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Assessment of nanosilver particles fungicidal activity toward *Candida* spp. isolated from the feet of adult human carriers

Ocena działania grzybobójczego nanosrebra wobec
Candida spp. izolowanych ze stóp dorosłych osób

Summary. The present studies aimed at: 1) species identification of the isolates collected from the skin of the feet; 2) evaluation of the fungicidal properties of nanosilver towards isolated strains of the *Candida* genus.

The studies included 61 healthy individuals aged 20–60 who were assessed in relation to the occurrence of *Candida* yeast carriage. Fungal culture and identification were conducted according to the standard procedures. Evaluation of fungicidal operation of nanosilver was carried out against the isolated strains of *Candida* genus and the reference strain. During the study, the modified dilution-neutralization method was applied according to the PN-EN 1275.

Swab samples collected from the skin of the lower extremities of 10 people (15.8%) was positive for the presence of fungi. The present studies showed that among the recovered yeasts, the presence of *C. albicans* and *C. guiliermondii* was observed as well as single cases of *C. parapsilosis*, *C. famata* and *C. lusitaniae*. All the tested strains proved to be susceptible to nanosilver application, i.e. all the studied strains exhibited the reduction in viable cell counts within 60 min of exposure time.

Key words: nanosilver, disinfection, *Candida* spp.

INTRODUCTION

Mycotic infection of skin and appendages is among the most common global disorders. It constitutes a major epidemiological, therapeutic and social problem [Ioannidou *et al*. 2006, Khaled and Hamad 2012]. Generally, mycotic infections are exogenous and have an external source. Fungi invade human organism via the following portals of entry:

the respiratory tract, gastrointestinal tract or broken skin [Richardson and Warnock 2012]. High numbers of fungi infect organism only at extreme conditions, most frequently in immunocompromised hosts. Recently, the number of mycosis episodes has shown the upward trend. Chronic fungal infection prevalence rate in the population of the temperate climatic zone is 10–20%, while 40% of population worldwide [Khaled and Hamad 2012].

Fungi, which are responsible for the skin diseases, are characterized by great diversity and variability. Currently, out of 250 thousand of the described fungal species, only about 200–300 fungi are known to cause diseases of humans and simply several species are clinically relevant [Richardson and Warnock 2012].

The yeast *Candida* have emerged as a major cause of fungal diseases, they induce as much as 55–90% mycotic infections [Richardson 2005]. Yeast-like fungi of *Candida* genus compose the saprophytic flora colonizing the gastrointestinal tract, genitourinary tract, skin and mucous membranes of upper airways. Superficial candidal infections affect the mucosal surfaces and skin where *Candida* organisms grow as commensals. Among the *Candida* species, *C. albicans* and *C. tropicalis* belong to the most invasive pathogens and may also play a role in pathogenesis of interdigital ulceration on hand or foot and even sepsis [Luo *et al*. 2011].

Footwear protection against microbial, and particularly fungal, contamination is an essential procedure in the prophylaxis of foot mycoses. This can be achieved by using of suitable chemical to disinfect feet and/or footwear. Nanosilver can make a promising alternative for rising resistance of fungi to commonly used antifungal substance.

Detailed mechanisms underlying the nano-Ag biocidal activity still remain unknown. Notably, it was found that its physicochemical parameters, namely specific surface area, high adsorption ability, chemical reactivity and catalytic properties improve its antimicrobial capacity [Banach *et al*. 2007, Li *et al*. 2010]. Nanostructured silver size and shape, a synthesis method and exposure time to nanosilver directly influence the biocidal effect of silver nanoparticles [Chmielowiec-Korzeniowska *et al*. 2013].

The oligodynamic microbicidal action of nano-Ag includes perforation of the cell wall and membrane [Xu *et al*. 2013], enzyme and protein denaturation, influence on metabolic and biochemical processes of cells and binding with DNA phosphate radicals [Luo *et al*. 2011]. Biocidal activity of nanoscale silver, in the case of fungi, is determined by a developmental stage of fungus and nanosilver concentration [Falkiewicz-Dulik and Macura 2006, Kim *et al*. 2008].

There is a large body of literature addressing bactericidal properties of nanosilver particles [Sondi and Salopek-Sondi 2004, Pal *et al.* 2007, Kim *et al*. 2008, Xu *et al*. 2013]. Biological activity is therefore a very important factor, so that nanosilver is used where asepsis is highly desirable. Mostly, this applies in biomedical engineering, cosmetology, food packaging industry, textile industry, crops, animal husbandry etc. [Czyż *et al*. 2013, Banach *et al.* 2016].

Still, biocidal effects of nanostructed silver towards fungal pathogens of the skin, including *Candida* spp. isolates, have not been analyzed so far. The present paper discusses the studies on fungal carriage state, mainly *Candida* genus, amongst healthy individuals and assessment of nanosilver effectiveness towards the isolated species of *Candida* spp.

The present studies aimed at: 1) species identification of isolates collected from feet; 2) evaluation of fungicidal properties of nanosilver towards isolated strains of *Candida* genus.

MATERIAL AND METHODS

The studies included 61 healthy individuals aged 20–60 years who were assessed in relation to occurrence of *Candida* yeast carriage.

Material for bacteriological evaluation was collected from the interdigital spaces of the left and right foot with a sterile swab at the end of the day. The feet were not washed or disinfected.

Species identification of isolated fungi

Fungal culture and identification were conducted according to the standard procedures of diagnostic mycology. The material for the study were swabs interdigital with the left foot and right, taken sterile swab moistened with sterile saline solution. Cultures were grown on the Sabouraud solid medium with chloramphenicol and incubated for 2–4 days at 37°C temperature.

With the purpose of obtaining pure cultures of a given strain, strain differentiation was made based on morphology. A pure culture was multiplied under the aforementioned conditions. After 48 h, the reading of culture was taken and Gram stain performed to provide preliminary identification. The final identification was conducted using a standardized system API 20C AUX (bioMérieux, Inc., Cyon, France) with biochemical tests.

Assessment of fungicidal activity of nanosilver particles

Evaluation of fungicidal operation of nanosilver was carried out against the isolated strains of *Candida* genus and the reference strain provided by the ATCC collection (Microbiologics Inc. St. Cloud, Minnesota, USA). During the study, the modified dilution-neutralization method was applied according to the PN-EN 1275 "Basic fungicidal activity".

Prior to the test, the fungal strains were multiplied on the Sabouraud medium at 37°C for 24 h. After incubation, the obtained cultures were used to form in sterile distilled water the test suspension of constant cell concentration $1{,}5 \times 10^6$ cfu/ml. Further, the suspension was diluted using a 10-fold serial dilution protocol. For precise quantification of cell numbers in the initial suspension (Z) from the successive dilutions Z^{-5} and Z^{-6}, the appropriate agar media were inoculated with 0,1 ml volume of the microorganism suspension. Neutralization mixture was prepared by adding to 8 ml neutralization medium 1m sterile distilled water. Next, from the first dilution Z^{-1}, 1 ml microbial cell suspension was placed in each of 4 test tubes with 9 ml nanosilver.

The experiment was performed for 5, 15, 30 and 60 min exposure of microorganisms to nanosilver. After the required time, 1 ml solution of suspension and nanosilver was put to the 9 ml neutralization solution prepared before. The 5-minute neutralization process was followed by surface inoculation on the Sabouraud medium. Neutralizing Broth (Scharlau, S.L., Spain) was used as a neutralizing agent in the present studies.

Neutralization solution validation process was carried out making the following mixtures: one containg 8 ml neutralizer, 1 ml sterile distilled water and 1 ml suspension of microorganisms tested Z^{-5} and the other three mixtures, each composed of 8 ml neutralizer, 1 ml nanosilver and 1 ml suspension of microorganisms studied Z^{-5}. Then 1 ml of inoculum was taken from each test tube and spread onto two plates with Sabouraud medium. All the plates were incubated at 37°C for 24–48 h and then the numbers of all

colonies visible at the media were estimated; the counting range of the acceptable number of colonies per plate was 15–300 cfu.

Analysis of biocidal activity of nanosilver was based on the calculation of:

1) weighted mean fungal count (N) from the formula:

$$N = \frac{c}{(n_1 + 0{,}1n_2)d}, \text{ where:}$$

N – weighted mean fungal count (cfu/ml);
c – sum of colonies calculated on all plates included in calculations;
n_1 – number of plates included in calculations from 1st dilution;
n_2 – number of plates included in calculations from 2nd dilution;
d – dilution factor corresponding to 1st dilution included in calculations.

2) numbers of fungal suspension (Na) from the formula:

$$N_a = \frac{c}{n \times d \times V}, \text{ where:}$$

N_a – number of viable fungi exposed to nanosilver activity, cfu/ml;
c – sum of colonies counted at both plates;
n – number of plates included in calculations;
d – dilution factor corresponding to 1st dilution included in calculations;
V – sample volume; 1 ml for dilution-neutralization protocol, validation procedure and fungal suspension.

For each identified organism, the reduction in fungal viability (fungal viable count) was calculated and reported.

3) Reduction in fungal viable count (R) was calculated from the formula:

$$R = \frac{N \times 10^{-1}}{N_a}, \text{ where:}$$

R – reduction in fungal viable count (%);
N – weighted mean fungal count;
N_a – number of viable fungi exposed to nanosilver solution activity.

Characteristics of nanosilver preparation

The present studies tested colloidal nanosilver of 50 ppm concentration. The suspension was obtained by a chemical reduction process in liquid phase. A source of silver ions was silver nitrate ($AgNO_3$). The solutions were prepared using deionized water. The reduction process was carried out in a 4525 PARR pressure reactor at 70–80°C at elevated pressure. With application of sodium pyrophosphate as a stabilizer and

glucose as a reducing agent, nanoscale silver was obtained. The suspension contained monodisperse poloyhedral silver particles of 10 nm dimensions at average and electrokinetic potential $\zeta = -31{,}5$ mV [Banach *et al*. 2012].

RESULT AND DISCUSSION

Species identification of isolates collected from feet

The swab samples collected from the lower extremities skin of 10 people (15,8%) displayed the fungal presence. The mycological evaluation showed the occurrence of 8 isolates, in that 6 identified as yeast-like fungi of the genus *Candida* and *Saccharomyces*, including *S. cerevisiae* (Fig. 1). The relatively pathogenic ones encompassed the identified dermatophyte *Microsporum canis* and alga *Prototeca wickerhamii*, which is known to cause protothecoses in human [Jagielski 2006]. In the present studies, among the recovered yeasts, the presence of *C.albicans* and *C. guiliermondii* was stated as well as single cases of *C. parapsilosis*, *C. famata* and *C. lusitaniae*. Shamim *et al*. [2003] reported that the most frequently isolated yeasts were *Candida albicans*, *C. glabrata* and *C. tropicalis*. The studies of Raz-Pasteur *et al*. [2011] on a human skin model for *ex vivo* evaluation showed that *C. albicans* exhibited far higher adherence potential to skin sections as compared to *C.* non-*albicans*. However, as all of them displayed strong adhesion properties, they can be isolated from the human skin and mucous membranes and that may play a key role in pathogenesis of superficial candidiases.

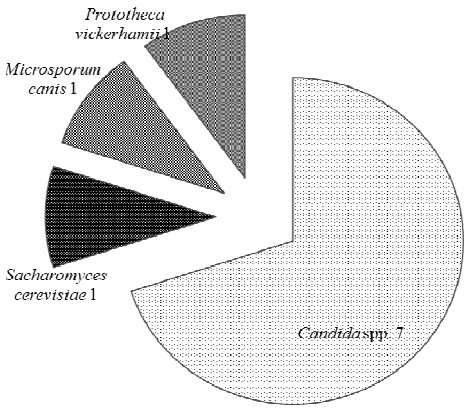

Fig.1. Number and genus of fungi isolated from feet skin
Rys. 1. Liczba i rodzaj grzybów izolowanych ze skóry stóp

Until recently, *C. albicans* accounted for 70–90% isolates recovered from yeast-infected individuals, whereas other *Candida* species were rarely isolated from clinical specimens [Anane *et al*. 2007].

Assessment of antifungal activity of nanoscale silver

The results of assessment of tested nanomaterial fungicidal properties together with validation outcomes are presented in Table 1 and 2.

Table 1. Results of antifungal activity of nanosilver
Tabela 1. Wyniki działania grzybobójczego nanosrebra

Test organism Organizm testowy	Fungal viable count (cfu/ml) in studied mixture (Na) in time Liczba zdolnych do życia komórek grzybów (cfu/ml) w badanej mieszaninie (Na) w czasie			
	5 min	15 min	30 min	60 min
Candida albicans ATCC	0,0	0,0	0,0	0,0
Candida albicans	$9{,}5 \times 10^3$	$5{,}5 \times 10^2$	0,0	0,0
Candida guilliermondii	$5{,}0 \times 10^1$	0,0	0,0	0,0
Candida parapsilosis	$8{,}7 \times 10^3$	$5{,}0 \times 10^1$	0,0	0,0
Candida famata	0,0	0,0	0,0	0,0
Candida lusitaniae	$7{,}7 \times 10^4$	$4{,}5 \times 10^3$	0,0	0,0
Reduction of fungal viable count within studied time Redukcja liczby zdolnych do życia komórek grzybów w badanym czasie				
Candida albicans ATCC	$> 10^4$	$> 10^4$	$> 10^4$	$> 10^4$
Candida albicans	$7{,}5 \times 10^1$	$1{,}3 \times 10^3$	$> 10^4$	$> 10^4$
Candida guilliermondii	$3{,}8 \times 10^4$	$> 10^4$	$> 10^4$	$> 10^4$
Candida parapsilosis	$6{,}8 \times 10^1$	$1{,}2 \times 10^4$	$> 10^4$	$> 10^4$
Candida famata	$> 10^4$	$> 10^4$	$> 10^4$	$> 10^4$
Candida lusitaniae	$1{,}6 \times 10^1$	$2{,}9 \times 10^2$	$> 10^4$	$> 10^4$

Table 2. Verification of methodology and validation of dilution-neutralization method for a product under study
Tabela 2. Weryfikacja metodyki i walidacja metody rozcieńczania-neutralizowania dla badanego produktu

Test organism Organizm testowy	Fungal viable count / Liczba zdolnych do życia komórek grzybów(cfu/ml)			
	test fungal suspension testowa zawiesina grzybów (N)	fungal suspension zawiesina grzybów (Nv)	control of neutralizer toxicity kontrola toksyczności neutralizatora (Nx)	control of dilution-neutralization kontrola rozcieńczania-neutralizowania (Ny)
Candida albicans ATCC	$6{,}4 \times 10^6$	$6{,}5 \times 10^2$	$5{,}5 \times 10^3$	$7{,}8 \times 10^3$
Candida albicans	$7{,}1 \times 10^6$	$6{,}9 \times 10^2$	$6{,}5 \times 10^3$	$7{,}6 \times 10^3$
Candida guilliermondii	$1{,}9 \times 10^7$	$1{,}9 \times 10^3$	$2{,}3 \times 10^4$	$2{,}1 \times 10^4$
Candida parapsilosis	$5{,}9 \times 10^6$	$5{,}6 \times 10^2$	$7{,}6 \times 10^3$	$5{,}9 \times 10^3$
Candida famata	$8{,}5 \times 10^6$	$8{,}8 \times 10^2$	$1{,}1 \times 10^4$	$1{,}0 \times 10^4$
Candida lusitaniae	$1{,}3 \times 10^7$	$1{,}3 \times 10^3$	$1{,}7 \times 10^4$	$1{,}8 \times 10^4$

According to the PN-EN 1275 applied to assess the efficacy of biocidal activity of chemical disinfectants, a preparation is consistent with the requirements of applicable standards if it causes a 10^4-fold or higher reduction in fungal viable count after 60 min

(or shorter) contact time at 20°C temperature. The present studies have shown that all determined strains proved to be susceptible to nanosilver application, i.e. all the studied strains exhibited the reduction in viable cell counts within 60 min exposure time (Tab. 1).

However, the assessment indicated that not all the strains are equally susceptible to nanoscale silver. In the case of the reference organism *Candida albicans* ATCC and the strains *C. famata* and *C. guiliermondii* isolated from the foot skin, the required reduction (10^4-fold) in the fungal viable cell counts occurred as early as after 5 min. Whereas, yeast *C. albicans*, *C. parapsilosis* and *C. lustaniae* appeared to be more resistant strains as the disinfecting activity of nanosilver was achieved not until the 30-minute contact with the preparation.

The present studies results are consistent with those reported by other authors. The experimental study of Nozari *et al*. [2012] compared the antifungal activity of fluconazole and silver nanoparticles against the following yeasts isolated from candidal vulvovaginitis: *Candida albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. famata*. The studies confirmed the same biocidal effectiveness of both preparations. Besides, the activity of fluconazole in combination with nanosilver particles increased the antifungal effects. Ravindran *et al*. [2013] highlighted nanosilvers potent activity comparable with that of amphotericin B used to treat severe systemic infections. Kim *et al*. [2008] pointed to the therapeutic potential of nanosliver for successful treatment of infectious fungal diseases. The authors proved that silver nanoparticles exhibit low hemolytic activity and it is essential with regard to safe application of preparations.

Biocidal properties of silver nanoparticles, in the case of fungi, are dependent on a fungal developmental stage and nanosilver concentration [Kim *et al*. 2008]. Special attention should be drawn to the reports discussing silver nanoparticles as potential inhibitors of biofilm formation whose complex structure makes it resistant to a wide range of pharmaceuticals [Monteiro *et al*. 2009, Soumitra *et al*. 2012]. It is of primary importance for control of fungi *Candida* genus, whose adherence properties make the key factor deciding about their pathogenicity.

CONCLUSIONS

The present studies have confirmed antifungal activity of nanosilver. A preparation applied was affective against the isolated from the foot skin yeasts *Candida* genus which are known to induce superficial fungal infections. Therefore, nanosilver can be successfully used in the textile industry for impregnation of socks and shoe liners and in the medicine as an anti-infection agent and in therapy of yeast-induced mycoses.

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Streszczenie. Celem przeprowadzanych badań była: 1) identyfikacja gatunkowa izolatów pobranych ze skóry stóp; 2) ocena właściwości grzybobójczych nanosrebra wobec wyizolowanych szczepów z rodzaju *Candida*.

Badaniami objęto 61 zdrowych osób w przedziale wiekowym 20–60 lat, u których oceniano występowanie nosicielstwa drożdżaków z rodzaju *Candida.* Hodowlę i identyfikację przeprowadzano według standardowych procedur. Ocena grzybobójczego działania nanosrebra przeprowadzona została dla wyizolowanych szczepów z rodzaju *Candida* oraz szczepu referencyjnego. Badania prowadzono zmodyfikowaną metodą rozcieńczania-neutralizowania zgodnie z polską normą PN-EN 1275.

W wymazach pobranych ze skóry kończyn dolnych u 10 osób (15,8%) stwierdzono obecność grzybów. Wśród izolowanych drożdży zidentyfikowano *C. albicans* i *C. guilliermondii* a w pojedynczych przypadkach *C. parapsilosis*, *C. famata* i *C. lusitaniae*. Wszystkie testowane szczepy były wrażliwe na użyte nanosrebro, tj. wszystkie wykazały redukcję komórek zdolnych do życia w czasie 60 min.

Słowa kluczowe: nanosrebro, dezynfekcja, *Candida* spp.