Antifungal activity of silver nanoparticles in a murine model of oral candidiasis

Abstract

Objective: The present study assessed the antifungal activity of silver nanoparticles against oral candidiasis induced, in immunosuppressed male rats, using Candida albicans (ATCC 90028).

Materials and Methods: One hundred and ninety two rats were assigned into six groups of 32 rats each. Rats in Group 1 (normal control) were immunosuppressed, but not infected with C. albicans. Those in Group 2 (negative control) were infected with the fungus. Rats in Groups 3 and 4 (test groups) were infected with C. albicans and treated topically with 50 µg/ml and 100 µg/ml silver nanoparticle solution, respectively. Those in Groups 5 and 6 (positive controls) were infected with the fungus, and treated topically with 2% miconazole oral gel and 2% miconazole aqueous suspension, respectively. All treatments were applied topically once daily for 2 weeks. Macroscopic evaluation of the candidal lesions, microbial counting and histopathological changes were evaluated on day 7, day 14 and the follow up assessment was performed at day 28.

Results: Silver nanoparticles provided quicker and effective antifungal activity against mucosal C. albicans infection, compared to miconazole gel or suspension, at day 7. However, the silver nanoparticles were comparable to miconazole at the end of therapeutic period (day 14) and at the end of the follow up period (day 28). The microbiological data are corroborated by histological findings.

Conclusion: Silver nanoparticles may be a promising candidate for the treatment of oral candidiasis. Additional studies are nonetheless warranted.

Keywords: Antifungal effect, Candida albicans, Miconazole, Oral candidiasis, Rat, Silver nanoparticles.
Amaç: Bu çalışma, Candida albicans (ATCC 90028) kullanılarak immünsuprese erkek sıçanlarda indüklenen oral kandidiyaza karşı gümüş nanopartiküllerin antifungal aktivitesini değerlendirildi.


Sonuç: Gümüş nanopartiküller oral kandidiyazis tedavisi için umut verici bir aday olabilir. Bunuyla birlikte ek çalışmalar garantilidir.
Introduction

Oral candidiasis is an opportunistic infection caused mainly by *Candida albicans*, an ubiquitous fungus that is a part of the normal oral flora in up to 80% of healthy individuals\(^1\). Changes in the buccal cavity, or an imbalance in host defenses, may result in loal *C. albicans* overgrowth, and then the transition of the relationship between *C. albicans* and its oral environment from the commensal state to an infectious state\(^2\). The capacity of *C. albicans* to be truly pathogenic is related to ability to successfully adhere, colonize, produce enzymes and interfere with host defense mechanisms\(^3\). The treatment of initial oropharyngeal candidiasis is considered obligatory, irrespective of the fact that symptoms severity may vary between individuals. If untreated, progressive colonization can lead to increased discomfort. Furthermore, this may predispose patients to more invasive disease stages, including esophageal candidiasis\(^4\).

Usually topical antifungal agents are the drugs of choice for uncomplicated, localized candidiasis. They can be administered as suspensions (e.g. nystatin or amphotericin B), gels (e.g. miconazole), or lozenges (e.g. clotrimazole troches)\(^5\). Miconazole is well-tolerated and has a broad spectrum activity against several species of *Candida*\(^6\). Unfortunately, there is now evidence of the development of resistance to many conventional antifungal agents by *Candida albicans*. This has stark implications for morbidity and mortality and warrants an urgent need for the development of novel antifungal therapies\(^8,9\).

Nanoparticles are clusters of atoms in the size range of 1–100 nanometers. The use of nanoparticles in medicine is gaining traction and different types of nanomaterials such as copper, zinc, titanium, magnesium, gold and silver have been reported to be useful in managing different cases\(^10\). Silver nanoparticles have however received special attention because of its possible efficacy as anti-bacterial and anti-viral agents and its reported activity against eukaryotic micro-organisms\(^11-13\).
Different *in vitro* studies indicate that therapy with nanosilver solution have lethal effects on yeasts of the *Candida* genus\textsuperscript{14-18}. However, only few studies have investigated the effects of silver nanoparticles in animal models\textsuperscript{19-22} of some diseases, but not oral candidiasis. It is therefore important to assess the anti-fungal property of silver nanoparticles against *Candida albicans* in a rat model. The aim of the present study was therefore to test the topical antifungal activity of silver nanoparticles in the treatment of experimentally-induced oral candidiasis in rats.

**Materials and methods**

**Drugs and chemicals**

Tetracycline hydrochloride was kindly provided by Chemical Industries Development Company (CID, Giza, Egypt). Prednisolone was obtained from Sanofi-Aventis (Cairo, Egypt). Xylazine chloride (Xyla-Ject\textsuperscript{®} vial) was purchased from ADWIA Pharmaceutical Company (10\textsuperscript{th} of Ramadan City, Egypt) and ketamine (Ketamine vial\textsuperscript{®}) was purchased from Sigma–Tec Pharmaceutical Industries (Egypt). Oral 2% (w/w) miconazole gel (Daktarin\textsuperscript{®} Oral Gel, Janssen Pharmaceutica NV, Beerse, Belgium) was purchased from the market. Micronized miconazole base in a powdered form was kindly provided by Medical Union Pharmaceuticals (MUP, Ismailia, Egypt). A 2% miconazole suspension was prepared by suspending 2 g of miconazole powder in 100 ml of distilled water. Synthesized silver nanoparticle aqueous solution was purchased from Nano-Tech Company (The sixth of October city, Egypt) and prepared in two different concentrations (50 and 100 μg/ml). Sabouraud dextrose agar plates were purchased from Difco Microbiology Systems (Franklin, NJ, USA).

**Characterization of silver nanoparticles**
The preliminary detection of silver nanoparticles was carried out by visual observation of the color change of the cell filtrates. Also, the optical properties of the prepared silver nanoparticles were investigated by recording the plasmon band for silver nanoparticles using the UV-Visible absorption spectra. The shape and size of the prepared silver nanoparticles were recorded by the transmission electron microscopy (HR-TEM) images.

The UV absorbance was measured on a PERKIN-ELEMER LAMBDA 4B spectrophotometer using 1-cm matched quartz cells and scanning the spectra between 250 and 800 nm. The transmission electron microscopy (HR-TEM) images were captured using a JOEL JEM-2100 system, equipped with a Gatan digital camera (Erlangshen ES500).

**In vitro susceptibility test**

For determination of the minimal inhibitory concentrations (MIC) for silver nanoparticles, two-fold dilutions of silver nanoparticles were added in sabouraud dextrose broth (SDB) medium. Then, *C. albicans* (ATCC 90028) was inoculated in SDB tube and incubated for 48 h at 37 ºC. Thereafter the density of the suspension was adjusted to 1 McFarland standard (3×10⁸ CFU/ml) with sterile saline. The silver nanoparticles suspensions (0.5 ml each) were added to respective tubes containing the fungal suspensions. The lowest concentration of silver nanoparticles that caused complete inhibition of the growth of *C. albicans in vitro* was taken as the MIC. The MIC experiments were replicated five times.

**In vivo antifungal activity**

**Experimental animals**

One hundred and ninety two 12-weeks old, female, specific-pathogen free, Wistar albino rats weighting 200–250 g, were obtained from the Center of Experimental Animals, Ministry of Health, Helwan, Cairo, and used in the study. Animals were acclimatized for two weeks before starting the experiment. During the acclimatization and experimental periods, rats
were housed in large polycarbonate plastic cages (maximum of 5 rats per cage). Rats were given *ad libitum* access to feed and water, and exposed to a normal dark/light cycle. Care was taken to minimize animal suffering or pain through appropriate handling. The study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Suez Canal University, Faculty of Pharmacy, Ismailia, Egypt.

**Induction of experimental candidiasis**

To the rats, 1000 mg/L tetracycline hydrochloride was given in drinking water. This treatment was initiated seven days prior to the inoculation of a *C. albicans* (ATCC 90028) suspension and was maintained up to the end of the experiment [25]. Then, rats were immunosuppressed with two subcutaneous injections of prednisolone (100 mg/kg) one day before and 3 days after infection with the organism. Forty eight hours before inoculation, animals were sampled by rolling a sterile cotton swab over the oral cavity, to confirm the absence of *C. albicans* in the oral cavity.

A suspension of *C. albicans* (ATCC 90028) containing $5 \times 10^8$ viable cells/ml was prepared according to the method described by Reed *et al.* [26]. For the inoculation of this suspension, rats were sedated via intramuscular injection of xylazine chloride solution and ketamine (1:0.5 ml, 85 and 13 mg/kg of body weight, respectively). The *C. albicans* (ATCC 90028) suspension (0.2 ml) was delivered into the mouths of the rats with a 1-ml syringe. Next, the suspension was spread on the tongue dorsum with a swab. This procedure was repeated for three consecutive days [27] (starting from day 9 to day 11). Seven days after the last inoculation all groups were sampled as described earlier to check for the presence of the fungi and to quantify the number of colony forming units (CFU) in the oral cavity before the starting the different therapeutic regimens.
Study groups

After the disease induction, rats were blindly randomized into six groups of 32 rats each as shown in Table 1 below.

Table 1: Protocol for randomizing rats into groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Description of group/treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control</td>
<td>Rats without oral candidiasis (immunosuppressed but not infected or drug treated)</td>
</tr>
<tr>
<td>2</td>
<td>Negative control</td>
<td>Rats with oral candidiasis (immunosuppressed and infected but not drug-treated)</td>
</tr>
<tr>
<td>3</td>
<td>Nanosilver 50 µg/ml</td>
<td>Rats with oral candidiasis and treated topically with silver nanoparticle solution (50 µg/ml)</td>
</tr>
<tr>
<td>4</td>
<td>Nanosilver 100 µg/ml</td>
<td>Rats with oral candidiasis and treated topically with silver nanoparticle solution (100 µg/ml).</td>
</tr>
<tr>
<td>5</td>
<td>Positive control (gel)</td>
<td>Rats with oral candidiasis treated topically with 2% miconazole oral gel</td>
</tr>
<tr>
<td>6</td>
<td>Positive control (suspension)</td>
<td>Rats with oral candidiasis and treated topically with 2% miconazole aqueous suspension.</td>
</tr>
</tbody>
</table>

Ten ml/kg body weight (bw) of all therapies were applied topically daily for two weeks. Distilled water (10 ml/kg bw) daily was applied to the tongue of rats in the normal and negative control groups for two weeks. Miconazole was used as a reference drug and thus served as positive control agent.

Study design

Baseline assessment was conducted on day 0 (just before treatment). Evaluation of the candidal lesions during drug treatment was conducted on days 7, 14 and on day 28 when post-treatment follow up assessment was conducted. At all-time points, macroscopic
evaluation was performed by oral examination to determine the extent of the oral candidal lesions. Direct examination of fungal cultures was performed at all of the time points. Eight rats from each experimental group were sacrificed at the four different time points above, and their tongue biopsies were taken for histological comparisons between groups.

Evaluation of the *Candidal* infection

**Macroscopic evaluation**

Macroscopic evaluation of the infection was done and a score from 0 to 4 assigned on the basis of the extent and severity of whitish, curd-like patches on the tongue surface (TS). A score of 0, 1, 2, 3, and 4 represent normal, white patches over less than 20% of the TS, white patches over more than 20% but less than 21% of the TS, white patches over more than 91% of the TS, and thick white patches appearing like pseudo membranes over more than 91% of the TS, respectively. Evaluation was performed by a trained observer blinded to the treatment groups.

**Microbiological analysis**

Collected samples were suspended in 5 mL of sterile saline. Then, 10 μl of each suspension was dropped in duplicate, after serial tenfold dilution on sabouraud dextrose agar plates containing cyclohexamide [(500 mg/L) in 5 mL acetone] and 0.05 % chloramphenicol [(50 mg/L) in 5 mL ethanol] to prevent sporelated fungi. All plates were incubated at 37 °C for 48 h, and the colonies were counted. The number of viable cells was determined to calculate the log of the CFU/ml. Counting was performed using a colony counter. In addition, each swab was cultured on a second media –CHROMagar Candida (CHROMagar Microbiology, Mumbi, India). Initial determination of species was screened by color on CHROMagar Candida.
Blood collection and determination of liver enzyme activities, serum creatinine and blood urea nitrogen

On the last day of the study, animals were anesthetized with xylazine-ketamine mixture (1:0.5 ml), at a dose of 85 mg/kg and 13 mg/kg bw, respectively. Blood samples for sera preparations were collected from the orbital sinus of each rat into plastic centrifuge tubes. Serum samples were separated from the clot by centrifugation at 1500 × g for 15 min using a bench top centrifuge (MSE Minor, England), pipetted into sterile plain tubes, and stored frozen at −20 °C until analyzed. Serum aspartate amino transferase (AST), serum alanine amino transferase (ALT), blood urea nitrogen (BUN) and serum creatinine were selected as indicators of toxicity\textsuperscript{29,30}. These parameters were determined using standard spectrophotometric methods.

Histopathological examination

For microscopic examination, the tongues were cut and fixed in 10% formalin for 24 h and hemisected in the sagittal plane. Next, the tissue samples were mounted in paraffin, and sectioned at 4-μm and stained with hematoxylin–eosin (H&E) and periodic acid–Schiff (PAS). To evaluate the degree of epithelium colonization by Candida, the semi quantitative method described by Junqueira et al. was used\textsuperscript{27}. Five histological fields of the tongue dorsum in each section, in the antero-posterior direction, were examined with a × 40 objective lens under a light microscope. A score from 0 to 4 was given for each histological field, representing absence of colonization, one to five hyphae, six to fifteen hyphae, sixteen to fifty hyphae, and more than fifty hyphae, respectively.

The intensity of the lesions was evaluated by examining epithelial alterations and the inflammatory response of the connective tissue. First, for the epithelial tissue, five tissue alterations were analysed: epithelial hyperplasia, disorganization of the basal layer, loss of filiform papillae, hyperparakeratosis, and the formation of intra-epithelial micro-abscesses.
Each epithelial alteration was classified as absent, discrete, or accentuated, such that the scores attributed were: 0 = absence of epithelial lesion, 1 = discrete epithelial lesion and 2 = accentuated epithelial lesion, with each rat tongue dorsum receiving a score varying from 0 to 10 points. In relation to chronic inflammatory infiltrate of the connective tissue, the following scores were attributed: 0 = absence of inflammatory cells, 1 = discrete inflammatory infiltrate and 2 = accentuated inflammatory infiltrate.

Photomicrographs were captured from tongue specimens stained with PAS or H&E from the experimental groups and photos (taken from different animals) were utilized to show the specific pathological changes presented by each stain.

Statistical analysis

Data derived from semi-quantitative systems were expressed as median. Statistical significance between groups was analyzed at each time point using Kruskal–Wallis test (a non-parametric ANOVA) and Dunn’s multiple comparisons’ test. However, quantitative variables were expressed as mean ± SEM and statistically analyzed using one-way ANOVA. The statistical software, SPSS version 16 (SPSS Inc., Chicago, IL) was employed for statistical analysis. Values of $P < 0.05$ were considered to be statistically significant.

Results

Characterization of silver nanoparticles

The purchased preparation of synthesized silver nanoparticles showed a characteristic surface plasmon peak at 425 nm (Figure 1-A). Further, silver nanoparticles were spherical and in the size range of 15-40 nm (Figure 1-B) as revealed by high resolution transmission electron microscopy.
microscopy. The particle size was in range of 12.5 to 40.5 nm, with an average size 20 nm. The nanoparticles were found to be stable for more than twelve months at room temperature if stored in dark brown glass bottles away from light.

**Determination of in vitro minimal inhibitory concentration**

*In vitro* determination of MIC for nanosilver against the *C. albicans* strain was performed before evaluation of the therapeutic efficacy of drugs. The MIC$_{50}$ for silver nanoparticles on *C. albicans* was 12.5 µg/L whereas the MIC$_{100}$ was 25 µg/L. Therefore, the double of this dose was used for *in vivo* testing of the anticandidal effect of nanosilver as described previously.

**In vivo antifungal activity of silver nanoparticles**

Each group in the *in vivo* experiment started with thirty two rats and there were no mortalities in all the experimental groups throughout the course of the experiment. In the present study, analysis of variance indicated a difference in the macroscopic scores among the test groups at day zero [$F_{(5,42)} = 20.76$], day 7 [$F_{(5,42)} = 25.61$], day 14 [$F_{(5,42)} = 31.54$] and day 28 [$F_{(5,42)} = 78.21$, $P < 0.05$]. Post-hoc analysis at day zero revealed that clinical scores in all test groups were different from the normal control group. Rats treated with 2% miconazole gel/suspension (positive controls) as well as those treated with nanosilver (50 or 100 µg/ml) solutions showed lower clinical scores (reduction in lesion size) compared to the negative control group at day 7. Further, rats treated with nanosilver (50 or 100 µg/ml) showed lower clinical scores compared to both positive controls. However, there was no significant difference between the two concentrations of nanosilver solutions. At day 14 (end of therapeutic period) and at day 28, (end of follow up period), all the treatment regimens improved the macroscopic score in comparison to the negative control group. There was no significant difference among the two test groups and the two positive control groups ($P < 0.05$, Fig. 2).
Regarding the microbial count, ANOVA indicated a difference in the microbial count among the test groups at day zero \([F(5,42)= 34.28]\), day 7 \([F(5,42)= 81.04]\), day 14 \([F(5,42)= 38.91]\) and day 28 \([F(5,42)= 45.77, P < 0.05]\). Post-hoc analysis at day zero indicated that the microbial counts in all the test groups were different from the normal control group. At day 7, all the treatment regimens decreased the microbial counts (reduction in log CFU/ml) compared to the negative control group. The log CFU/ml in rats treated with nanosilver (50 or 100 µg/ml) solutions was less than that calculated in rats in the positive control groups. Comparing the effect of the low and high concentrations of nanosilver solution highlighted a non-significant difference between the two groups. At day 14 and day 28, all the treatment regimens resulted in a decrease in the microbial count in comparison to the negative control group and there was no significant difference among the test groups and the positive control groups (Fig. 3).

Assessment for effect of nanosilver solutions on the activity of liver enzymes, blood urea and creatinine are shown in Table 1. Analysis of variance indicated non-significant differences between the test and the positive control groups regarding these parameters \((P < 0.05)\).

Histopathological examination for sections stained with PAS stain revealed that there was a difference in the colonization by *Candida* between the test and positive control groups at day zero \([F(5,42)= 85.14]\), day 7 \([F(5,42)= 68.62]\), day 14 \([F(5,42)= 54.31]\) and day 28 \([F(5,42)= 31.95, P < 0.05]\). Post-hoc analysis at day zero highlighted that all the test groups were different from the normal control group. At day 7, 14 and 28, all the treatment regimens showed lower score for colonization by *Candida* in comparison to the negative control group. At day 7 and day 14, the score obtained by rats treated with nanosilver (50 or 100 µg/ml) solutions was found reduced to a much greater extent compared to the positive control groups. Indeed, at day 28, both test groups produced a decrease in the score for colonization by candidal hyphae compared to the negative control but not the positive control groups (Fig. 4A&B).
Furthermore, histopathological examination for sections stained with H&E indicated a difference in the epithelial alterations among the test groups at day zero \( F(5,42) = 73.28 \), day 7 \( F(5,42) = 54.62 \), day 14 \( F(5,42) = 59.92 \) and day 28 \( F(5,42) = 59.34, P < 0.05 \). Post-hoc analysis at day zero showed that all the test groups were different from the normal control group. At the subsequent three time points, all the treatment regimens reduced the score for epithelial alterations. At day 7, the score obtained by rats treated with nanosilver (100 µg/ml) was lower than that obtained by rats in the positive control groups. At day 14, the score observed in rats treated with nanosilver (50 or 100 µg/ml) solutions was lower than that observed in both positive control groups. Differently, at day 28, the test groups and the positive control groups showed comparable scores for epithelial alterations (Fig. 5A&B).

In addition, analysis of variance demonstrated a difference in the score of inflammation among the test groups at day zero \( F(5,42) = 18.23 \), day 7 \( F(5,42) = 11.26 \), day 14 \( F(5,42) = 17.34 \) and day 28 \( F(5,42) = 19.72, P < 0.05 \). Post-hoc analysis at day zero indicated that all test groups were different from the normal control group. At the other three time points, all the treatment regimens reduced the inflammatory infiltrate compared to the negative control group. At day 7, rats treated with nanosilver (50 or 100 µg/ml) solutions exhibited lower scores compared to miconazole gel/suspension (positive control) groups. At day 14 and day 28, there was no significant difference between the test and positive control groups (Fig. 5A&C).

**Discussion**

Silver nanoparticles present a large surface area, which provides better contact with microorganisms\(^{28}\). The effective concentration of silver nanoparticles found in the present study was lower than that reported previously by other authors\(^{16,17}\). Compared with other
metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells\textsuperscript{29-33}.

Therapeutic efficacies of drugs used in this study for controlling oral candidiasis were evaluated by assessing the morphological changes in the tongue, microbiological examination on number of colony forming units in the lesions of the oral cavity, and histopathological examination of tongue tissues. The procedure adopted in the present study was successful as there was a progressive increase in the number of colonies after inoculation. The mean log CFU per swab was similar to that obtained by other authors\textsuperscript{31, 32} with the same inoculum size (3x10\textsuperscript{8} CFU/ml). The histological results obtained corroborate the microbiological data, as abundant mycelium penetration was observed in the tongue epithelium. Seven days of treatment with silver nanoparticle solutions (50 or 100 µg/ml) resulted in a significant reduction in the total CFU. At this time point, the topical antifungal effect of silver nanoparticles was superior to that produced by miconazole. After 14 days of topical treatment (the end of therapeutic period), the treatment regimens successfully reduced the oral infection relative to the negative control.

For histological results, on day zero, the tongues showed areas of atrophic papillae covered with orthokernized epithelium with mild chronic inflammatory infiltrate of lamina propria. Candidal hyphae and spores were prominent and confined to keratin layer and did not penetrate the prickle cell layer (PAS stain). This was in contrast to histological sections which showed few hyphae in the surface keratinized layer of tongue papillae with loss of filiform papillae, hyperparakeratosis, exocytosis, disorganized epithelium and chronic inflammatory infiltrate in the lamina propria. Similar observations were reported by previous authors\textsuperscript{29-33}.

Epithelial tissue alterations appeared to be directly affected by fungal infection. Experimental groups treated topically with silver nanoparticle solutions exhibited fewer epithelial
alterations. This may be attributed to the *Candida* infection itself which led to epithelial changes that remained even with absence of the candidal hyphae. Other virulence factors may include the presence of pathogenic organisms, factors (adherence, hydrolytic enzymes, and morphological alternation) or microbial toxins after clinical healing of *C. albicans* lesions.

In the present *in vivo* study, comparison between the antifungal effects of silver nanoparticle solutions versus the standard antifungal agent (2% miconazole gel/suspension) showed that silver nanoparticles was superior to miconazole. The current results are in agreement with an *in vitro* study that used the same *C. albicans* (ATCC 90028) strain, the authors of which concluded that silver nanoparticles exhibited a more potent antifungal activity than fluconazole, an antifungal agent that is widely used against many fungal infections\(^\text{17}\). Moreover, in yet another *in vitro* study where fluconazole, was used as positive control for comparison with Ag-NPs, the antifungal activity of fluconazole (against *Phoma glomerata*, *Phoma herbarum*, *Fusarium semitectum*, *Trichoderma sp.*, and *Candida albicans*) increased significantly in presence of Ag-NPs. The maximum antifungal activity was observed against *C. albicans* followed by *Trichoderma sp.* and *P. glomerata*. However, no enhancement of the antifungal activity was recorded in the cases of *F. semitectum* and *P. herbarum*\(^\text{34}\).

Regarding toxicity, silver nanoparticles provoked no acute toxicity in rats as confirmed by normal ALT and AST activities as well as normal serum creatinine and BUN after treatment. These data were different from those demonstrated by other studies using silver nanoparticle solution in rats or mice\(^\text{35-38}\). Importantly, it was reported that the toxicity of silver towards bacteria and fungi were greater than that towards human cells\(^\text{39}\). A recent clinical research studied the effects of commercially available nanoparticles in healthy volunteers and concluded that no toxicity was detected with the utilized comprehensive assays and tests. The authors rightfully stated that further studies are definitely warranted. Studies like this are needed for a more widespread application of nanomedicine\(^\text{40}\). Furthermore, additional testing
of silver nanoparticles in other species of Candida is required before conclusive statements can be made. Additional studies are therefore warranted.

In conclusion, analysis of data provided evidence that silver nanoparticles provided quicker clearance against mucosal *C. albicans* infection over that provided by miconazole at day 7. However, the silver nanoparticles were comparable to miconazole at day 14 (end of therapeutic period) and day 28 (end of the follow up period). Therefore, silver nanoparticles may be a promising antifungal agent in patients with oral candidiasis once an ideal clinical protocol for therapy is established. Further studies are still required to determine the mode of action by which silver nanoparticles exert their fungicidal effect.

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**Conflict of interests**

None to declare

**Author contributions**

Conception and design of experiments: AAA, AAM, CECCE, SAZ, MMA, ANMR, SMA, MMH;
Performance of the experiments: AAA, SAZ, MMA, PAH;
Analysis the data: AAA, SAZ;
Contribution of reagents/materials/analysis tools: AAA, SAZ, MMA;
Manuscript preparation: AAA, CECCE, AAM, SAZ, MMA;
Graphics: AAA, SAZ, CECC;
Critique of initial draft of manuscript: ANMR, EAM, SMA, MMH
References


Figure legends

Figure 1. A) UV-Visible absorption spectra of the silver nanoparticles at 425 nm. B) Transmission electron micrograph for silver nanoparticles showing their spherical shape with average size of 20 nm.

Figure 2. The median value for the clinical scores at different time points, day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period) and day 28 (the end of the follow up period). Data are expressed as median and analyzed at each time point using Kruskal–Wallis test and Dunn’s multiple comparisons’ test at $P<0.05$. *Compared to normal group, #Compared to control group, §Compared to 2 % miconazole gel group, ¶Compared to 2% miconazole suspension group, $n=8$.

Figure 3. Microbial counts expressed as log CFU/ml for the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period, and day 28 (the end of the follow up period). CFU: colony forming units. Data are expressed as median and analyzed at each time point using Kruskal–Wallis test and Dunn’s multiple comparisons’ test at $P<0.05$. *Compared to normal group, #Compared to control group, §Compared to 2 % miconazole gel group, ¶Compared to 2% miconazole suspension group, $n=8$.

Figure 4. A) Histopathological sections of tongue of rats showing normal bristle-like papillae with normal epithelial layers (arrow), normal connective tissue appearance (double arrow). After inoculation with *C. albicans*, candidal hyphae and spores covering the epithelum (interrupted arrow), with disorganization of basal cell layer (double arrow) and mild inflammatory cell infiltration (elbow double arrow). After treatment with nanosilver (50 or 100 µg/ml) or miconazole there were normal appearance of both epithelium (arrow) and connective tissue (double arrow) [PAS x 20]. B) Colonization by Candida in the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period, and day 28 (the end of the follow up period). Data are expressed as median and analyzed at each time point using Kruskal–Wallis test and Dunn’s multiple comparisons’ test at $P<0.05$. *Compared to normal group,
Compared to control group, $\text{Compared to 2 \% miconazole gel group, } ^{\text{*}}\text{Compared to 2\% miconazole suspension group, } n=8$.

**Figure 5.A**) Histopathological sections of tongue of rats showing normal bristle-like tongue papillae with normal epithelium (arrow), normal connective tissue appearance (double arrow). After inoculation with *C. albicans*, acanthosis and hyperplasia of the epithelum (linear arrow), with disorganization of basal cell layer (double arrow) and inflammatory cell infiltration (elbow double arrow). After treatment with nanosilver (50 or 100 µg/ml) or miconazole there were normal appearance of both epithelium (arrow) and connective tissue (double arrow) [H&E x 40]. **B**) Epithelial alterations and **C**) Inflammatory infiltrates in the tongue of the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period, and day 28 (the end of the follow up period). Data are expressed as median and analyzed at each time point using Kruskal–Wallis test and Dunn’s multiple comparisons’ test at $P< 0.05$. $^{\text{*}}\text{Compared to normal group, } ^{\#}\text{Compared to control group, } ^{\text{\$}}\text{Compared to 2 \% miconazole gel group, } ^{\text{\textasteriskcentered}}\text{Compared to 2\% miconazole suspension group, } n=8$.

**Table 2:** Effect of test silver nanoparticles solutions on serum AST, ALT, creatinine and urea in rats with oral candidiasis.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (Unit/L)</th>
<th>AST (Unit/L)</th>
<th>Creatinine (mg/dL)</th>
<th>BUN (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>20.8 ± 2.8</td>
<td>73.4 ± 13.5</td>
<td>1.40 ± 0.65</td>
<td>6.7 ± 1.08</td>
</tr>
<tr>
<td>Negative Control</td>
<td>23.5 ± 4.6</td>
<td>78.2 ± 16</td>
<td>1.16 ± 0.01</td>
<td>7.4 ± 4.8</td>
</tr>
<tr>
<td>Silver nanoparticles (50 µg/ml)</td>
<td>21.7 ± 2.7</td>
<td>73 ± 10</td>
<td>1.17 ± 0.02</td>
<td>7.2 ± 2.2</td>
</tr>
<tr>
<td>Silver nanoparticles (100 µg/ml)</td>
<td>17.8 ± 2.3</td>
<td>76 ± 12</td>
<td>1.18 ± 0.01</td>
<td>6.6 ± 2.1</td>
</tr>
<tr>
<td>Positive (gel) Control</td>
<td>21.4 ± 2.5</td>
<td>70 ± 21</td>
<td>1.19 ± 0.01</td>
<td>6.6 ± 2.3</td>
</tr>
<tr>
<td>p-value (tests vs normal control)</td>
<td>(p &gt; 0.05)</td>
<td>(p &gt; 0.05)</td>
<td>(p &gt; 0.05)</td>
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ALT: alanine aminotransferase, AST: aspartate aminotransferase, BUN (mg/dl): blood urea nitrogen. Results are expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni’s post-hoc test.
Fig. 1: A) UV-Visible absorption spectra of the silver nanoparticles at 425 nm. B) Transmission electron micrograph of the silver nanoparticles used in this work showing spherical in shape with average size of 20 nm.
**Fig. 2:** Bar chart describing the mean value for the clinical scores at different time points, day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period, and day 28 (the end of the follow up period).
Fig. 3: Microbial counts for the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period), and day 28 (the end of the follow up period).
Fig. 4: A) Histopathological sections of tongue of rats stained with periodic acid–Schiff stain after inoculated with Candida albicans and treated with nanosilver (50 or 10 µg/ml) or miconazole [x 20 magnification]. B) Colonization by Candida in the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period), and day 28 (the end of the follow up period).
Fig. 5: A) Histopathological sections of tongue of rats stained with H&E after inoculated with Candida albicans and treated with nanosilver (50 or 100 µg/ml) or miconazole [x 40 magnification]. B) Epithelial alterations and C) Inflammatory infiltrates in the tongue of the experimental groups at day zero (before initiation of treatments), day 7 (the middle of the therapeutic period), day 14 (the end of the therapeutic period), and day 28 (the end of the follow up period).