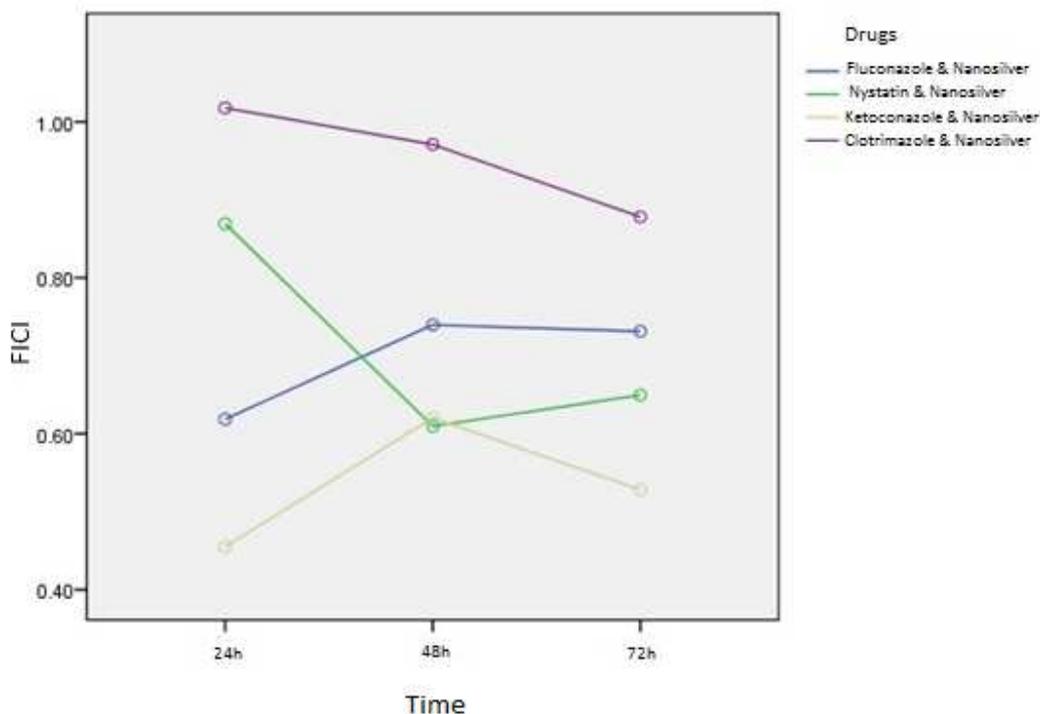
Potential synergistic effects of each drug in combination with nanosilver were estimated by logistic analysis and are shown in table 3.

Table 3: Demonstration of potential synergistic effect of drugs with nanosilver

drug	Potential synergistic effect
Ketoconazole	0.93
Nystatin	0.80
Fluconazole	0.78
Clotrimazole	0.43

In this study, it was found that the common drugs combined with nanosilver particles show higher anti fungal effect and may partially or totally inhibit the yeast growth. The MIC values of these drugs against candida species ranged from 4 to 128 µg/ml. In this study, 5.26% of *Candida albicans* isolated from patients were sensitive to fluconazole, and in another research 7.4% of *Candida albicans* species were sensitive to fluconazole, which was close to results achieved in the present study. Unlike Kim et al who showed that the MICs of nanosilver on candida species ranged

from 1 to 25 µg/ml, in our study, the MICs mean of nanosilver was 37 µg/ml at 72 hours; this difference could be caused by the discrepancy in standard strain that had been used in the two studies [5, 14].



The MICs mean of ketoconazole and nystatin on candida species were 49.6 µg/ml and 75.5 µg/ml, respectively. 26.6% of candida species were sensitive to ketoconazole but all species were resistant to nystatin. Accordingly, Klein indicated candida species were resistant to ketoconazole and nystatin [4]. In another study, Suarez showed candida species' resistance to clotrimazole (MIC: 0.22 mg/ml) [17]; however, in our study, all candida species were resistant to clotrimazole that it is probably due to self-treatment and abuse of the drug.

According to the results of the study, combination of ketoconazole with nanosilver had the maximum synergistic effect (FICI mean: 0.52) on candida species isolated from chronic vaginitis at 72 hours, while combination of clotrimazole with nanosilver had the minimum synergistic effects (FICs mean: 0.87).

The chi-square test used to evaluate relationship between the type of drug combination and synergistic effects was significant. As shown in table 2, the combination of fluconazole with nanosilver had synergistic effects on 50% of candida species and partial synergistic effects on 26.7% of species, and there were not any interactions between other candida species. Gajbhiye et al indicated that the combination of fluconazole with nanosilver produced by fungi had been effective on standard strain of *Candida albicans* [9], the results of which were compatible with ours.

The combination of nystatin with nanosilver had synergistic effects on 36.7% of candida species, partial synergistic effects on 43.3% of species and additive effects on 10% of species, but there was not any interaction on 10% of candida species. In other study, indicated the synergistic effects of nystatin in combination with Nanosilver particles on *Candida* species [14].

The combination of ketoconazole with nanosilver had synergistic effects on 63.3% of candida species and relative synergistic effects on 30% of species, but there was not any interference in 2% of candida species. In addition, Khodavandi et al indicated that the combination of ketoconazole with allicin had synergistic effects on candida species, such as *C. albicans*, *C. glabrata* and *C. tropicalis* [11].

The combination of clotrimazole with nanosilver had synergistic effects on 13.3% of candida species, partial synergistic effects on 30% of species and additive effects on 3.3% of species, but there was not any interaction on 53.3% of candida species, which indicates that this drug acts weaker than others.

Finally, as shown in table 3, the logistic regression showed that the combination of ketoconazole with nanosilver had the maximum (93%) and the combination of clotrimazole with nanosilver had the minimum (43%) synergistic effects, which is probably due to self-treatment and abuse of clotrimazole by patients.

CONCLUSION

proper diagnosis of infection in laboratory, performing in vitro susceptibility tests, and administration of topical formulations of nanosilver particles in combination with current drugs can be useful for more effective treatment of vaginal candidiasis and prevention of the disease recurrence.

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